Environmental Fate of Selected Anticancer Drugs: Photodegradation, Biodegradation and Toxicity of Cyclophosphamide, 5-Fluorouracil, Methotrexate and Imatinib

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4) **Carlos Alexandre Lutterbeck**; Deivid Ismael Kern; Ênio Leandro Machado; Klaus Kümmerer. (2015). Evaluation of the toxic effects of four anticancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. *Chemosphere* 135 (2015) 403–410. http://dx.doi.org/10.1016/j.chemosphere.2015.05.019

5) **Carlos Alexandre Lutterbeck**; Marcelo Luís Wilde; Ewelina Baginska; Ênio Leandro Machado; Klaus Kümmerer. (2015) 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. *Science of the Total Environment* 527–528 (2015) 232–245. http://dx.doi.org/10.1016/j.scitotenv.2015.04.111

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This cumulative thesis and the publications listed on the following page were submitted to the Faculty of Sustainability of Leuphana University Lüneburg to earn the academic degree of Doctor of Natural Science (Dr. rer. nat.)

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Dedication

I would like to dedicate this thesis to my parents Lira and Bruno, and especially to my wife Paula, for all the love, affection and encouragement transmitted during this work.

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List of symbols and abbreviations

5 - FU 5 - Fluorouracil

AI Acute inhibition

ANOVA Analysis of variance

AOPs Advanced oxidation processes

CBT Closed bottle test

CI Chronic inhibition

CP Cyclophosphamide

DOC Dissolved organic carbon

EIC Extracted ion chromatogram

ESI-IT-MS^n Mass spectrometry with electrospray ionization and ion trap detector in tandem

Fe^{2+} Ferrous ions

GI Growth inhibition

H_2O_2 Hydrogen peroxide

HO• Hydroxyl radical

HPLC (LC) High performance liquid chromatography

IC Inhibitory concentration

IM Imatinib

LC-MS/MS(LC-MSn) Liquid chromatography tandem mass spectrometry

LI Luminescence inhibition

LOD Limit of detection

LOEC Lowest observed effect concentration

LOQ Limit of quantification

m/z Mass to charge ratio

min Minute

MP Millipore water

MS Mass spectrometry

MTX Methotrexate

NPOC Non purgeable organic carbon

OD Optical density

OECD Organization for economic co-operation and development

QSAR Quantitative structure–activity relationship

TP Transformation product

Rs Resolution

RSD Relative standard deviation

RSM Response surface methodology

S/N Signal to noise ratio

ThOD Theoretical oxygen demand

TIC Total ion chromatogram

TiO_2 Titanium dioxide

TOC Total organic carbon

TP Transformation product

t_R Retention time

UP Ultrapure water

UV Ultraviolet

V. fischeri *Vibrio fischeri*

Xe Xenon

WWTPs Waste water treatment plants

λ Wavelength

Summary

In the discourse on pharmaceuticals in the environment, hardly any attention has been paid to anticancer drugs. Because of their none-selective modes of action, that is, because they affect both cancerous and healthy cells, these drugs are regarded as potentially carcinogenic, genotoxic, mutagenic, and teratogenic substances. It is, however, not known how and to what extent these substances affect organisms and the environment in the long run. For this reason, this dissertation evaluated, addressing several endpoints and using organisms from different trophic levels and *in silico* predictions, the fate (bio- and photo degradation) and ecotoxicity of these substances. Four anticancer drugs (cyclophosphamide (CP), 5-fluorouracil (5-FU), methotrexate (MTX), and imatinib (IM) were selected.

None of these anticancer compounds can be classified as "readily biodegradable," a classification that indicates that biodegradation will only play a minor role in the elimination of these compounds and that they cannot be removed by the conventional processes used in sewage treatment plants and will most likely remain in the water cycle. Despite the high degrees of mineralization achieved in advanced (photo)oxidation processes, it was not possible to fully mineralize the compounds, a result that indicates that transformation products were created during these reactions.

The ecotoxicity assays performed with *V. fischeri* indicated that 5-FU was, of all the substances tested, likely to be the most toxic (very toxic), followed by MTX (toxic) and IM (toxic/harmful), whereas CP was nontoxic. MTX presented the highest phytoxicity activity in the *Lactuca sativa* assay, followed by 5-FU, IM, and CP. The results of the tests performed with *A. cepa* showed cytotoxic (5-FU, MTX, and CP) and genotoxic effects (5-FU, CP, and IM) and mutagenic activity (5-FU, MTX, CP, and IM) of the compounds. Photo transformation products (PTPs) of CP, MTX, and 5-FU were nontoxic towards *V. fischeri*. However, some PTPs formed during the photodegradation of 5-FU led to positive mutagenic and genotoxic alerts in several *in silico* models.

Not one of the compounds examined in this dissertation is likely to be fully eliminated from the water cycle by (natural) photolysis and/or advanced oxidation. Moreover, some of the treatments resulted in the formation of stable intermediates that were even less biodegradable than parent compounds. This finding shows that it is not enough to focus on primary elimination because TPs are not necessarily better biodegradable than their respective parent compounds. As indicated by the genotoxic and mutagenic positive alerts presented by different *in silico* models,

the PTPs observed here are likely to require, despite their lower toxicity in comparison to the parent compounds, screening after treatments.

Zusammenfassung

Innerhalb des Themas "Arzneimittel in der Umwelt" haben die sogenannten Zytostatika-Medikamente bis jetzt nur wenig Aufmerksamkeit erhalten. Aufgrund ihrer fehlenden oder aufgrund der nicht vorhandenen selektiven Wirkungsweisen, die nicht nur Krebszellen, sondern auch gesunde Zellen betreffen, sind Zytostatikamedikamente als potenziell carcinogene, genotoxische, mutagene und teratogene Substanzen bekannt. Daher basiert die vorliegende Dissertation auf der Bewertung der Destination (Bio- und Foto-Abbau) und der Ökotoxizität bei Organismen verschiedener trophischer Ebenen anhand der Messung mehrerer Endpunkte, um gezielte *in silico* Vorhersagen vornehmen zu können. Vier Zytostatika-Medikamente (cyclophosphamide (CP), 5-fluorouracil (5-FU), methotrexate (MTX) and imatinib (IM)) wurden ausgewählt.

Keiner der untersuchten Zytostatika-Substanzen konnte als "leicht biologisch abbaubar" klassifiziert werden; dies zeigt, dass die biologische Abbaubarkeit nur eine kleine Rolle bei der Elimination diesen Substanzen spielen wird, und dass sie nicht durch konventionelle Kläranlagen entfernt werden und somit wahrscheinlich im Wasserkreislauf bleiben werden. Trotz der hohen Mineralisierungsgrade, die in den fortgeschrittenen (Foto) Oxidationsprozessen erreicht wurden, hat keine der Behandlungen dazu geführt oder geholfen, die Medikamente vollständig zu mineralisieren, was auf die Erzeugung von Transformationsprodukten während der Reaktionen hindeutet.

Die mit *V. fischeri* durchgeführten Ökotoxizitätsversuche ergaben, dass 5-FU das giftigste Medikament (sehr giftig) war, gefolgt von MTX (giftig) und IM (giftig / gesundheitsschädlich), während CP ungiftig war. MTX zeigte die höchste Phytotoxizität-Aktivität in den *Lactuca sativa* Versuchen, gefolgt von 5-FU, IM und CP. Die Ergebnisse der Untersuchungen, die mit *A. cepa* durchgeführt wurden, zeigten zytotoxische (5-FU, MTX und CP) und gentoxische Wirkungen (5-FU, CP und IM) und mutagene Aktivität (5-FU, MTX, CP und IM) der untersuchten Medikamente. Fotoabbauprodukte (FAPs) von CP, MTX und 5-FU waren im *V. fischeri*-Test ungiftig gegen *V. fischeri*. Jedoch zeigten einige FAPs, die während des Fotoabbaus von 5-FU gebildet wurden, in mehreren *in-silico* Modellen mutagene und genotoxisch positive Ergebnisse.

Daher ist nicht davon auszugehen, dass die untersuchten Medikamente umfassend vom Wasserkreislauf durch (Natur-)Photolyse und/oder fortgeschrittene Oxidation beseitigt werden. Darüber hinaus haben einige der verwendeten Behandlungen zur Bildung von stabilen

Nebenprodukten geführt, die noch weniger bioabbaubar und toxischer als die Muttersubstanz waren. Dies zeigt, dass die bloße Kenntnis über primäre Eliminationen nicht ausreichend ist, um eine geeignete Behandlungsmethode auszuwählen und dass die FAPs generell nicht besser biologisch abbaubar sind als die Muttersubstanz. Außerdem zeigen die positiven gentoxischen und mutagenen Ergebnisse/Warnungen einiger der *in silico* Modellen erzeugten FAPs, dass trotz der geringeren Toxizität im Vergleich zu den Muttersubstanzen Überprüfungen nach den Behandlungen empfehlenswert sind.

1. Introduction and motivation

In recent years, the interest in the presence of organic micro-pollutants in wastewaters, surface waters, and ground waters has grown (Kümmerer, 2011). Most of these pollutants are unregulated contaminants, which might, depending on their properties, pose a risk to human health and the environment. Like other substances, pharmaceutically active compounds are now recognized as micro-pollutants, which have also received attention of late (Kümmerer, 2011). Pharmaceuticals are ubiquitous substances, and several studies have reported their presence in wastewaters (Escher et al., 2011; Kümmerer, 2001; Radjenović et al., 2009), surface waters (Buerge et al., 2006; Fatta-Kassinos et al., 2011; Kümmerer, 2008; Lindqvist et al., 2005), drinking waters (de Jongh et al., 2012; Heberer, 2002; Mompelat et al., 2009), and ground waters (Fram and Belitz, 2011; Heberer, 2002; López-Serna et al., 2013).

While the number of studies on pharmaceuticals in the environment has increased considerably in recent years, one important class has, somewhat surprisingly, been overlooked in this discourse: anticancer drugs. Nowadays, approximately 4,000 active pharmaceutical compounds, used in human and veterinary drugs, are available on the European market (Mompelat et al., 2009), and among them are approximately 100 anticancer drugs (Kümmerer et al., 2014). Due to the increasing demand for these substances for chemotherapy treatment, the consumption of these compounds is expected to more than double over the next ten years (Nussbaumer et al., 2011). Consequently, they are also likely to be even more present in the drinking and wastewater than they are today.

The first studies confirming the presence of anticancer drugs in aquatic systems were already published in the 1980s (Aherne et al., 1985; Richardson and Bowron, 1985). Further studies were released in the 1990s (Aherne et al., 1990), and these kinds of investigations continue to be conducted to this day (Besse et al., 2012; Rowney et al., 2009). Nevertheless, there is still a lack of knowledge concerning the environmental fate of the drugs and their metabolites after excretion and concerning possible risks resulting from their presence in aquatic environments (Kümmerer et al., 2014).

Because of their none-selective mode of action, that is, because they affect both cancerous and healthy cells, anticancer drugs have been regarded as potential carcinogenic, genotoxic, mutagenic and teratogenic compounds (Allwood et al., 2002; Eitel et al., 1999). Despite the fairly low consumption rates and low environmental concentrations, especially in comparison to other groups of pharmaceuticals (e.g. antibiotics, beta-blockers), it is not possible to establish threshold

values for lowest effect concentrations of these compounds (Kosjek and Heath, 2011). Some authors believe that, due to the combination of toxicological properties and poor biodegradability (Toolaram et al., 2014), anticancer drugs might have adverse effects on any growing eukaryotic organism, even at very low concentrations (Besse et al., 2012; Johnson et al., 2008).

After administration, anticancer drugs and other pharmaceutical compounds are, depending on their properties, metabolized in the human body in different degrees and excreted as metabolites or unchanged parent compounds, and they can then reach the water bodies. After reaching surface waters, parent compounds and their metabolites undergo several physico-chemical/biological processes (e.g., dilution, hydrolysis, biodegradation, photolysis, and sorption to bed sediments) that can lead to the formation of transformation products (TPs), which might be more harmful and persistent than their parent compounds (Mahmoud et al., 2013; Pérez-Estrada et al., 2008).

Since biodegradation often plays only a minor role in the elimination of the pharmaceuticals, photolysis in natural waters under solar irradiation has been suggested as a degradation pathway of these compounds. Photochemical degradation is regarded, especially compared to other environmental transformation processes, as one of the most relevant processes determining the environmental fate of these compounds and the ecological risks that they are likely to represent (Buth et al., 2010; Fatta-Kassinos et al., 2011). UV-based technologies have been widely used in the treatments for remove organic contaminants, such as pharmaceuticals, from the water bodies (De la Cruz et al., 2012; Kim and Tanaka, 2009; Kim et al., 2009; Köhler et al., 2012; Yuan et al., 2011).

Cyclophosphamide (CP), methotrexate (MTX), 5-fluorouracil (5-FU), and imatinib (IM) are among the most frequently administered anticancer drugs worldwide. These four compounds are, based on their mechanisms of action, classified in different subgroups. CP is an alkylating agent that has been in clinical use since the late 1950s to treat various forms of cancer (bronchial, breast, and ovarian cancer, lymphomas, leukemia) and is also used as an immunosuppressant for autoimmune diseases and after organ transplantations (Buerge et al., 2006). It acts by inhibiting and altering DNA replication. MTX is an antimetabolite widely used at high doses as chemotherapy for various forms of cancer (bronchial, breast, and ovarian cancer, lymphomas, leukemia). It has been sold since the 1940s (Rubino, 2001) and acts by blocking enzyme activity and disrupting DNA synthesis. The most commonly used anticancer drug is 5-FU (Kosjek and Heath, 2011), an antimetabolite that works by inhibiting DNA synthesis and by slowing tumor

growth. It has been used for more than fifty years to treat solid tumors, for example colorectal, breast, skin, bladder, and lung cancers (Mahnik et al., 2007). Imatinib (IM)(Glivec® or Gleevec®) is a protein tyrosine kinase inhibitor that was developed in the 1990s and is used to treat chronic myeloid leukemia and gastrointestinal stromal tumors (Bianchi et al., 2013). It selectively inhibits BCR-ABL tyrosine kinase activity, which is involved in the regulation of many biological processes (cell growth, migration, survival).

The four compounds examined in this dissertation are known not to be fully removed by biological and oxidative treatments (Besse et al., 2012; Booker et al., 2014; Henschel et al., 1997; Steger-Hartmann et al., 1997; Yu et al., 2006) and have already been detected in the different water matrices in ranges from ng to µg/L concentrations (Aherne et al., 1985; Castiglioni et al., 2005; Gómez-Canela et al., 2013; Mahnik et al., 2007; Negreira et al., 2014; Ternes, 1998; Weissbrodt et al., 2009; Yin et al., 2010).

Because the elimination of anticancer drugs and other pharmaceuticals by conventional wastewater treatments is often incomplete and inefficient (Wang and Lin, 2014; Zhang et al., 2013), other alternatives need to be tested. Advanced oxidation processes (AOPs) may yield good results in terms of elimination. They represent a promising alternative, since they can be used in combination with biological treatments for wastewater remediation, as a pre-treatment, increasing the biodegradability by a partial oxidation, or as a post-treatment to degrade persistent compounds (De la Cruz et al., 2012). Advanced (photo) oxidation processes are based on the generation and use of powerful transitory species, mainly the hydroxyl radical ($HO^{\bullet}$), which can be generated photo-chemically (including solar light) or by other forms of energy, and can very effectively oxidize organic matter (Glaze, 1987; Glaze et al., 1987). In recent years, many studies have examined 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; Arslan-Alaton and Gurses, 2004; De la Cruz et al., 2012; Elmolla and Chaudhuri, 2010; Maroga Mboula et al., 2012; Sleman et al., 2012). However, often incomplete mineralization results in the formation of unwanted TPs of unknown properties.

As anticancer drugs allow people to live longer and to achieve higher standards of living, the consumption of these compounds is expected to increase, and consequently, they are more likely to be present in higher concentrations in the different environments. In light of their toxicological properties and the lack of safe threshold values for the lowest effect concentrations for anticancer drugs, it is necessary to assess the risks that these substances and their metabolites

(TPs) may pose to human health and other organism when they are present in different environments.

Studies on the (eco) toxicity of anticancer drugs are still scarce, and most of the studies that have been published have evaluated the acute effects in organisms belonging to different trophic levels (i.e., algae, zooplankton, and other invertebrates and fish) (Santos et al., 2010). Unfortunately, acute tests have almost no environmental relevance and can underestimate the toxicity of chemicals that show mainly long-term toxicity, for example antibiotics or anticancer drugs (Backhaus and Grimme, 1999; Froehner et al., 2000; Henschel et al., 1997). For this reason, there is a need for a more comprehensive toxicological assessment of anticancer drugs and their TPs in general and for studies involving mainly long-term assays (chronic) and tests in particular, which may provide further insights concerning the cytotoxic, genotoxic, and mutagenic potentialities of these compounds. It is crucial to understand the behavior of anticancer drugs in the environment (sorption, hydrolysis, biodegradation, natural photolysis) and to look for alternative treatments that could remove these compounds, improve their biodegradability, and/or reduce their toxicity.

2. Aim and Objectives

This dissertation investigated the environmental fate of four commonly used anticancer drugs in aquatic environments through a combined approach that involved toxicological evaluations, natural forms of attenuation, and potential removal techniques for these compounds. The specific objectives of this dissertation were

- to assess the toxicological properties of the four anticancer drugs mentioned above through ecotoxicological, genotoxic, mutagenic, cytotoxic and phytotoxic assays using experimental systems and *in silico* predictions;
- to evaluate the biodegradation rates of these pharmaceuticals;
- to compare the effectiveness of different photolytic treatments regarding the primary elimination and mineralization of cytotoxic compounds;
- to asses the potential of photodegradation treatments to improve the biodegradability of the four anticancer drugs and to reduce possible toxic effects by using a combination of experimental and *in silico* tools;
- to investigate the possible formation of TPs during photodegradation experiments;

- and to identify and elucidate the structures of TPs formed during treatments and to propose tentative pathways of degradation reactions.

3. Research approach

This scholarly work done to fulfill the degree requirements focused on the four anticancer drugs. These compounds were selected based on specific criteria that were relevant for the five studies described below: a) CP, 5-FU, and MTX are among the most commonly used cytostatic compounds worldwide, have been sold since the middle of the twentieth century, and are constantly detected in different water matrices. b) Although IM can be considered a relatively new compound, high amounts of this pharmaceutical have been used to treat different forms of cancer. c) Several studies have shown that anticancer drugs are compounds that are very toxic for different organisms. d) There is a lack of information concerning the toxicological properties of anticancer drugs and its TPs. e) Little is known about the biodegradation of cytostatic compounds and about its TPs.

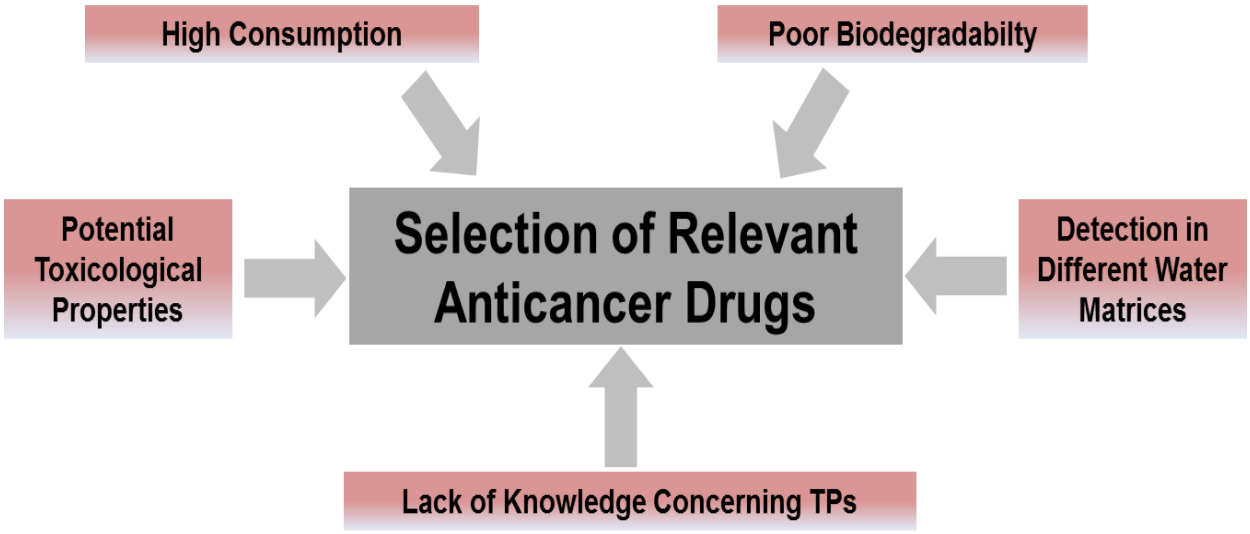

Fig. 1 – Criteria used to select the anticancer drugs investigated in this dissertation.

The **first paper** investigated the primary elimination and mineralization of cyclophosphamide by three different advanced photo treatments: UV/H_2O_2, UV/H_2O_2/Fe^{2+}, and UV/TiO_2. CP is a cytotoxic compound that was introduced as a pharmaceutical in the late 1950s and that is still often used today to treat several types of cancer. The efficiencies of the three processes were compared based on the degree of mineralization. The experiments were performed with an UV polychromatic lamp during 256 min and samples were collected at regular

time points (2, 4, 8, 16, 32, 64, 128 and 256 min) to measure the primary elimination and mineralization of CP and to investigate the formation of transformation products. LC-MS/MS (ion trap) analyses were performed to measure the elimination of the parent compound and to investigate the formation of TPs. Toxicity assays with the samples collected at all-time points were performed with the bioluminescent bacteria *Vibrio fischeri* (Menz et al., 2013).

In the **second paper**, the focus was on MTX, an antimetabolite that has been sold since the 1940s and that is widely used at high doses to treat various types of cancer and some autoimmune diseases. This paper investigated the elimination and mineralization efficiencies of MTX by three advanced (photo) oxidation treatments (UV/H_2O_2, $UV/H_2O_2/Fe^{2+}$, and UV/TiO_2) and the formation of TPs during the reactions. Furthermore, the aerobic biodegradation and the bacterial toxicity of MTX and the photo-TPs were tested in the Closed Bottle Test (CBT(OECD, 1992)) and with the Kinetic Luminescent Bacteria Test (Menz et al., 2013), respectively. The samples were tested before (0 min) and after the treatments (256 min) in order to evaluate the potential of these treatments to improve biodegradability and/or to reduce the toxicity of MTX and its photo-TPs.

The **third paper** investigated photodegradtion of CP and 5-FU by UV and simulated sunlight irradiation. The experiments were performed with two different irradiation sources: an UV TQ 150 W medium pressure lamp and a Xe TXE 150 W lamp. Liquid chromatography tandem mass spectrometry (LC-IT-MS/MS) analyses were done in order to monitor the elimination of parent compounds and to identify TPs formed during the photodegradation experiments. Aerobic biodegradation (Closed Bottle Test, OECD 1992) and ecotoxicity (Kinetic Luminescent Bacteria Test, Menz et al., 2013) of parent compounds and of photolytic mixtures were tested before (0 min) and after (256 min) the experiments that involved UV and the Xe lamps. QSAR analyses using a set of different models provided by the software CASE Ultra 1.5.2.0 were performed to assess possible mutagenic and genotoxic activities of 5-FU and the generated TPs. It was possible to assess the potential of both lamps to improve biodegradability and/or to reduce the toxicity of CP and 5-FU.

The **fourth paper** focused on the toxicological evaluation of the parent compounds of the four anticancer drugs (CP, MTX, 5-FU, and IM). These drugs are compounds with a considerable environmental impact because they have been identified, even at low concentrations, as potentially cytotoxic, genotoxic, mutagenic, carcinogenic, or teratogenic substances in non-target organisms (Alwood et al., 2002; Kümmerer et al., 2014). Therefore, this paper investigated the

toxicity of the four compounds in plant bioassays. The phytotoxicity was evaluated with seeds of lettuce (*Lactuca sativa*) by adapting the method proposed by Sobrero and Ronco, (2004). The germination of seeds and the growth of the seedlings were the endpoints considered in these assays. The seeds of onion (*Allium cepa*) were used to assess the cytotoxic, genotoxic, and mutagenic effects of the compounds. The cytogenetic evaluation was carried out using the methodological adaptations proposed in Fiskesjö (1985, 1995) for the mitotic index, by Grant (1982) for chromosome aberrations, and by Ma et al. (1995) for the analysis of micronuclei.

The **fifth paper** dealt with the application of three advanced (photo) oxidation treatments (UV/H_2O_2, $UV/H_2O_2/Fe^{2+}$, and UV/TiO_2) for the primary elimination and mineralization 5-FU. 5-FU is the most widely used anticancer drug (Kosjek and Heath, 2011) and has been frequently detected in several water matrices in concentrations ranges from ng to µg/L (Zhang et al., 2013). The concentrations of H_2O_2 and TiO_2 for the UV/H_2O_2 and UV/TiO_2 experiments were optimized by performing prescreening tests, whereas the $UV/H_2O_2/Fe^{2+}$ process was optimized by means of response surface methodology. The effects of three independent variables (pH and H_2O_2 and Fe^{2+} concentrations) were evaluated in order to optimize the experimental setting. 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). Moreover, biodegradability (OECD, 1992) and ecotoxicity of 5-FU and of the photolytic mixture formed after the reactions were assessed. The mutagenicity and genotoxicity of 5-FU and of the TPs created during the advanced (photo) oxidation treatments were assessed by a set of *in silico* predictions using the software CASE Ultra V.1.5.2.0. It was possible to monitor not only the degradation of the parent compound, but also to evaluate the efficiency of the processes to improve biodegradability and/or to reduce toxicity effects.

4. Results and Discussion

The results of the **first paper** indicated a complete elimination of the parent compound of in all the treatments wherein $UV/Fe^{2+}/H_2O_2$ was the fastest process, followed by UV/H_2O_2 and UV/TiO_2. All the reactions obeyed pseudo-first order kinetics. Despite the fast elimination of CP in all the treatments, not one of the processes achieved full mineralization, indicating the formation of stable compounds. Degrees of mineralization higher than 85 % were observed in the $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2 reactions, whereas UV/H_2O_2 achieved 72.5 % of DOC removal. LC-MS/MS analysis revealed the generation of five TPs during the reactions. All the TPs formed

during the photocatalytic processes were already reported in previous studies as human metabolites that are formed during the degradation process of CP in the human body. Not one of them showed significant toxicity against *V. fischeri* in the three endpoints (acute luminescent inhibition, chronic luminescent inhibition, and growth inhibition).

All the irradiation experiments easily eliminated the parent compound of MTX **(paper 2)**. The UV/TiO_2 led to the fastest reaction, followed by UV/Fe^{2+}/H_2O_2 and UV/H_2O_2. In the photo-catalysis process, MTX was almost completely eliminated after 4 min, whereas in the photo-Fenton and UV/H_2O_2 reactions, these times were 8 min and 16 min, respectively. 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. TPs were preliminarily identified by LC-UV–MS/MS. MTX was only partially biodegraded in the closed bottle test (CBT). A threshold of less than 70 % mineralization of MTX (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 and ecotoxicological tests on the photo treated solutions. Therefore, according to the results of the photo treatments, only samples from the UV/H_2O_2 reactions were collected and tested in the CBT experiments. The UV/H_2O_2 process demonstrated not to be a good treatment option, since the formed TPs were 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. This could be seen as an advantage since the persistence of the formed TPs is a clear disadvantage.

The results of the experiments with the Xe lamp indicated that the direct photolysis due to sunlight will not be degrade and therefore limit the presence of CP in aquatic environments. Similarly, photodegradation due to only UV irradiation seems also not to be a suitable treatment for removal. These results can be attributed to the very low absorbance of CP (UV max absorbance at 193 nm) in the wavelengths ranges of the Hg lamp (200-600 nm) and the Xe lamp (200-800). 5-FU was only partially degraded by the Xe lamp (32.2%), whereas the UV radiation completely eliminated the parent compound of 5-FU after 32 min. However, despite the complete elimination of the parent compound, only 18% mineralization was achieved after 256 min. Three TPs were formed during the photodegradation experiments and identified. The results of the CBT indicated that not one of the parent compounds (CP and 5-FU) reached a biodegradation level of 60% ThOD and therefore cannot be classified as ready biodegradable substance. As already

expected, no improvement of the biodegradability of CP was observed for the samples that were submitted to 256 min of photodegradation. The samples submitted to the treatments with the Xe lamp presented no significant differences in the toxicity of 5-FU for the three endpoints (AI, CI and GI). An explanation for this finding can be the partial transformation of the parent compound (only 32% of primary elimination) and the formation of stables that exhibit a similar toxicity to 5-FU, since no mineralization was registered using this lamp. In contrast, samples photolysed with the UV lamp registered high reductions in chronic toxicity. The results indicated that the 24 h luminescence inhibition at this endpoint was reduced from 50% to 5% in the assays performed with *V. fischeri*. Furthermore, while 5-FU showed positive mutagenic and genotoxic alerts in three models of the *in silico* predictions, only one TP presented a positive alert for a genotoxic activity. Hence, the photolysis using the UV radiation might have the potential to be applied as a pre-treatment for degradation of 5-FU, improving its biodegradability and reducing the toxicity.

The results of additional experiments performed with IM and MTX, which have yet to be published, showed that IM did not undergo any degradation in response to simulated sunlight and was only partially degraded by UV radiation, whereas MTX was partially degraded in response to simulated sunlight radiation and completely eliminated after 128 min of UV radiation. As indicated by the CBT results, IM and MTX cannot be classified as readily biodegradable compounds, and not one of the photodegradation treatments (UV or Xe) increased biodegradability. However, UV radiation significantly reduced the toxicity of MTX. Therefore, it can be concluded that TPs, although not biodegradable, are less toxic than their respective parent compounds.

The results of the fourth paper, which dealt with *A. cepa* assays, showed that the compounds are likely to exhibit cytotoxic, genotoxic, and mutagenic properties. Statistically significant differences ($p < 0.0001$) of the mitotic indexes were observed for MTX, 5-FU, and CP (291%, 26.5% and 13.9%, respectively), indicating cytotoxicity of these cytostatics. MTX was the most mutagenic compound forming the highest percentage of micronucleated cells (98.1%), followed by 5-FU, CP, and IM (97.8%, 83.4%, and 81.7%, respectively). Statistically significant increases ($p < 0.0001$) of chromosome aberrations were found in cells exposed to 5-FU, CP, and IM (61.8%, 51.3%, and 36.7%, respectively). An increased prevalence of apoptotic cells was observed for cells exposed to samples of MTX and 5-FU. Not one of the selected compounds, even at the highest concentrations, significantly affected the seed germination of *L. sativa*. Therefore, it cannot be considered a good endpoint to evaluate the toxic effects of the anticancer

drugs analyzed here. Measuring growth inhibition on *L. sativa* seedlings seems, however, to be a useful tool to evaluate the sublethal effects of different pollutants. The results showed that MTX was the most phytotoxic compound (IC_{25} = 1.1 mg/L), followed by 5-FU, IM, and CP (IC_{25} values of 9.8, 55.3, and 69.9 mg/L, respectively). Seeds exposed to MTX displayed dun spots on the seedlings roots, which indicated necrosis of tissues. In summary, although it is unlikely that the pharmaceuticals concentrations measured in the environment could cause lethal effects in plants, the results obtained in this study indicate that these compounds may affect the growth and normal development of plants.

The results of dose response ecotoxicological experiments (not yet published) showed toxic effects of the four compounds varying from µg to mg/L. Chronic assays performed with *C. dubia* indicated that 5-FU was the most toxic drug (EC_{50} 13.09 µg/L), followed by MTX (EC_{50} 1.15 mg/L), IM (EC_{50} 1.33 mg/L), and CP (EC_{10} 4.18 mg/L). Only IM had significant toxic effects in the acute assays involving *V. fischeri* (EC_{50} 11.6 mg/L), whereas 5-FU (EC_{50} 450 µg/L) showed the highest toxicity in the chronic test, followed by MTX (EC_{50} 4.07 mg/L), IM (EC_{50} 12.71 mg/L), and CP (EC_{10} 74.1 mg/L). Considering the growth endpoint, 5-FU showed the highest inhibition followed by MTX and IM (with EC_{50} values at 1.31, 5.32, and 47.72 mg/L, respectively). CP was not toxic for this endpoint. The low toxicity of CP can be attributed to its mode of action: CP is a prodrug that is not active itself unless it undergoes metabolic activation in the liver. The EU Directive 93/67/EEC (Commission of the European Communities, 1996) classifies substances based on their EC_{50} values and the related effects on aquatic organisms in different classes: < 1 mg/L (very toxic to aquatic organisms), 1-10 mg/L (toxic to aquatic organisms), and 10-100 mg/L (harmful to aquatic organisms). Substances with EC_{50} values above 100 mg/L are not be classified. Based on this directive, 5-FU can be classified as a very toxic compound, MTX as toxic, IM as toxic towards *C. dubia* and harmful to *V. fischeri*, whereas CP cannot be classified. Therefore, the ecotoxicological assays involving *V. fischeri* and *C. dubia* turned out to be efficient tools for an initial toxicological screening of these substances in aquatic environments.

Similar to CP and MTX, the primary elimination of 5-FU occurred very quickly in all three treatments. This and related results were published in the fifth paper**.** The $UV/Fe^{2+}/H_2O_2$ process removed the parent compound in less than 2 min, whereas in the UV/H_2O_2 and UV/TiO_2 reactions, these times were 8 and 32 min, respectively. The highest degrees of mineralization were reached in photo Fenton and photocatalysis reactions (> 70%), whereas the UV/H_2O_2

process was less efficient (42%). It was found that the concentrations of H_2O_2 and Fe^{2+} significantly affected the efficiency of the photo Fenton reactions. The results of the Surface Response Methodology indicated optimal concentrations of 525 mg/L and 120 mg/L for hydrogen peroxide and ferrous ions, respectively. However, the results also indicated that variation of the pH did not significantly affect these reactions. Six TPs were formed during the advanced (photo) oxidation processes and identified. 5-FU was characterized by very low biodegradation in the CBT experiments. Using the same methodology to test for biodegradation of MTX, only samples with less than 70% DOC removal were tested in the CBT. In contrast to the results obtained with MTX, the photolytic mixture resulting from the UV/H_2O_2 presented a noticeable improvement of biodegradability in CBT. As the parent compound was easily degraded after 8 min during the UV/H_2O_2 photodegradation, these results are likely to suggest the formation biodegradable by-products. The results of the acute ecotoxicological assays indicated that there were no differences in terms of toxicity of the samples before and after the UV/H_2O_2 reactions. Nevertheless, statistically significant differences were found in the toxicities of the other two endpoints. Chronic toxicity was completely eliminated after 256 min (from 51 to -3%), with $p \leq 0.0001$, while growth inhibition decreased from 20 to -1% ($p \leq 0.001$). The *in silico* predictions showed that the parent compound of 5-FU presented positive alerts for mutagenic and genotoxic activities, whereas most of the formed TPs did not show any positive alert, indicating potentiality of the AOPs examined here to reduce the genotoxicity and mutagenicity of 5-FU. One exception was TP 146, an OH-derivative of 5-FU; in this case, the QSAR analysis indicated that the mutagenicity of the parent compound was either retained or altered.

5. Summary

With an increased prevalence of cancer worldwide, it expected that consumption of anticancer drugs will keep rising, and as a result, these drugs will be present in different environments. It is believed that each living organism may potentially be affected by their different modes of action and by the fact that they are expected to exert effects at very low concentrations, so that is not possible to establish safe threshold values for lowest effect concentrations of these compounds.

The results of the studies presented in this thesis demonstrated that anticancer drugs can have different toxic effects on plants. However, since the toxic effects will depend on the type of

compound, its concentration and the plant species, with distinct metabolism and uptake characteristics, a battery of different plant assays should be performed for testing environmental contaminants and screening of treated waste water intended for irrigation.

In accordance with some results already reported in the literature, not one of the selected compounds was classified as readily biodegradable in the experiments. Moreover, not one of the substances studied here was eliminated in experiments using, simulated sunlight irradiation. Therefore, it can be assumed that once released in the environment aquatic, natural attenuation (biodegradation and photolysis) will not narrow the presence of these compounds in different water matrices.

The photodegradation experiments performed with the UV lamp and the advanced (photo) oxidation treatments led to contradictory results, not allowing for a general conclusion about the efficiency of the treatments and showing that a compound and treatment specific assessment is necessary. On the one hand, CP was not characterized by elimination, and IM was only partially degraded with the UV lamp. On the other hand, 5-FU and MTX were completely eliminated during the treatments. Moreover, whereas for IM neither the biodegradability was improved nor the toxicity was reduced, the primary elimination of MTX and 5-FU led to the formation of less toxic byproducts and in the specific case of 5-FU more biodegradable byproducts.

Despite the easy elimination of the parent compounds of CP, MTX. and 5-FU in all the advanced (photo) oxidation treatments, not one of the processes yielded complete mineralization, which indicates the formation of TPs. The UV/Fe^{2+}/H_2O_2 was the most efficient process for the three compounds, achieving the highest mineralization degrees and consequently minimizing the generation of TPs. Considering specifically CP, no significant difference regarding the toxicity was observed during the treatments, that is, as well as the parent compound not one of the transformations products formed during the reactions was toxic against *V. fischeri*. The results of the UV/H_2O_2 experiments showed the formation of less toxic TPs for MTX, and 5-FU. In contrast, while for 5-FU a noticeable enhancement of the biodegradability was observed after the UV/H_2O_2 treatment, for the MTX the formed TPs were less biodegradable than the parent compound.

So, the combined application of different treatment alternatives 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. However, one should be aware that TPs are not generally better biodegradable than a given parent and can be even more toxic. This again reinforces the need a compound and treatment specific assessment.

In summary, the results presented in this thesis demonstrate that at least for practical application knowledge on primary elimination is insufficient to select the appropriate treatment method and treatment time. So, the combination of several analytical methods is necessary to get a more complete understanding of the dynamics of the processes. Additionally, this kind of knowledge is urgently needed to conduct treatment in a way that formation of TPs is minimized, since sometimes they can be even more recalcitrant and toxic than the parent compounds. 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. The results presented in this thesis also reinforce the importance of the identification of the TPs formed during the processes by different analytical methods. Furthermore, since little is known about the generated TPS, the combination of the experimental data and *in silico* approaches such as QSAR models can give valuable insights into the environmental fate, behavior and risk of such compounds.

6. Conclusions and future perspectives

This thesis has shown that anticancer drugs can exhibit toxic effects against different living organisms. Although it is unlikely that the concentrations of pharmaceuticals measured in the environment could have lethal effects in plants, the results obtained in this study indicate that these compounds may affect the growth and normal development of these plants. Furthermore, the (few) (eco) toxicological data concerning the anticancer drugs are based on acute or in vitro-assays. In this sense, a broader approach, involving different organisms (from different trophic levels) and focusing in long exposure (chronic assays) and *in vivo* assays is necessary for a better understanding of the toxicological effects of anticancer drugs and their metabolites. Moreover, as the residues of these compounds in the environment often occur as complex mixtures, testing for

mixtures to verify possibly synergistic or antagonistic effects of molecules with similar mode of action is also an important issue for a more detailed risk assessment.

According to the results presented in this thesis, it was found that once released in the aquatic environment, the studied compounds will probably remain in the water cycle, since neither biodegradation nor natural photolysis will play an important role on their degradation.

Although 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, rarely comparative studies of treatment methods are performed. In the present thesis, different treatment options were tested. The results demonstrate that at least for practical application knowledge on primary elimination is insufficient to select the appropriate treatment and that the combination of several analytical methods is necessary to get a more complete understanding of the dynamics of the processes. Such knowledge is urgently needed to conduct treatment in a way that high mineralization degrees were achieved and the formation of TPs is minimized. Moreover, taking into account the scarce information concerning the toxicological properties of the byproducts, the identification of such TPs is of crucial importance. So, the strategy of combining suitable experimental and *in silico*-tools can bring important contributions to recognizing the environmental fate, behavior and risk of such compounds and the resulting TPs.

Finally, considering the increased demand for existing and new anticancer drugs worldwide and the lack of safe threshold limits for the toxic effects that these compounds can pose to the human health and to other non-target organisms, the presence of anticancer drugs and its metabolites in the environment should be a theme of concern not only for the scientific community but also for the general public.

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Curriculum Vitae

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List of Publications:

1) **Carlos Alexandre Lutterbeck**; Ênio Leandro Machado; Klaus Kümmerer (2015). Photodegradation of the antineoplastic cyclophosphamide: A comparative study of the

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2) **Carlos Alexandre Lutterbeck**; Ewelina Baginska; Ênio Leandro Machado; Klaus Kümmerer. (2015) Removal of the anti-cancer drug Methotrexate from water by advanced oxidation processes: aerobic biodegradation and toxicity studies after treatment. *Chemosphere* 141 (2015) 290–296. http://dx.doi.org/10.1016/j.chemosphere.2015.07.069
3) **Carlos Alexandre Lutterbeck**; Marcelo Luís Wilde; Ewelina Baginska; Ênio Leandro Machado; Klaus Kümmerer. (2015) Degradation of Cyclophosphamide and 5-Fluorouracil by UV and simulated sunlight treatments: Assessment of the enhancement of the biodegradability and toxicity. *Environmental Pollution* xxx (2015) 1-10 http://dx.doi.org/10.1016/j.envpol.2015.10.016
4) **Carlos Alexandre Lutterbeck**; Deivid Ismael Kern; Ênio Leandro Machado; Klaus Kümmerer. (2015). Evaluation of the toxic effects of four anticancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation. *Chemosphere* 135 (2015) 403–410. http://dx.doi.org/10.1016/j.chemosphere.2015.05.019
5) **Carlos Alexandre Lutterbeck**; Marcelo Luís Wilde; Ewelina Baginska; Ênio Leandro Machado; Klaus Kümmerer. (2015) 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. *Science of the Total Environment* 527–528 (2015) 232–245. http://dx.doi.org/10.1016/j.scitotenv.2015.04.111
6) Deivid Ismael Kern; Rômulo de Oliveira Schwaickhardt; **Carlos Alexandre Lutterbeck**; Lourdes Teresinha Kist; Eduardo Alexis Lobo Alcayaga; Ênio Leandro Machado. Ecotoxicological and Genotoxic Assessment of Hospital Laundry Wastewaters. *Arch Environ Contam Toxicol* (2015) 68: 64–73 DOI 10.1007/s00244-014-0072-0
7) **Carlos Alexandre Lutterbeck**; Rômulo de Oliveira Schwaickhardt; Alexandre Straatmann; Lourdes Teresinha Kist; Eduardo Alexis Lobo Alcayaga; Ênio Leandro Machado. Electrooxidation Combined with Ozonation in Hospital Laundry Effluents Treatment. *Clean – Soil, Air, Water* (2014), 42 (5), 601–608 DOI: 10.1002/clen.201200580

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Poster presentation:

1) **Carlos Alexandre Lutterbeck**, Ewelina Bagisnka, Ênio Leandro Machado, Klaus Kümmerer. Assessment of the biodegradability and ecotoxicity of the anti-cancer drug Imatinib Mesylate after UV and simulated sunlight irradiation treatments. SETAC Europe 25th Annual Meeting. 3-7.05.2015, Barcelona, Spain.
2) **Carlos Alexandre Lutterbeck**, Deivid Ismael Kern, Ênio Leandro Machado, Klaus Kümmerer. Assessment of the Toxic Effects of Four Anticancer Drugs in Plant Bioassays. SETAC North America 35th Annual Meeting. 9-13.11.2014, Vancouver, BC, Canada.
3) **Carlos Alexandre Lutterbeck**, Ênio Leandro Machado, Klaus Kümmerer. Photodegradation of Methotrexate and 5-Fluorouracil: Ecotoxicological Studies Before and After Treatment. SETAC North America 35th Annual Meeting. 9-13.11.2014, Vancouver, BC, Canada.
4) **Carlos Alexandre Lutterbeck**, Deivid Ismael Kern, Ênio Leandro Machado, Klaus Kümmerer. Ecotoxicological evaluation of selected anticancer drugs. 50th Congress of the European Societies of Toxicology (EUROTOX). 7-10.09.2014, Edinburgh, Scotland**.**
5) **Carlos Alexandre Lutterbeck**, Deivid Ismael Kern, Ênio Leandro Machado, Klaus Kümmerer. Evaluation of the effects of four cytotoxic compounds against plant seedlings 50th Congress of the European Societies of Toxicology (EUROTOX). 7-10.09.2014, Edinburgh, Scotland.
6) Jana Weiss, Ewelina Baginska, **Carlos Alexandre Lutterbeck**, Richard Bolek, Benoit Roig, Klaus Kümmerer, Marja Lamoree, Véronique Boireau. Formation of stable transformation products of pharmaceuticals in the water treatment cycle. SETAC North America 34th Annual Meeting. 17–21.11.2013, Nashville, Tennessee, USA.
7) Jana Weiss, Ewelina Baginska, **Carlos Alexandre Lutterbeck**, Richard Bolek, Benoit Roig, Klaus Kümmerer, Marja Lamoree, Véronique Boireau. Formation of stable transformation products of pharmaceuticals in the water treatment cycle. 13th International Conference on Environmental Science and Technology (CEST). 5-7.09.2013, Athens, Greece.
8) **Carlos Alexandre Lutterbeck**, Richard Bolek, Kham Dieu Huynh, Ênio Leandro Machado, Klaus Kümmerer. Removal of Methotrexate, 5 Fluorouracil and

Cyclophosphamide from water by UV, UV/H2O2 and UV/Fe2+/H2O2 processes. PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?). 2-3.06.2013, Nimes, France.

9) Anju P. Toolaram, Ewelina Baginska, **Carlos Alexandre Lutterbeck**, Kham Dieu Huyn, Klaus Kümmerer. Environmental fate of Methotrexate: photodegradation, biodegradability and mutagenicity assessment PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?). 2-3.06.2013, Nimes, France.

10) Ewelina Baginska, **Carlos Alexandre Lutterbeck**, Klaus Kümmerer. Biodegradability of cytostatic drugs: Cyclophosphamide, 5-Fluorouracil and Methotrexate and their photo-transformation resulting from treatment with UV and simulated sunlight radiation products in Closed Bottle Test. PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?). 2-3.06.2013, Nimes, France.

Declaration 1

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

Carlos Alexandre Lutterbeck
Lüneburg, 22 October 2015

Authors' contributions to the articles:

Article	Short Tittle	Specific contributions of all authors	Author status	Weighting factor	Publication status	Conference contributions
1	Photodegradation of the antineoplastic cyclophosphamide: A comparative study of the efficiencies of UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 processes	**Carlos Alexandre Lutterbeck**; Ênio Leandro Machado; Klaus Kümmerer	Co-author with predominant contribution [Überwiegen der Anteil	1.0	Chemosphere 120 (2015) 538–546. http://dx.doi.org/10.1016/j.chemosphere.2014.08.076	PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?). Nimes, France: 2-3.06.2013
2	Removal of the anti-cancer drug Methotrexate from water by advanced oxidation processes: aerobic biodegradation and toxicity studies after treatment	**Carlos Alexandre Lutterbeck**; Ewelina Baginska; Ênio Leandro Machado; Klaus Kümmerer	Co-author with predominant contribution [Überwiegen der Anteil	1.0	Chemosphere 141 (2015) 290–296. http://dx.doi.org/10.1016/j.chemosphere.2015.07.069	PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?).Nimes, France: 2-3.06.2013
3	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 predominant contribution [Überwiegen der Anteil	1.0	Environmental Pollution xxx (2015) 1-10 http://dx.doi.org/10.1016/j.envpol.2015.10.016	PHARMAS Conference (Pharmaceuticals in the environment: is there a problem?). Nimes, France, 2-3.06.2013 and SETAC North America 35th Annual Meeting. 9-13.11.2014: Vancouver, BC, Canada.
4	Evaluation of the toxic effects of four anticancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation.	**Carlos Alexandre Lutterbeck**; Deivid Ismael Kern; Ênio Leandro Machado; Klaus Kümmerer	Co-author with predominant contribution [Überwiegen der Anteil	1.0	Chemosphere 135 (2015) 403–410. http://dx.doi.org/10.1016/j.chemosphere.2015.05.019	50th Congress of the European Societies of Toxicology (EUROTOX). 7-10.09.2014: Edinburgh, Scotland and SETAC North America 35th Annual Meeting. 9-13.11.2014: Vancouver, BC, Canada
5	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	Co-author with predominant contribution [Überwiegen der Anteil	1.0	Science of the Total Environment 527–528 (2015) 232–245. http://dx.doi.org/10.1016/j.scitotenv.2015.04.111	Pharmaceuticals in the environment: is there a problem? PHARMAS Conference, Nimes, France; 06/2013
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Declaration of Authorship: Authors' contributions to the articles:

	Paper 1	Paper 2	Paper 3	Paper 4	Paper 5
Conception of research approach	CAL, ELM, KK	CAL, EB, ELM, KK	CAL, MLW, EB, CL, ELM, KK	CAL, DIK, ELM, KK	CAL, CL, MLW, EB, ELM, KK
Development of research methods	CAL, KK	CAL, EB	CAL, MLW, EB	CAL, DIK	CAL, MLW, EB
Data collection and data preparation	CAL	CAL, EB	CAL, MLW, EB	CAL, DIK	CAL, MLW, EB
Execution of research	CAL	CAL, EB	CAL, MLW, EB	CAL, DIK	CAL, MLW, EB
Analysis/ interpretation of data	CAL, ELM, KK	CAL, EB	CAL, MLW, EB, CL	CAL, DIK	CAL, MLW, EB, CL
Writing of the manuscript	CAL	CAL, EB	CAL, MLW, EB	CAL, DIK	CAL, MLW, EB
Internal revision of manuscript	CAL, ELM, KK	CAL, EB, ELM, KK	CAL, CL, MLW, EB, ELM, KK	CAL, DIK, ELM, KK	CAL, CL, MLW, EB, ELM, KK

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Appendices

Paper 1

Photodegradation of the antineoplastic cyclophosphamide: A comparative study of the efficiencies of UV/H_2O_2, $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2 processes.

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Photodegradation of the antineoplastic cyclophosphamide: A comparative study of the efficiencies of UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 processes

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HIGHLIGHTS

- AOPs were compared with respect to degree and kinetics of degradation and mineralization of CP.
- The treatments showed full elimination of CP in all the experiments.
- Complete mineralization was not achieved, indicating the formation of stable compounds.
- Transformation products were detected during two treatments.
- None of the transformation products was toxic against *Vibrio fischeri*.

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ABSTRACT

Anticancer drugs are harmful substances that can have carcinogenic, mutagenic, teratogenic, genotoxic, and cytotoxic effects even at low concentrations. More than 50 years after its introduction, the alkylating agent cyclophosphamide (CP) is still one of the most consumed anticancer drug worldwide. CP has been detected in water bodies in several studies and is known as being persistent in the aquatic environment. As the traditional water and wastewater treatment technologies are not able to remove CP from the water, different treatment options such as advanced oxidation processes (AOPs) are under discussion to eliminate these compounds. The present study investigated the degradation of CP by three different AOPs: UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2. The light source was a Hg medium-pressure lamp. Prescreening tests were carried out and afterwards experiments based on the optimized conditions were performed. The primary elimination of the parent compounds and the detection of transformation products (TPs) were monitored with LC–UV–MS/MS analysis, whereas the degree of mineralization was monitored by measuring the dissolved organic carbon (DOC). Ecotoxicological assays were carried out with the luminescent bacteria *Vibrio fischeri*. CP was completely degraded in all treatments and UV/Fe^{2+}/H_2O_2 was the fastest process, followed by UV/H_2O_2 and UV/TiO_2. All the reactions obeyed pseudo-first order kinetics. Considering the mineralization UV/Fe^{2+}/H_2O_2 and UV/TiO_2 were the most efficient process with mineralization degrees higher than 85%, whereas UV/H_2O_2 achieved 72.5% of DOC removal. Five transformation products were formed during the reactions and identified. None of them showed significant toxicity against *V. fischeri*.

1. Introduction

During the last decades, advances in medicine allowed for an increase in the life expectancy of people worldwide. Pharmaceuticals are substances especially developed for this purpose and are therefore part of the modern life of humans. On one hand pharmaceuticals contribute to our well-being, high life expectancy and economic prosperity. On the other hand they are often incompletely metabolized and released into the environment and can

Abbreviations: AOP, advanced oxidation process; CP, cyclophosphamide; DOC, dissolved organic carbon; DOM, dissolved organic matter; HPLC, high performance liquid chromatography; LC, liquid chromatography; mM, mili-molar; MS, mass spectrometry; nm, nanometer; TOC, total organic carbon.

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create great harm to the aquatic environments (Halling-Sørensen et al., 1998; Heberer and Ternes, 2006; Kümmerer, 2008). Pharmaceutically active compounds are now recognized as micro-pollutants and their study has been a topic for several years already (Kümmerer, 2011).

Cytotoxic drugs are substances widely used to treat different forms of cancer. Because of the increasing aging of the human population which is associated with an increased prevalence of cancer it is expected that anti-cancer treatment will more than double over the next 10 years (Hoppe-Tichy, 2010). Although the measured environmental concentrations of these compounds are low compared to other drugs (Kosjek and Heath, 2011), the anti-cancer drugs are of particular environmental concern because they are potentially genotoxic, carcinogenic, mutagenic and teratogenic even at low concentrations (Kummerer et al., 2000; Zounkova et al., 2010).

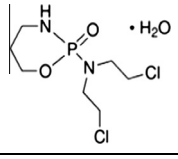
The alkylating agent Cyclophosphamide (CP, chemical structure see Table 1) is more than 50 years after its introduction still one of the most used cytotoxic compounds in anti-cancer therapy. It acts by inhibiting or altering the DNA replication. It has been extensively used to treat a broad spectrum of solid tumors (carcinomas of the bronchus, breast, ovary, and various sarcomas), chronic lymphocytic leukemia, and lymphomas (Nussbaumer et al., 2011). After application about 20% of the parent compound is released unchanged through renal excretion (Rowney et al., 2009). Presence and behavior of CP in the environment has been studied several times. The results reveal that CP is only poorly biodegraded (Kummerer et al., 1996; Steger-Hartmann et al., 1997) and underwent no direct photolysis (Buerge et al., 2006; Lin et al., 2013b). Accordingly, CP is a compound with a high persistence in the environment and was expected in the water cycle. Furthermore, it has a low octanol–water partition coefficient (K_{ow}) (Wang et al., 2009) and is highly soluble in water (Table 1).

Considering the fact that the UV absorption of CP in wavelengths above 200 nm is almost negligible and that its degradation in this range is unexpected to occur by direct photolysis, its elimination seems only to be possible through different alternatives. The removal of CP and other pharmaceuticals by conventional wastewater treatment is often incomplete and inefficient (Zhang et al., 2013; Wang and Lin, 2014). So, different advanced treatment options are under discussion. Advanced oxidation processes (AOPs) are attributed to remove pollutants. These processes are based primarily on the generation of hydroxyl radicals ($OH^{\cdot}$) which attack aggressively and almost non-selectively inorganic as well as organic compounds, including a variety of xenobiotics and micropollutants (Arslan-Alaton and Dogruel, 2004).

They represent an interesting alternative, since 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. However, it has also be found that only incomplete mineralization is reached resulting in the formation of recalcitrant and sometimes even more toxic unintended by-products (Lin et al., 2013b; Magdeburg et al., 2014). Among the AOP's, UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 have been extensively investigated for the degradation of wide range of pharmaceutical compounds (Arslan-Alaton and Dogruel, 2004; Arslan-Alaton and Gurses, 2004; Elmolla and Chaudhuri, 2010; De la Cruz et al., 2012; Maroga Mboula et al., 2012; Sleman et al., 2012; Wols et al., 2013).

The present study aimed to compare the efficiency and kinetics of cyclophosphamide degradation and the rate and degree of mineralization by UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 treatments as well as identify possible transformation products and evaluate its toxicity against the bioluminescent bacteria *Vibrio fischeri*. The impact of different parameters has been investigated, such as pH, hydrogen peroxide, ferrous and titanium dioxide concentration.

Table 1
Structure and main physico-chemical properties of cyclophosphamide.

Structure	MW	WS	Log K_{ow}	$t_{1/2}$*
	279.1 g mol^{-1}	5943 mg L at 25 °C[a]	0.63[b]	80 d[c]

MW: molecular weight; WS: water solubility; log K_{ow}: partition coefficient (octanol/water); $t_{1/2}$: half-life.
* In lake water incubated under dark conditions.
[a] Llewellyn et al. (2011).
[b] Wang et al. (2009).
[c] Buerge et al. (2006).

2. Materials and methods

Cyclophosphamide is classified as a potential carcinogenic substance and must be handled carefully. All the experiments conducted in our laboratories were carried out obeying strict safety precautions (Eitel et al., 1999; Allwood et al., 2002). The waste generated was disposed and treated as hazardous. All instruments and glassware used were carefully cleaned after usage.

2.1. Chemicals

All the chemicals used in this study were of analytical grade or higher. Acetonitrile (HiPerSolv CHROMANORM, LC–MS grade, BDH Prolabo) and formic acid (analytical grade) were purchased from VWR International GmbH (Darmstadt, Germany). Hydrogen peroxide (H_2O_2 30% w/w) was obtained from Merck-group (Darmstadt, Germany) whereas iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), catalase from bovine liver (2000–5000 units mg $protein^{-1}$), ammonium metavanadate (NH_4VO_3) and 1,10-phenantroline ($C_{12}H_8N_2$) from Sigma–Aldrich Biochemie GmbH (Hamburg, Germany). Sodium hydroxide (NaOH 1M) and sulfuric acid (H_2SO_4 2M, 98%) were acquired from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and Titanium dioxide (TiO_2 P25) was purchased from Evonik Degussa GmbH (Frankfurt, Germany). Cyclophosphamide monohydrate (Endoxan, Baxter Oncology, Round Lake, IL, USAI) was kindly provided 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 CP, all the solutions were prepared using ultrapure water (Q_1: 16.6 mΩ and Q_2: 18.2 mΩ).

2.2. Advanced treatment experiments in general

The experiments were carried out in an 800 mL batch photoreactor containing 600 mL of the CP solutions and with an initial concentration of 20 mg L^{-1}. The option for a higher initial concentration of CP (20 mg L^{-1}) was done in order to measure the extent of the mineralization with a high sensitivity (TOC instrument works with a higher sensitivity in the range from 1 to 20 mg carbon) instead of just the degradation of the parent compound and to generate information about the formation of photo-TPs (very low initial concentrations of CP could lead to the formation of photo-TP's below instrumental quantification limits). A medium-pressure mercury lamp (TQ150, UV Consulting Peschl, Mainz) with an ilmasil quartz immersion tube was used to irradiate the samples. The lamp exhibits a polychromatic spectrum and emits radiation from 200 to 600 nm. 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).

Magnetic stirring was used throughout the experiments. A circulating cooling system (WKL230, LAUDA, Berlin) was used to maintain the temperature between 20 ± 2 °C. The pH values were monitored during all the treatments. Photochemical reactions were run for 256 min and samples were taken at regular time points (2, 4, 8, 16, 32, 64, 128 and 256 min) to check the degradation and mineralization rates of CP.

Nonlinear regression analyses were performed using exponential decay models 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.

2.2.1. UV/H_2O_2-treatment

Preliminary experiments were performed in order to find the optimum H_2O_2 concentration. Three different H_2O_2 concentrations were tested (9.8 mM, 14.7 mM and 19.6 mM) and the evaluation of the performance was based on the mineralization rates. In order to avoid direct oxidation of CP by hydrogen peroxide, the lamp was turned on first and afterwards the hydrogen peroxide was added. The pH of the samples collected during the treatment was adjusted to 7 (± 1) with NaOH. Then 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) was added to remove residual H_2O_2. Samples were stored until analysis at 4 °C.

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

The photo-Fenton treatment was conducted based on the results of the UV/H_2O_2experiments. The initial concentration of H_2O_2 and $FeSO_4{\cdot}7H_2O$ were 9.8 mM and 1.56 mM, respectively, and the pH was adjusted to 5 with H_2SO_4 (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, in order to verify the effects of different concentrations of hydrogen peroxide and ferrous ions, two extras experiments were performed. After the collection, the pH of the samples was adjusted to 7 ± 1 with NaOH in order to precipitate the ferrous ions and catalase was used to destroy the residual H_2O_2. Then the samples were centrifuged at 4000 rpm for 5 min and filtered over 0.2-μm filter membranes (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey–Nagel, Germany) and stored until analysis at 4 °C. 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-treatment

Prescreening tests with different concentrations (100, 500 and 1000 mg L^{-1}) were carried out in order to observe the impact of the titanium dioxide concentration. Before starting the photo-catalytic test, solutions containing CP and TiO_2 were constantly stirred for 30 min in the dark to reach adsorption equilibrium on the TiO_2 surface. The samples taken at the different time points were centrifuged at 4000 rpm for 5 min and then filtered through 0.2-μm filter membranes (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey–Nagel, Germany) and stored until analysis at 4 °C.

2.3. Chemical analysis

The primary elimination of CP was monitored by high performance liquid chromatography (HPLC 1100 series, Agilent Technology, Waldbronn, Germany) consisting of a G1312A binary pump, a G1329A thermostatic autosampler, a G1316A column oven (set to 30 °C), and a G1322A degasser. The chromatographic separation of the sample was performed on a Nucleodur RP-18 endcapped 100–3, 2 μm (Macherey–Nagel, Düren, Germany) coupled to a precolumn, (Nucleodur C18 ec 4–2, 3 μm; 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 min^{-1}. Standards of CP (0.1, 1, 2.5, 5, 10 and 20 mg L^{-1}) 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 100 μg L^{-1} and 350 μg L^{-1}, respectively.

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 and 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.

Dissolved organic carbon (DOC) measurements in three replicates were done to determine the degree of mineralization using a TOC (total organic carbon) analyzer (TOC 5000, Shimadzu GmbH, Duisburg, Germany).

2.4. Toxicity analyses

The toxicity of CP and of the possible transformation products generated during the photodegradation experiments was evaluated using the marine bacterium *V. fischeri*. The assays were performed in accordance with a new automated method (Menz et al., 2013) that combines the conventional short-term luminescence inhibition test according to EN ISO 11348 and the *Photobacterium phosphoreum* growth inhibition test (DIN 38412-37).

In this test a defined amount of a diluted or undiluted sample is combined with the luminescent bacteria suspension on a microtiter plate (96 well-plates) and afterwards positioned in a platen reader and incubated for 24 h. At regular intervals, occurs a measurement of the optical density and luminescence so that a complete kinetics of light emission and the cell growth is recorded.

Three different endpoints were measured in each test: the acute luminescence inhibition (AI) after 30 min, chronic luminescence inhibition (CI) after 24 h and growth inhibition (GI) after 14 h. The concentrations (dilutions) of the compounds were tested in triplicate in every experiment and repeated independently in duplicate. In each assay, 4.5 mg L^{-1} 3,5-dichlorophenol and 0.05 mg L^{-1} chloramphenicol were used as positive controls for acute toxicity and chronic inhibition, respectively. The significance limits adopted for acute and growth inhibition tests were 20% while for the chronic test was 15%. A more detailed description and assessment of the kinetic LBT can be found elsewhere (Menz et al., 2013).

3. Results and discussion

In order to assess the kinetics of the photodegradation of CP the following relationship was used:

$$d[C]/dt = k_1[HO^{\cdot}][C]$$

$$k_1[HO^{\cdot}] = k_{obs}$$

$$-d[C]/dt = k_{obs} * [C]$$

where C is the concentration of the compound at a time point; k_{obs} is the observed rate constant; t is the reaction time. Half-lives were calculated by the following equation:

$$t1/2 = \ln\frac{2}{k}$$

The UV-only control experiment showed that CP did not undergo direct UV photolysis (Fig. S2). Furthermore, no degradation of the parent compound was observed after a period of 10 d in our hydrolysis tests (22 °C ± 1, pH 6.5).

3.1. UV/H_2O_2

The effects of the different concentrations of hydrogen peroxide on the DOC removal were tested. From the obtained results we observe that the process has an optimal H_2O_2 concentration at 9.8 mM (330 mg L^{-1}) achieving a mineralization of 72.5% within 256 min. Above this concentration, the efficiency decreases reaching to 65.2% and 52.8% of mineralization, 14.7 mM (450 mg L^{-1}) and 19.6 mM (660 mg L^{-1}), respectively (Fig. S3). Furthermore the kinetics of the processes also showed delays of the reactions at higher H_2O_2 concentrations (Table S1) This loss in the efficiency of the UV/H_2O_2 treatment can be attributed to the fact that most of the degradations are very dependent on the H_2O_2 concentration, increasing to an optimum value, beyond which an inhibitory effect takes place (Litter, 2005). When added in excess H_2O_2 acts as hydroxyl radical or hole scavenger to form the perhydroxyl radicals ($HO_2^{\cdot}$) which are much less reactive than hydroxyl radical (Eqs. (1) and (2)) (Behnajady et al., 2006).

$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + HO_2^{\cdot} \quad (1)$$

$$H_2O_2 + h^+ \rightarrow H^+ + HO_2^{\cdot} \quad (2)$$

Therefore it is important to the system avoid an excess of H_2O_2 that could retard the reaction rates. Venta et al. (2005) have investigated the degradation of CP by O_3/H_2O_2 treatment. The authors verified that by applying only ozone the oxidation of CP was very slow, with 96% of the parent compound remaining in the solution (pH 7) after 6 min reaction time. They also observed a greatly enhancement of the reaction rate by the addition of hydrogen peroxide, which in the presence of ozone favors the generation of hydroxyl radicals and which in turn are responsible for the degradation of cyclophosphamide. The results also showed the presence of an optimum hydrogen peroxide concentration for the experiments.

Fig. 1 presents the dynamics of the photolytic mineralization of CP by the three methods. From the UV/H_2O_2 treatment, it can be observed that about 65% of the mineralization of CP occurs during the first 128 min. Afterwards the mineralization rate slowed down relatively achieving 72.5% until 256 min. This behavior shows the presence of 2 steps in the mineralization of CP: 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 decrease could be explained by the formation of newly recalcitrant TP's. The application of a modified pseudo-first order kinetic model fitted well to the reaction and supports this assumption (Table 2). This model considers both the presence of oxidizable and non-oxidizable matter and attributes the remaining DOC of the mixture to the presence of refractory compounds (Martins et al., 2010).

The parent compound was quickly removed in 8 min. Venta et al. (2005) obtained similar results using the O_3/H_2O_2 treatment, with CP being almost completely degraded in 6 min. However, the authors observed a TOC removal of only 58.6% after 30 min of reaction, indicating the formation of by products that were only partially degraded to CO_2. The pH of the solution decreased from 6.5 (0 min) to around 3.5 after the addition of H_2O_2 and remained in this range until the end the reaction (256 min).

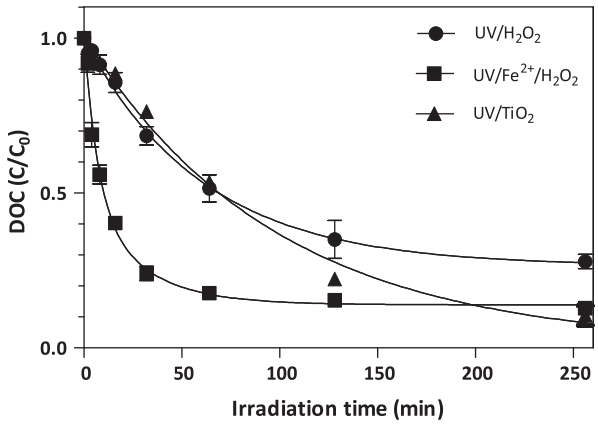


Fig. 1. Kinetics of the mineralization of CP under the three different treatment reactions. Values are the means ± SD from three independent experiments.

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

The data of the UV/H_2O_2 treatment were taken into account to set the proper concentration of H_2O_2 present in the other tests. The obtained results showed that although faster at the first minutes of the reactions the final degree of mineralization of CP is not much impacted by the concentrations of hydrogen peroxide and ferrous ions (Fig. S4 and Table S1). So we used the lowest concentrations, i.e., 9.8 mM and 1.55 mM of H_2O_2 and Fe^{2+}, respectively, to perform the assays.

From Fig. 1 one can observe the presence of two different phases in the mineralization of CP: one very fast until the first 32 min, resulting in a mineralization of 76% and the other much slower achieving 87% after 256 min. The obtained data fitted well to the two phase decay kinetic model and exhibited the presence of two rate constants: K_{fast} = 0.1489 min^{-1} and K_{slow} = 0.0366 min^{-1} (Table 2). One explanation for the presence of different phases can be the quickly consumption of hydrogen peroxide. Using the spectrophotometric method with ammonium metavanadate (λ_{max} = 450 nm) as described by Nogueira et al., we verified that more than 90% of the initial concentration was consumed in 2 min (Fig. S5), and after 32 min the presence of hydrogen peroxide remaining in the solution was under the detection limits of the method. Another possible explanation for this slowdown can be the formation of newly recalcitrant TP's.

According to De la Cruz et al. (2012) micropollutants present in water bodies can be degraded through the photo-Fenton process by different pathways: direct photolysis, or indirect photolysis through the DOM present, the attack of hydroxyl radicals after their generation by the photolysis of H_2O_2, Fenton and the photo-Fenton reactions, or the attack of other reactive oxygen species (ROS). Considering that the radiation absorption of CP in wavelengths between 200 and 600 nm is almost negligible and no dissolved organic matter was present in the solutions, we can assume that CP was mainly degraded through OH radicals, generated by the cleavage of H_2O_2, and Fenton reactions with oxidation of Fe^{2+} to Fe^{3+} by H_2O_2. Previous studies carried out in our laboratories, using only UV radiation (UV medium pressure lamp λ = 200–600 nm) and ultrapure water as medium, support this assumption, since no mineralization and degradation of CP were observed after 256 min. Kim and Tanaka, 2009 investigated the degradation of 30 Pharmaceuticals and Personal Care Products

Table 2
Apparent mineralization rate constants of cyclophosphamide under different AOP's and the corresponding $t_{0.5}$.

Process	K'_{fast} (min^{-1})	$t_{1/2fast}$ (min)	K'_{slow} (min^{-1})	$t_{1/2slow}$ (min)	R^2	SS
UV/H_2O_2	0.0170	40.82	–	–	0.997	0.0008
$UV/Fe^{2+}/H_2O_2$	0.1354	5.12	0.0308	22.52	0.992	0.0067
UV/TiO_2	0.0103	67.17	–	–	0.993	0.0067

(PPCP's). The authors compared the effectiveness of two lamps in pure water: an UV low pressure lamp (8 W) emitting light at 254 nm and an UV low pressure lamp (10 W) emitting both at 254 nm and 185 nm. The results showed CP as the most resistant substance among all the investigated compounds. However, it must be pointed out that the use of the lamp emitting at 254 nm and 185 nm increased to more than five times the degradation rates of CP when compared to the lamp emitting only at 254 nm. This enhancement can be attributed to the generation of OH radicals formed by the photolysis of water under wavelength of 185 nm. These findings reinforce again that CP is mainly degraded through OH radicals and not by direct photolysis.

The UV radiation can also contribute to the degradation rate due to the iron recycling caused by the photochemical reduction of ferric ions (Eq. (3)) or by the photolysis of Fe^{3+} (Eq. (4)), both leading to the production of $OH^{\cdot}$ and Fe^{2+} (Augugliaro et al., 2006). At 32 min Fe^{2+} reaches its lowest value (7.6 mg L^{-1}) and in the absence of H_2O_2 the mineralization rates strongly decrease (Fig. S6). After this time point a slow iron recycling happens, with a measured Fe^{2+} concentration of 12.7 mg L^{-1} at 256 min.

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\cdot} \quad (3)$$

$$Fe^{3+} + h\nu + H_2O \rightarrow Fe^{2+} + OH^{\cdot} + H^{+} \quad (4)$$

These reactions can explain the existence of the two different phases in the mineralization of CP, which occurred very fast in the presence of H_2O_2 and consequently generation of high amounts of $OH^{\cdot}$ until 32 min, and other (much slower) after this time point that remained happening due to the recycling of the ferrous ions (Supplementary Material). The fast oxidation is typical for Fenton reactions being indicative of a direct and fast $OH^{\cdot}$ oxidation step that slows down as soon as Fe^{3+} starts to accumulate (Arslan-Alaton and Dogruel, 2004). After the addition of H_2O_2 (at the beginning of the tests) the pH decreased from 5 to 3 and remained in this range until the end of the experiments.

3.3. UV/TiO_2

The results of the adsorption experiments (between CP and TiO_2) indicated that no adsorption occurred throughout 256 min reaction time with the three different concentration (100, 500 and 1000 mg L^{-1}) at pH 6. The effect of the TiO_2 loading on the photo-catalytic mineralization of CP was also studied. When the TiO_2 amount was increased from 100 mg to 500 mg a considerable enhancement in efficiency was noted for CP mineralization (58.4–89.6%). This can be explained by an increased number of catalyst active sites that are available for photo-catalytic reactions at higher TiO_2 concentrations. However, no improvement of the mineralization was observed by adding 1000 mg of TiO_2 (Fig. S7 and Table S1). This excess that occurs at higher catalyst concentrations, can mask part of the photosensitive surface and consequently hindering or even reflecting the penetrating light; these loses of photons lead usually the mineralization to a plateau or even to decrease (Hapeshi et al., 2010).

Similar observations were reported in other studies involving pharmaceuticals compounds (Yang et al., 2008; Elmolla and Chaudhuri, 2010; Giraldo et al., 2010; Hapeshi et al., 2010). Based on the results, the optimum catalyst loading for the mineralization of CP in our experiments is 500 mg and it was used to study the effect of other parameters.

The results presented in Fig. 1 shows that almost 80% of the mineralization happened during 128 min. Afterwards a decrease of the reaction rates occurs until 256 min where a mineralization of 89.6% was achieved. As can be observed in Table 2, the application of a modified pseudo-first order kinetic model also fitted well to the reaction. A decrease of the pH from 6.8 (0 min) to 5.2 (256 min) was observed in the experiments.

3.4. Process comparison

Although slower at the beginning the performance of UV/TiO_2 after 256 min was in the same range than $UV/Fe^{2+}/H_2O_2$, with DOC removals of 89.6% and 87.2%, respectively. The combined use of UV and H_2O_2 resulted in the lowest mineralization rate (72.2%). Table 2 presents the mineralization rate constants of CP. Based on the results it can be assumed that treatments obeyed pseudo-first order kinetics. 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).

Considering only the primary elimination of the parent compound which was 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. 2). In the photo-Fenton process, CP was completely eliminated in less than 120 s (2 min), whereas in the UV/H_2O_2 and UV/TiO_2 reactions more than 99% was removed in 480 s (8 min) and 1920 s (32 min) respectively. The faster degradation of CP under $UV/Fe^{2+}/H_2O_2$ and UV/H_2O_2 can be attributed to the quick generation of high amounts of hydroxyl radicals by Fenton and photo-Fenton reactions. As CP was removed in less than 120 s 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 0.0103 and 0.0024 s^{-1} and the half-life times were 67.3 and 288.8 s (Table S2 Supplementary Material). Venta et al. (2005) by applying the O_3/H_2O_2 reaction at pH 7 and with an optimized molar ratio (O_3/H_2O_2) verified rate constants and half-life times of 0.0081 s^{-1} and 86 s, respectively.

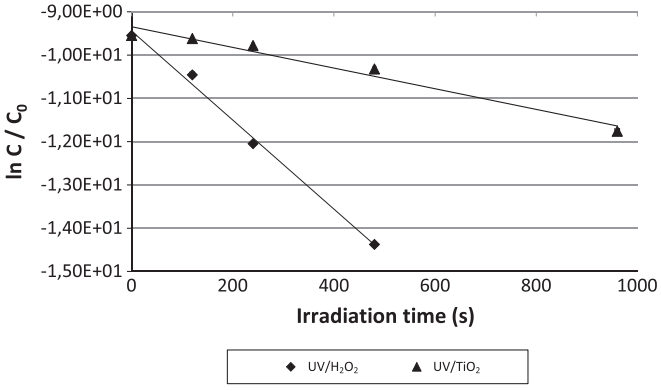


Fig. 2. Kinetics of the degradation of CP by UV/H_2O_2 and UV/TiO_2.

3.5. Identification of transformation products (TPs)

The appearance of new peaks during the UV/H_2O_2 and UV/TiO_2 processes is an indicative of the formation of TPs (Fig. 3). As mostly very little is known about the toxicological properties of the by-products generated during several treatment processes, the identification of the most relevant TPs is an important issue, once they can be recalcitrant and even more toxic than the parent compound.

In order to elucidate the molecular structures of the TPs formed during both processes (UV/H_2O_2 and UV/TiO_2), the new peaks generated during the reactions were, depending on their intensity, isolated and fragmented up to MS3. The total ion chromatogram (TIC) in LC–MS showed the development of five new peaks during the UV/H_2O_2 and four new peaks during the UV/TiO_2 reactions. These compounds are labeled as TP1, TP2, TP3, TP4 and TP5 in Fig. 4. The chromatographic behavior demonstrated that, with exception of the TP 2, all the TPs formed during the reactions have higher polarity than CP (T_R = 16.9), which is indicated by shorter retention time (Table S3).

The generation of TPs was divided in three steps. In the first one, the TPs were formed directly from de degradation of CP. The primary TPs were then further degraded and formed secondary TPs which in turn formed the tertiary TPs. Three PTs were generated through the primary degradation of CP (Fig. 5). TP1 (277.02 m/z) has 16 Da more than CP. Its fragmentation to MS/MS originated several product ions, which allowed for the elucidation of its structure and its identification. TP1 matches the human metabolite 4-hydroxycyclophosphamide, the main active metabolite of CP and its formation occurred through a hydroxylation (Baumann and Preiss, 2001). However, on the basis of the MS fragmentation, it is difficult to predict the exact position where the hydroxylation took place.

Another peak with less 2 Da (m/z 259) compared to m/z 261 of CP was obtained by photodegradation of CP. This is likely to be due to a dehydrogenation of CP or due to a dehydroxylation of TP1 (in this case TP 2 would be formed as a secondary TP) (Hemminki et al., 1987). The fragmentation originated several product ions, which allowed for the elucidation of its structure and its identification. The TP2 was identified as iminophosphamide, a nontoxic human metabolite of CP (Hemminki et al., 1987). The third primary photo-TP was TP3 (m/z 198.3), which was fragmented until MS_3 and formed only during the UV/H_2O_2 reactions. TP3 has 63 Da less than CP and was probably formed through the loss of chloroethane of the bis(2-chloroethyl)amine side chain. It was identified as 2-dechloroethylcyclophosphamide, an inactive metabolite of CP, which is formed in the human body as a second route of CP elimination (de Jonge et al., 2005).

TP4 was generated as a secondary TP of CP and when compared to m/z 261 of CP, has 14 Da more. As one can observe in Fig. 5, TP4 was probably formed through a dehydrogenation of TP1 (277.02 m/z) or by a hydroxylation of TP2 (m/z) 259.01. It matches well known the inactive metabolite of CP, 4-ketocyclophosphamide (Fernández et al., 2010; Medana et al., 2013). Lastly a new peak at m/z 138.03 was observed with a retention time of 12.5 min and labeled as TP5. Probably the formation of this TP occurred through the loss of the fragment bis(2-chloroethyl)amine from the TP4. TP5 was identified as (1-aminocyclopropyl)phosphonic acid.

3.6. Toxicity analyses

The ecotoxicological analyses showed that the parent compound of CP is not toxic to *V. fischeri* at the concentration used in our assays. These results were already expected since previous studies performed in our laboratories showed that CP has a lowest observed effect concentration (LOEC) for the chronic toxicity of 120 mg L^{-1} to *V. fischeri*. The absence of toxicity of CP can be attributed to its mode of action i.e. CP is a pro drug that is not active itself unless undergo metabolic activation on the liver.

Furthermore, as one can observe in Fig. 6, when considering the limits established by Menz et al. (2013) for the three endpoints, none of the transformations products generated during the UV/H_2O_2 and UV/TiO_2 treatments presented a significant inhibition to *V. fischeri*.

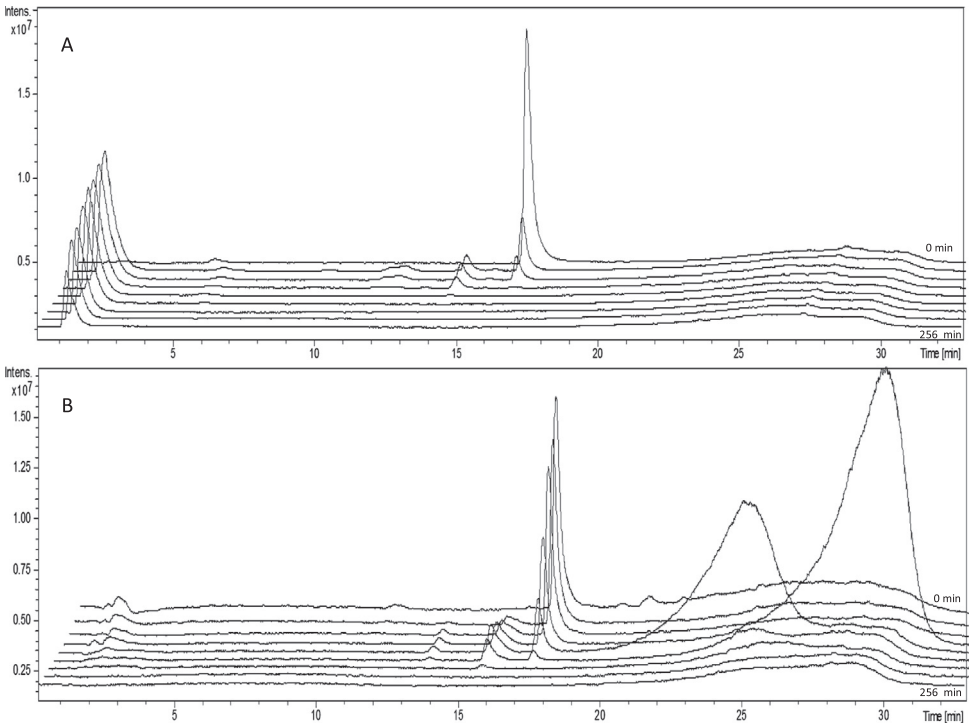


Fig. 3. Total ion chromatograms (TICs) of CP samples collected at different time points (0, 2, 4, 8, 16, 32, 64, 128 min and 256) using LC–ESI–MS in positive mode (initial concentration of 20 mg L^{-1}). (A) UV/H_2O_2 reactions and (B) UV/TiO_2 reactions.

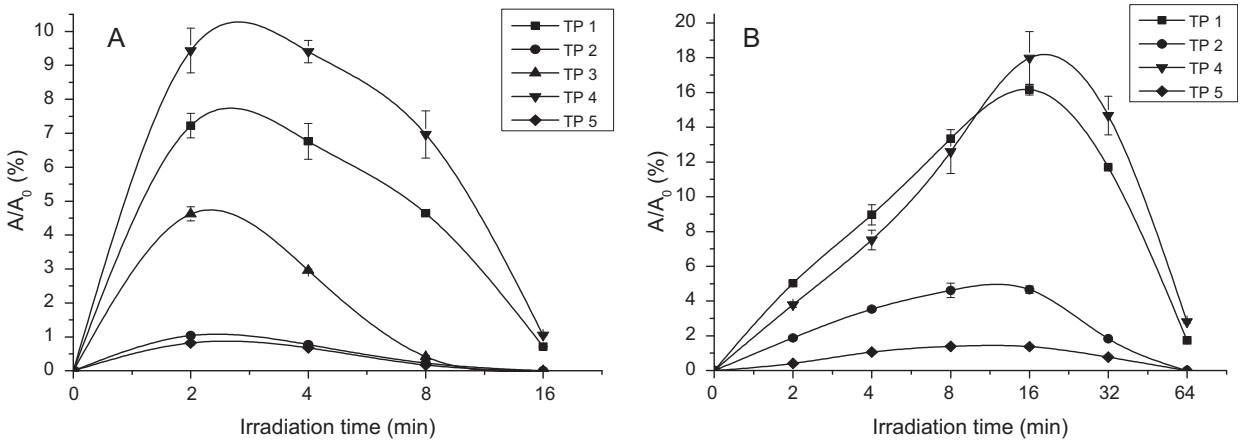


Fig. 4. Time course of appearance/disappearance of peak area of the photo-TPs of CP measured by LC–ESI–MS in positive mode. (A = TPs formed during the UV/TiO_2 reactions and B = TPs formed during the UV/H_2O_2 reactions) (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of CP at 0 min) (Initial concentration of CP = 20 mg L^{-1}; n = 3).

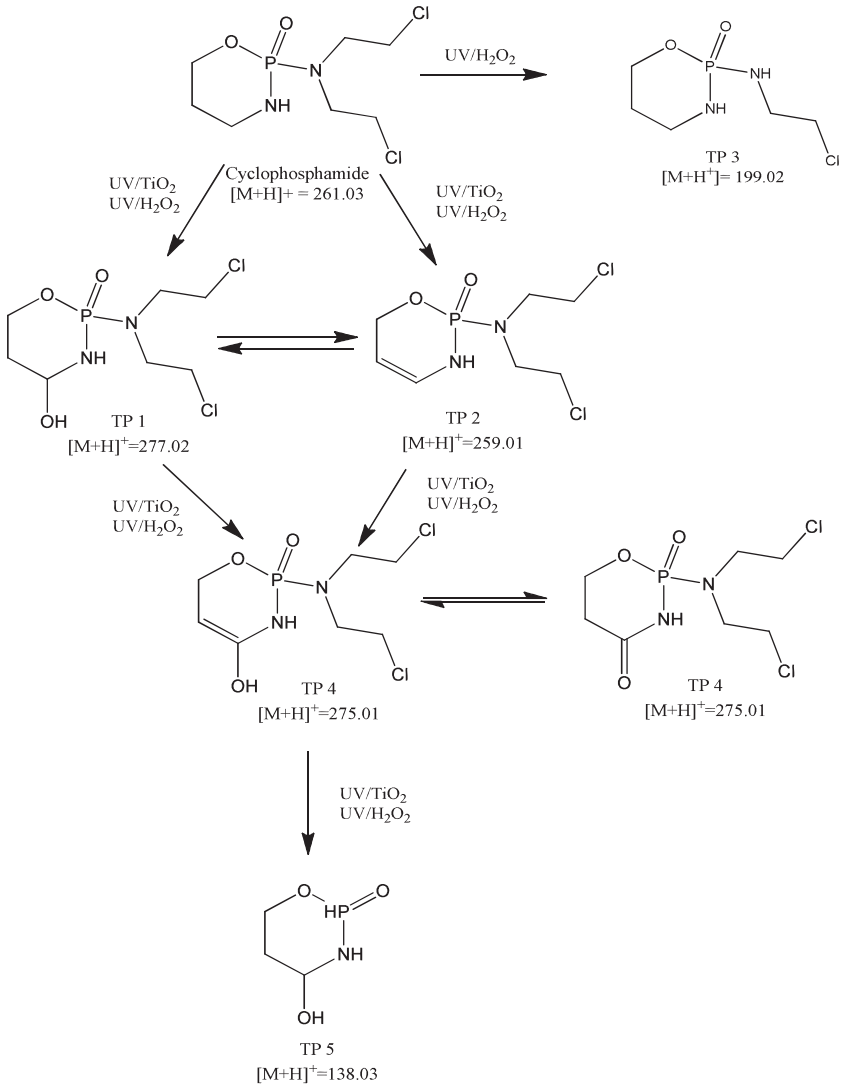


Fig. 5. Tentative pathway of the photodegradation of CP under UV/H_2O_2 and UV/TiO_2 treatments.

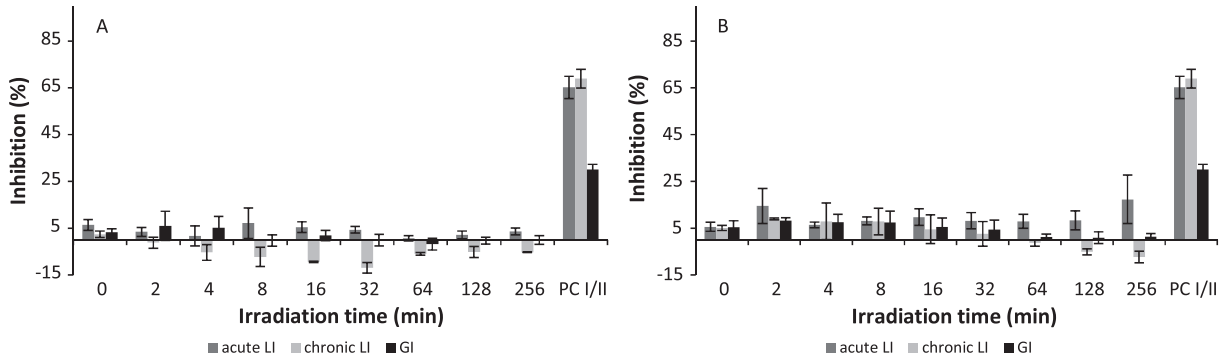


Fig. 6. Toxicity tests with *V. fischeri* ($n = 2$) considering three endpoints: acute luminescence inhibition after 30 min (acute LI), chronic luminescence inhibition after 24 h (chronic LI) and Growth inhibition after 14 h (GI) positive control I (PCI): 4.5 mg L^{-1} 3,5-dichlorophenol (acute LI), positive control II (PCII): 0.05 mg L^{-1} chloramphenicol (chronic LI, growth Inh.) A = analyses of the parent compound (CP) and of the different time points of the UV/H_2O_2 treatment and B = analyses of the parent compound (CP) and of the different time points of the TiO_2 treatment.

4. Conclusions

Several treatment procedures are under discussion for the removal of micro-pollutants from water. However, comparison of methods is rarely done. The degradation and mineralization of cyclophosphamide under three UV-based processes using the same conditions of radiation, pH and experimental set up was therefore investigated. Although all the reactions were effective in degrading CP, none of them achieved a complete mineralization, suggesting the presence of stable intermediates. The UV/Fe^{2+}/H_2O_2 treatment presented the highest rate constants, removing CP in less than 2 min and achieving 76% mineralization at 32 min. After this time point, the mineralization strongly slowed down so that to perform the reaction during 256 min seems not to be interesting. In the same way, the UV/TiO_2 and UV/H_2O_2 reactions should not be carried out more than 128 min, since only a low mineralization was verified after this time point. Five transformation products were detected during the UV/H_2O_2 reactions whereas four were found in the UV/TiO_2 treatments. All the TPs generated during the experiments were identified and are also formed during the degradation process of CP in the human body. None of them was toxic to *V. fischeri*. The combined application of the studied AOP's with a biological treatment should be investigated, in order to verify the feasibility of the processes to be used as pre or post-treatment and to ensure the complete mineralization of the compounds.

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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.2014.08.076.

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Supplementary Material

Photodegradation of the Antineoplastic Cyclophosphamide: a comparative study of the efficiencies of UV/H_2O_2, $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2 processes

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Text Supplementary Material S1:

A one phase decay model was adopted to calculate the rate constant of the mineralization of CP by the UV/H_2O_2 and UV/TiO2 processes. 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 adopted to calculate the rate constants of the mineralization of CP by the 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

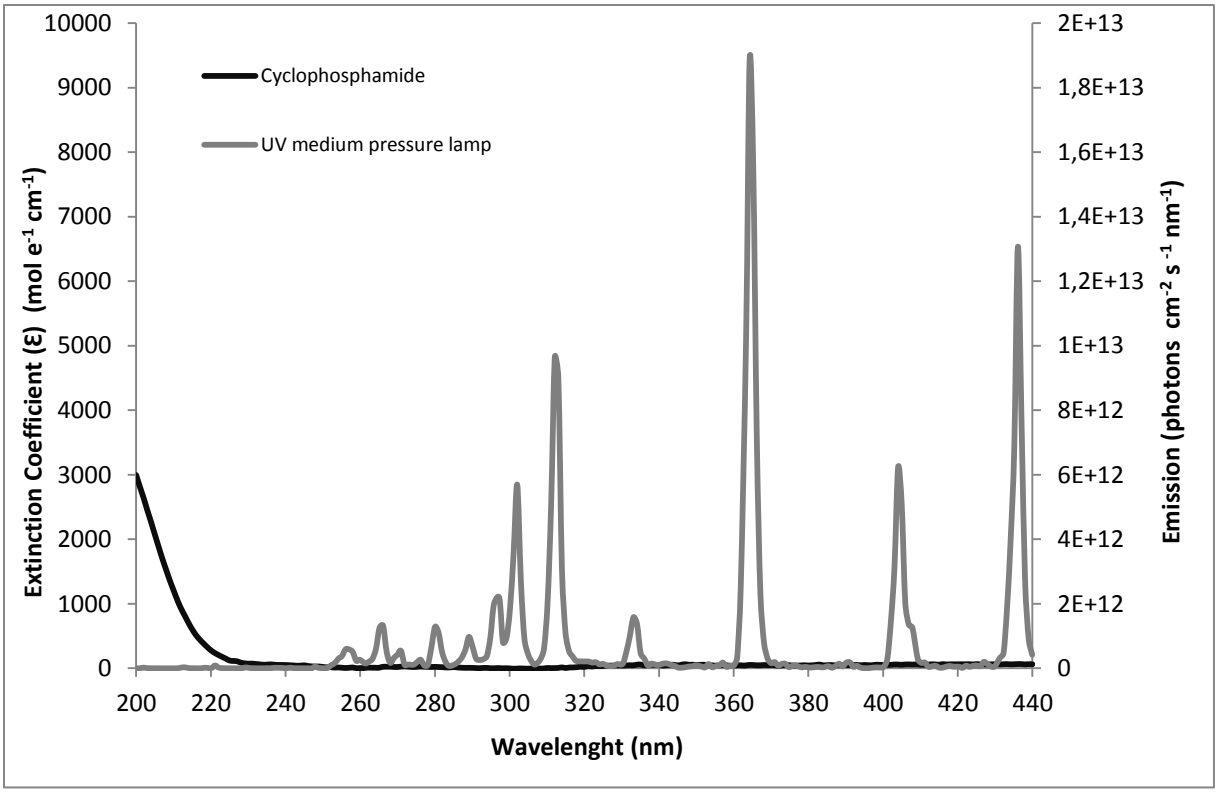


Fig. S1 – Intensity of light emitted by the used UV lamp and molar extinction coefficient of CP for the range of 200 nm to 440 nm. (Concentration of 10 mg/L)

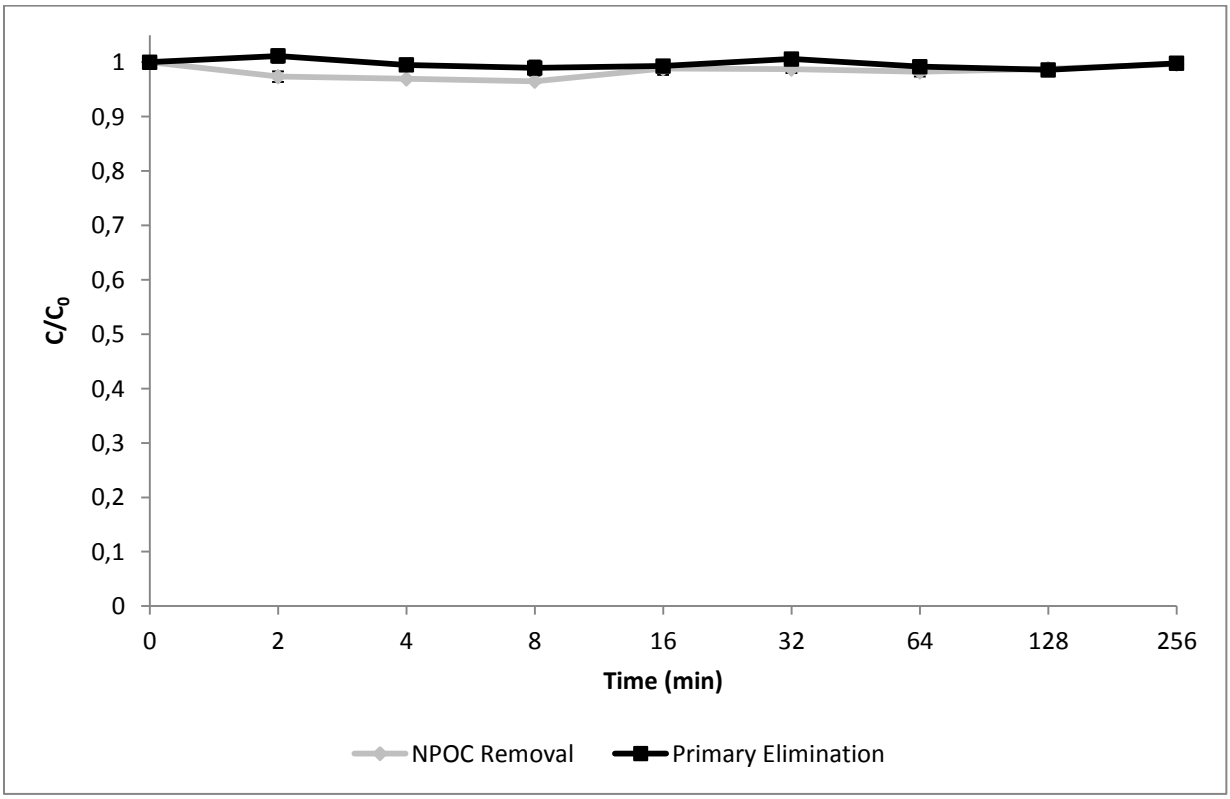


Fig. S2 – UV Photolysis of CP using a medium pressure Hg lamp. (Concentration of CP 20 mg/L)

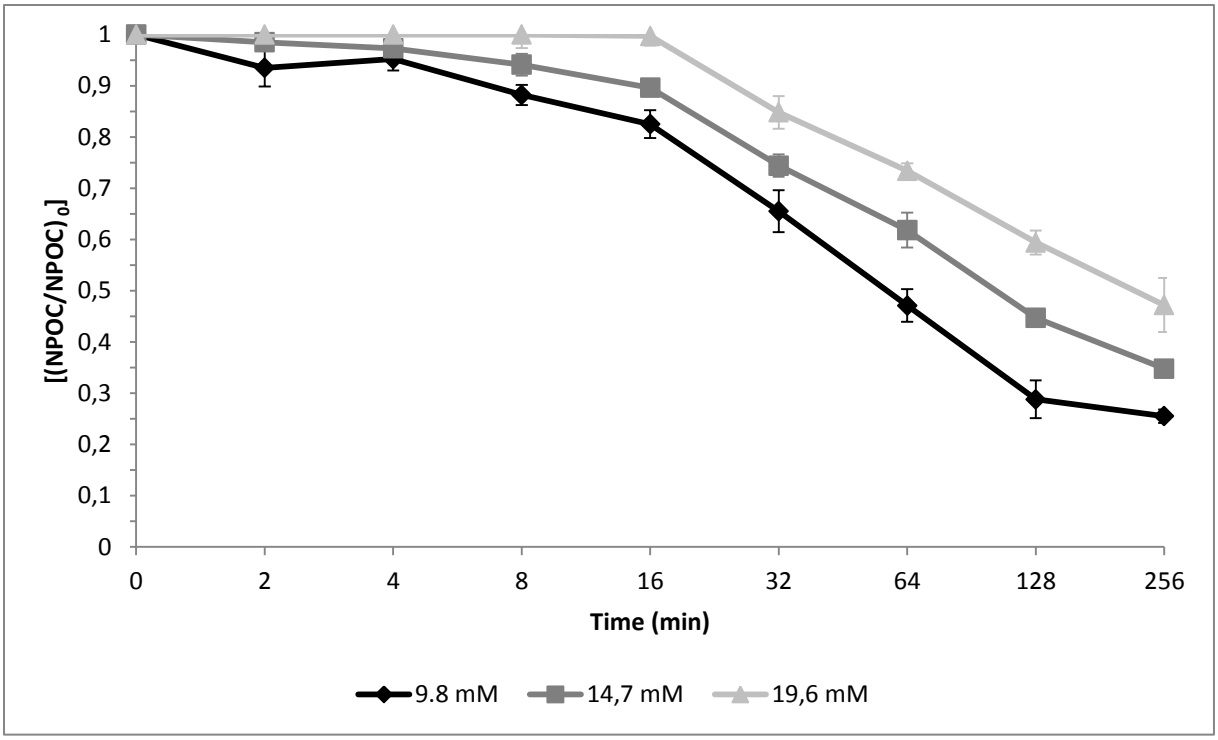


Fig. S3 - Effect of the H_2O_2 concentration on the mineralization rates of Cyclophosphamide. (Concentration of CP 20 mg/L)

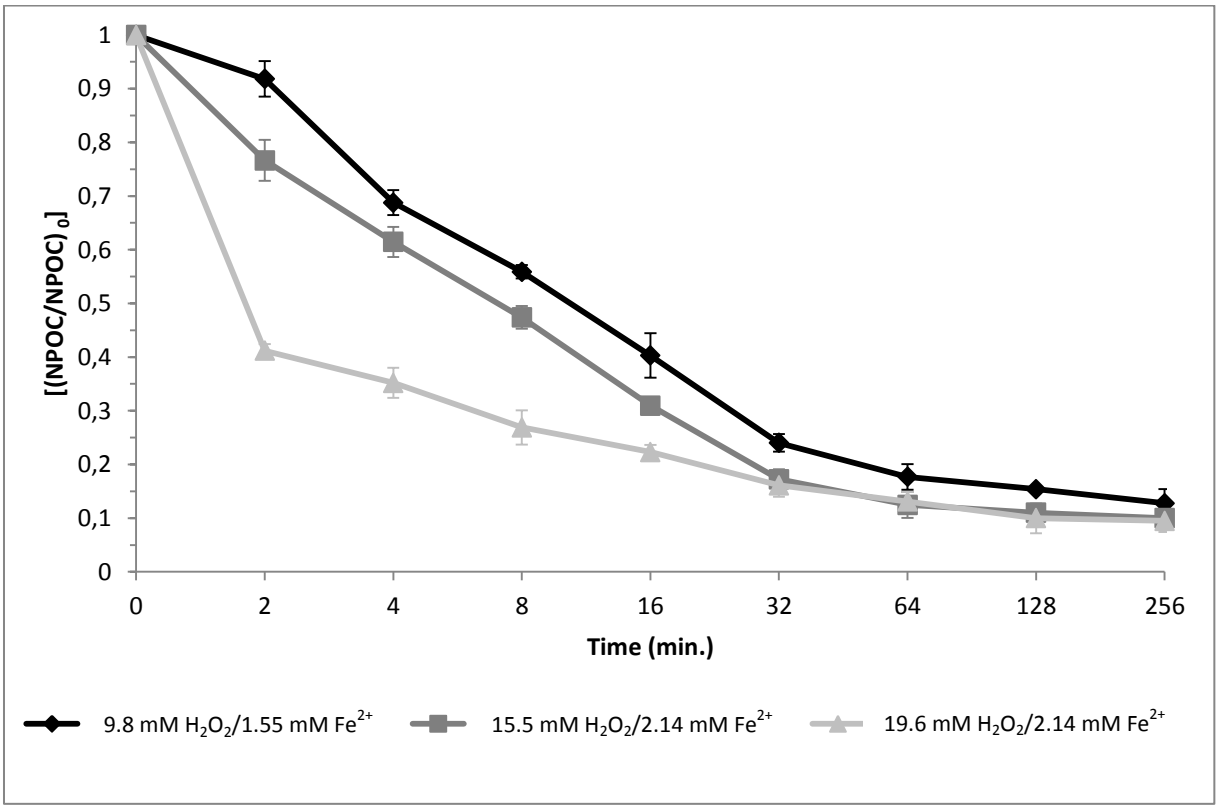


Fig .S4 - Mineralization of CP under UV/Fe^{2+}/H_2O_2, with different concentrations of H_2O_2 and Fe^{2+}. (Concentration of CP 20 mg/L)

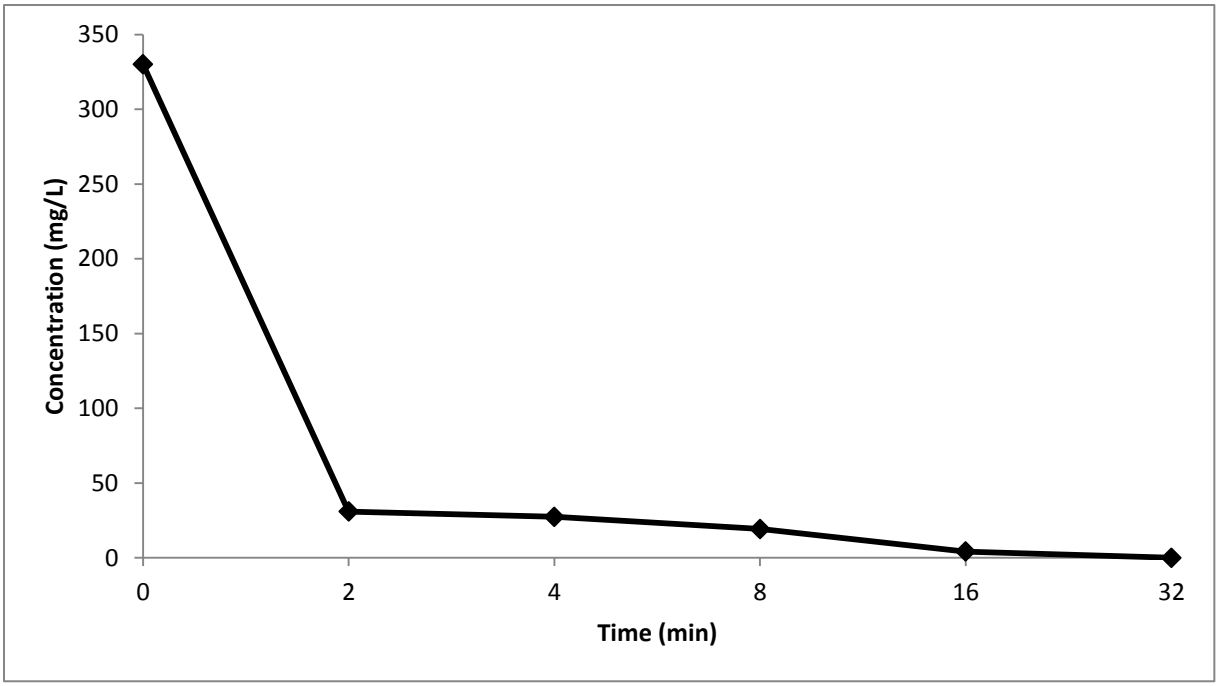


Fig. S5 – Consumption of H_2O_2 during the photo-Fenton treatment.

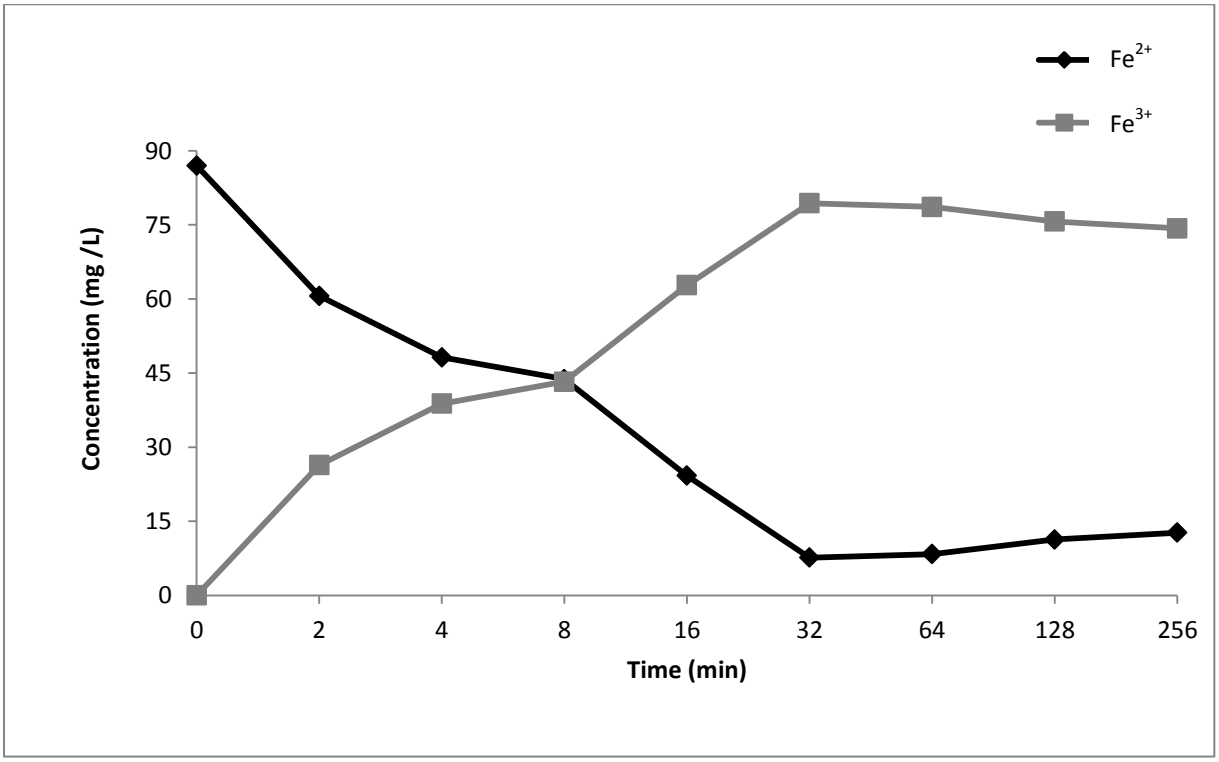


Fig. S6 – Consumption of ferric and ferrous ions during the UV/Fe^{2+}/H_2O_2 reactions.

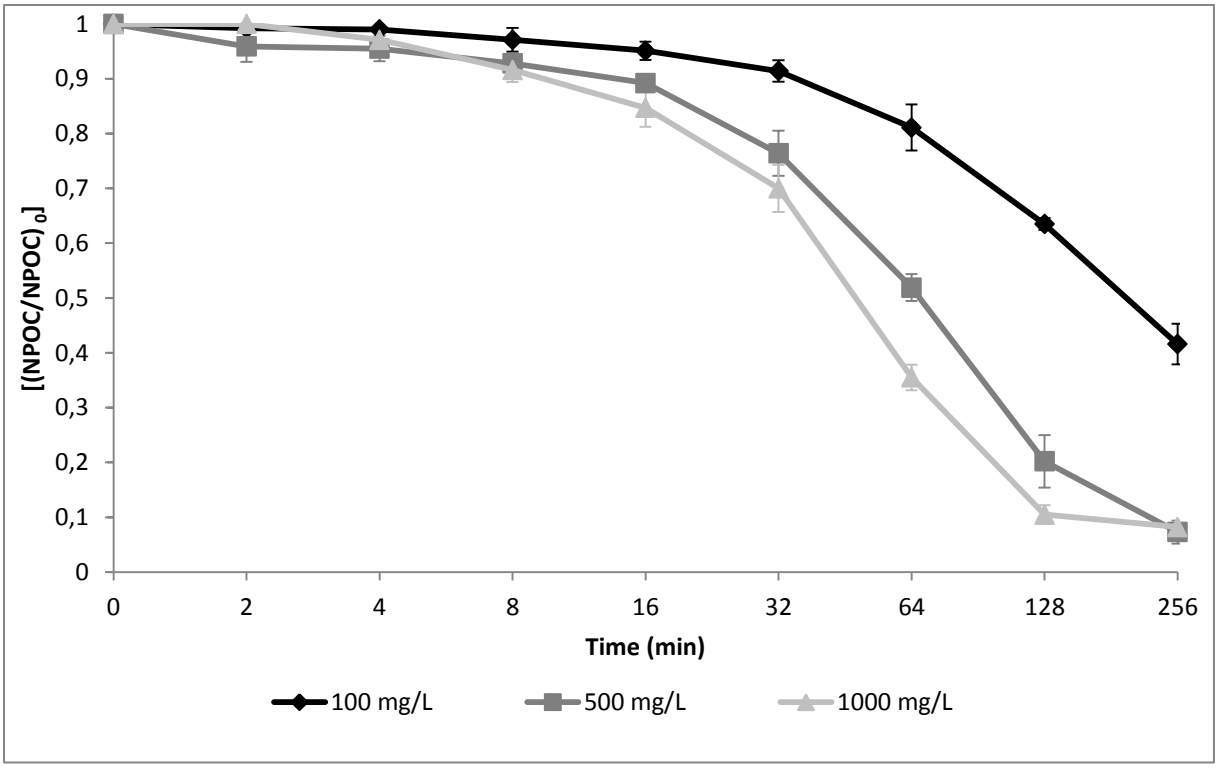


Fig. S7- Effect of catalyst loading on the mineralization rates of Cyclophosphamide. (Concentration of CP 20 mg/L)

Table S1 – Mineralization rate constant of the three processes using different catalyst concentrations.

Process	**Catalyst Concentrations**		
UV/H_2O_2 (K')	**9.8 mM (H_2O_2)**	**15.5 mM (H_2O_2)**	**19.6 mM (H_2O_2)**
	0.01826 min^{-1}	0.01361 min^{-1}	0.009551 min^{-1}
$UV/Fe^{2+}/H_2O_2$ (K')	**9.8 mM/1.55 mM (H_2O_2/Fe^{2+})**	**15.5 mM/2.14 mM (H_2O_2/Fe^{2+})**	**19.6 mM/2.14 mM (H_2O_2/Fe^{2+})**
	0.08610 min^{-1}	0.1113 min^{-1}	0.4670 min^{-1}
UV/TiO_2 (K')	**100 mg (TiO_2)**	**500 mg (TiO_2)**	**1000 mg (TiO_2)**
	0.003305 min^{-1}	0.01005 min^{-1}	0.01575 min^{-1}

Table S2 – Apparent degradation rate constants of cyclophosphamide under UV/H_2O_2 and UV/TiO_2 and corresponding $t_{0.5}$.

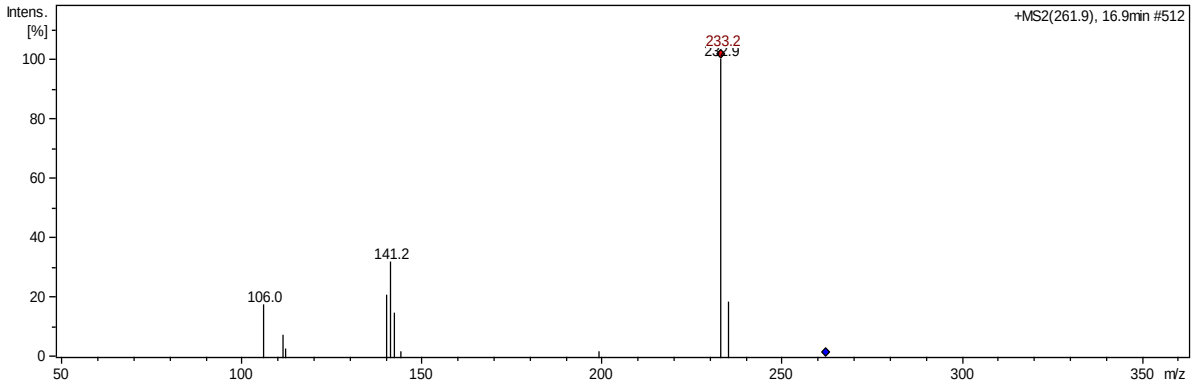
Process	**Time point (s)**	**k (s^{-1})**	**R^2**	**$t_{1/2}$ (s)**
UV/H_2O_2	480	0.0103	0.99	67.3
UV/TiO_2	1920	0.0024	0.97	288.8

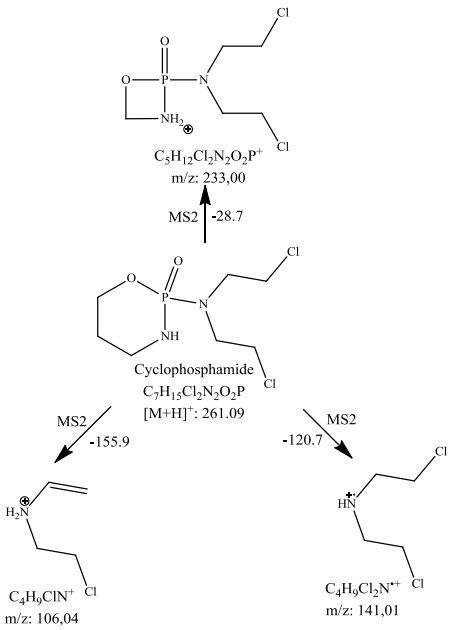
Table S3 – Mass spectrometer parameters for CP and its photolysis TPs in LC-UV–MS/MS using gradient method (positive mode; RT, retention time; m/z)

Compound	RT (min)	Precursor ions (m/z)	Product ions (m/z)
Cyclophosphamide	16.9	261.9	233 (100), 141 (33.7), 106 (18.3)
TP1	15.2	276.9	221 (100), 259 (20.85), 142 (8.73), 88 (3.1), 281 (4.25)
TP2	19.9	259.1	147 (100), 223 (10.6), 101 (37.1), 175 (7.83) 241 (39.45)
TP3	5.4	199.4	171 (100), 198.9 (50.35), 158 (6,82), 78.3 (100)
TP4	14.9	276	221 (100), 123.9(43.12), 159.9 (23.8), 239.1 (12.25), 142 (5.2)
TP5	12.5	138.1	92 (73.1), 107(58.5), 123 (84.6)

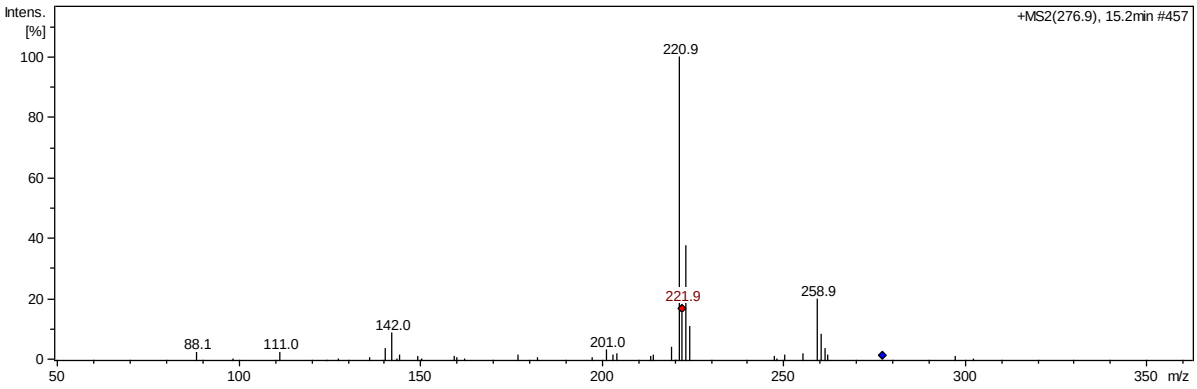
Text Supplementary Material S2: Proposed structures of the fragments ions formed during the MS2 and MS3 fragmentation of CP and the TPs formed during UV/H_2O_2 and UV/TiO_2 reactions.

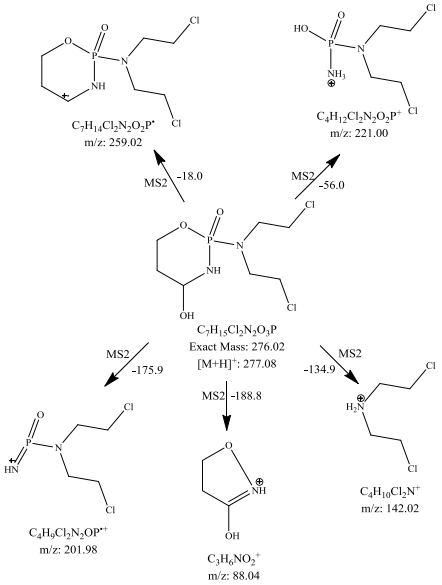
CYCLOPHOSPHAMIDE



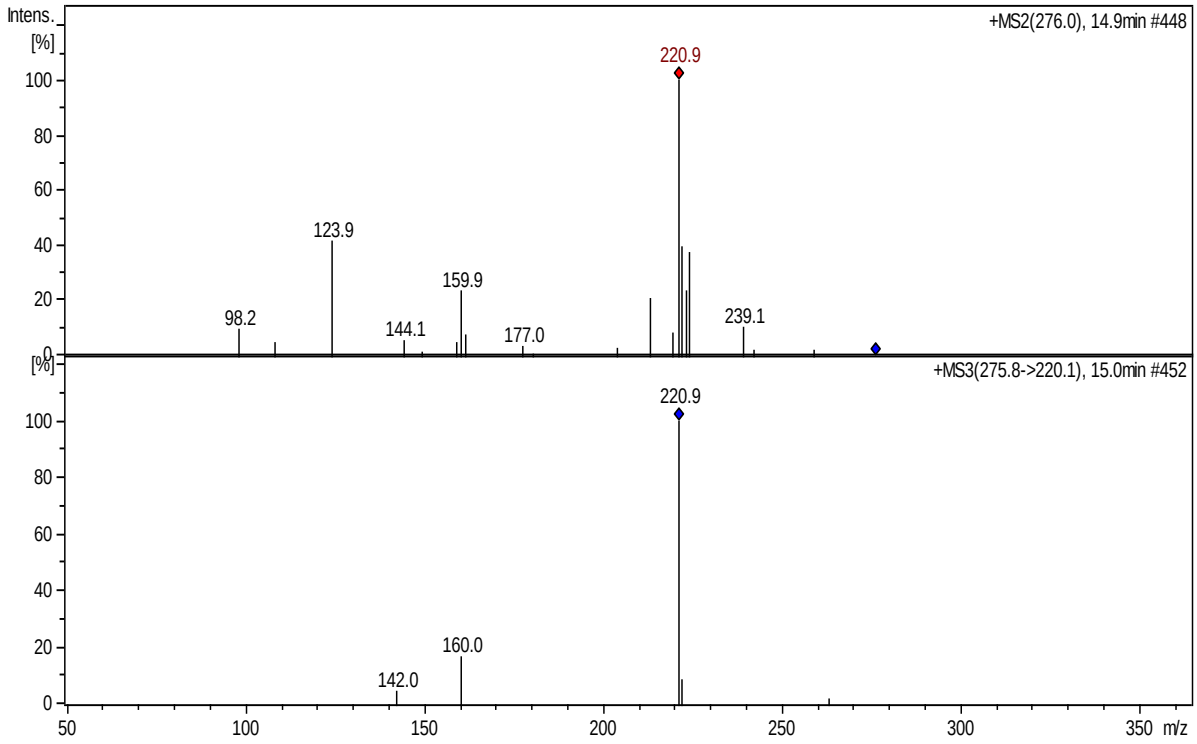


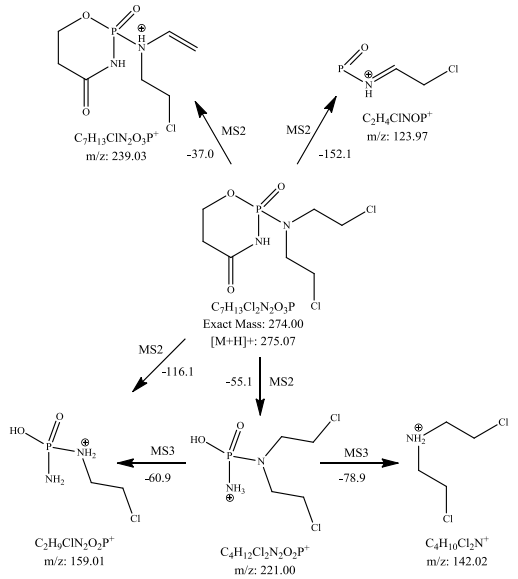
TP 277


Intens.
[%]
+MS2(276.9), 15.2min #457
220.9
221.9
258.9
142.0
88.1
111.0
201.0
m/z

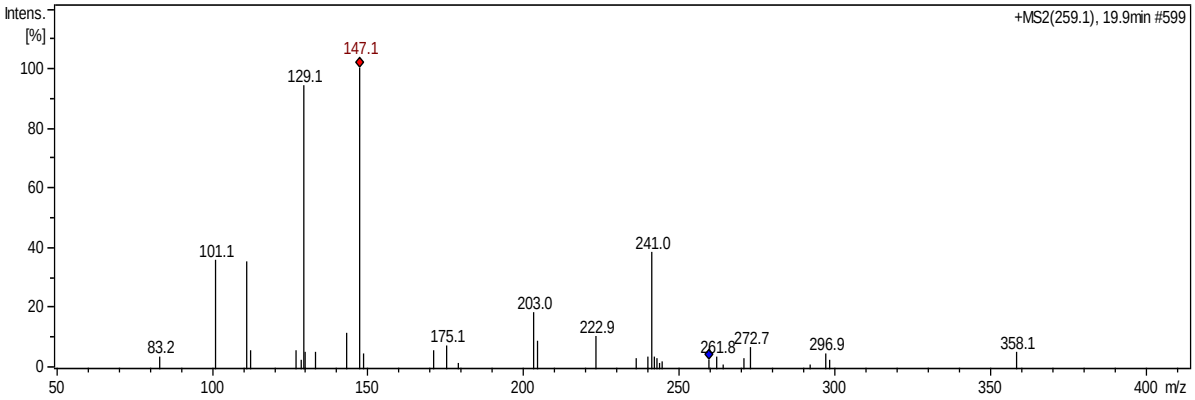

C7H14Cl2N2O2P+
m/z: 259.02
C4H12Cl2N2O2P+
m/z: 221.00
MS2 -18.0
MS2 -56.0
C7H13Cl2N2O3P
Exact Mass: 276.02
[M+H]+: 277.08
MS2 -175.9
MS2 -188.8
MS2 -134.9
C4H9Cl2N2OP+
m/z: 201.98
C3H6NO2+
m/z: 88.04
C4H10Cl2N+
m/z: 142.02

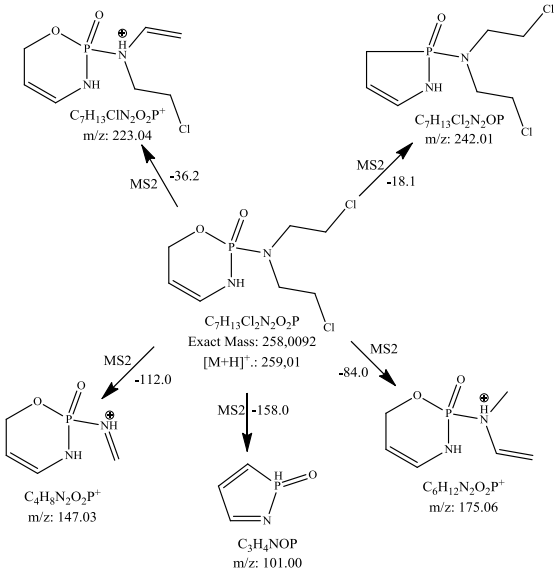
TP 275


Intens.
[%]
+MS2(276.0), 14.9min #448
220.9
123.9
159.9
98.2
144.1
177.0
239.1
+MS3(275.8->220.1), 15.0min #452
220.9
160.0
142.0
m/z

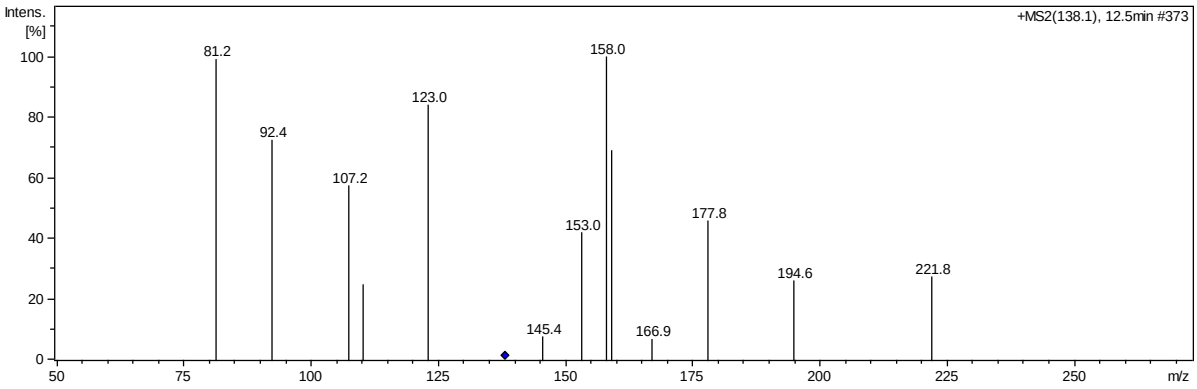

C7H13ClN2O3P+
m/z: 239.03
MS2
-37.0
MS2
-152.1
C2H4ClNOP+
m/z: 123.97
C7H13Cl2N2O3P
Exact Mass: 274.00
[M+H]+: 275.07
MS2
-116.1
-55.1
MS2
MS3
-60.9
MS3
-78.9
C2H9ClN2O2P+
m/z: 159.01
C4H12Cl2N2O2P+
m/z: 221.00
C4H10Cl2N+
m/z: 142.02

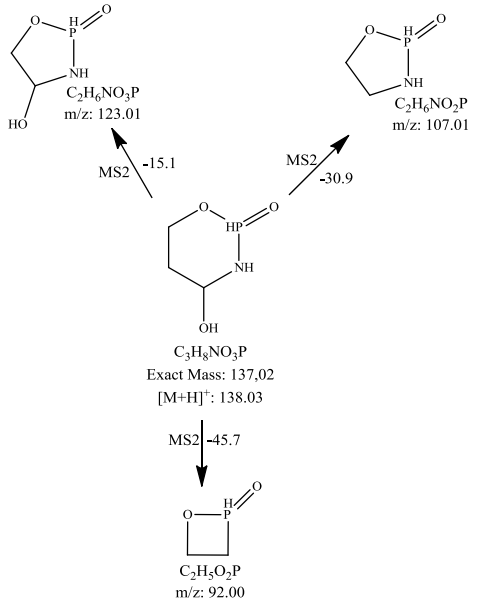
TP 259


+MS2(259.1), 19.9min #599
Intens.
[%]
147.1
129.1
101.1
83.2
175.1
203.0
222.9
241.0
261.8
272.7
296.9
358.1
m/z

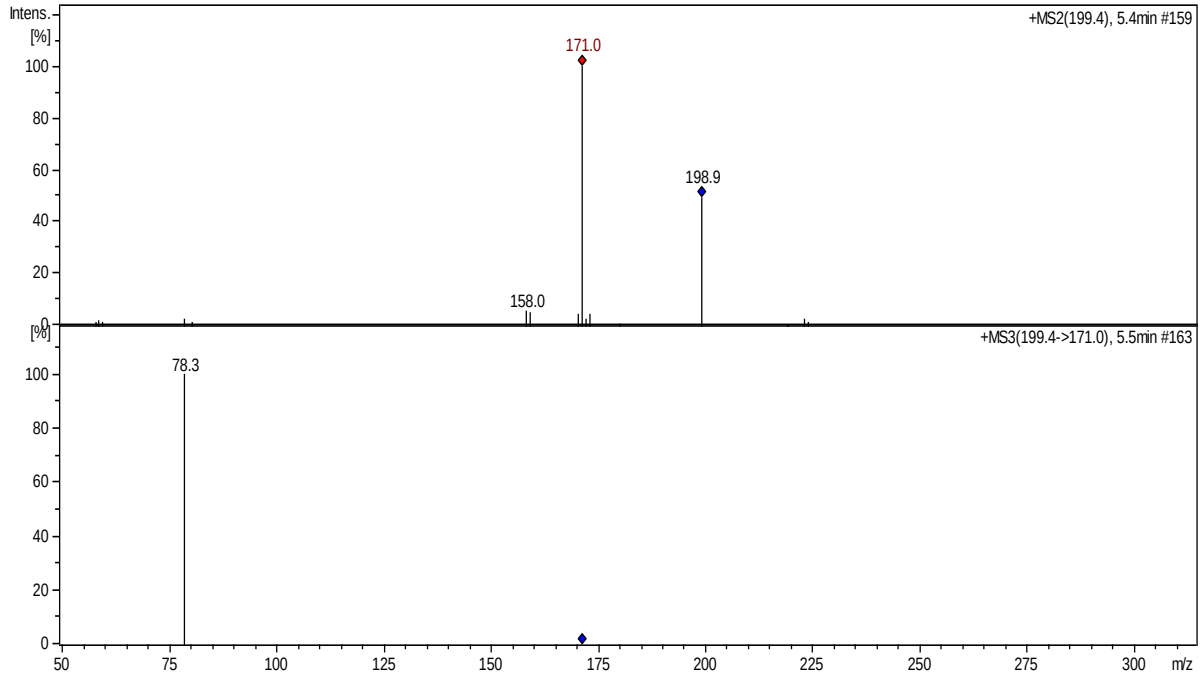

C7H13ClN2O2P+
m/z: 223.04
MS2 -36.2
C7H13Cl2N2OP
m/z: 242.01
MS2 -18.1
C7H13Cl2N2O2P
Exact Mass: 258,0092
[M+H]+.: 259,01
MS2 -112.0
MS2 -158.0
MS2 -84.0
C4H8N2O2P+
m/z: 147.03
C3H4NOP
m/z: 101.00
C6H12N2O2P+
m/z: 175.06

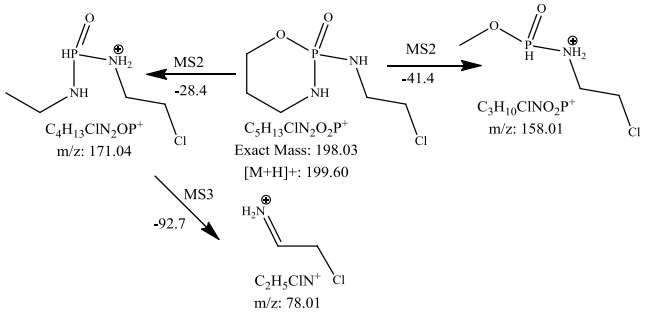
TP 138


Intens.
[%]
+MS2(138.1), 12.5min #373
81.2
92.4
107.2
123.0
145.4
153.0
158.0
166.9
177.8
194.6
221.8
m/z


C2H6NO3P
m/z: 123.01
MS2 -15.1
MS2 -30.9
C2H6NO2P
m/z: 107.01
C3H8NO3P
Exact Mass: 137,02
[M+H]+: 138.03
MS2 -45.7
C2H5O2P
m/z: 92.00

TP 199


Intens.
[%]
+MS2(199.4), 5.4min #159
171.0
198.9
158.0
+MS3(199.4->171.0), 5.5min #163
78.3
m/z


MS2
-28.4
MS2
-41.4
MS3
-92.7
$C_4H_{13}ClN_2OP^+$
m/z: 171.04
$C_5H_{13}ClN_2O_2P^+$
Exact Mass: 198.03
[M+H]+: 199.60
$C_3H_{10}ClNO_2P^+$
m/z: 158.01
$C_2H_5ClN^+$
m/z: 78.01

Paper 2

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 ($HO_2^{\cdot}$) 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):

$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + HO_2^{\cdot} \quad (1)$$

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.

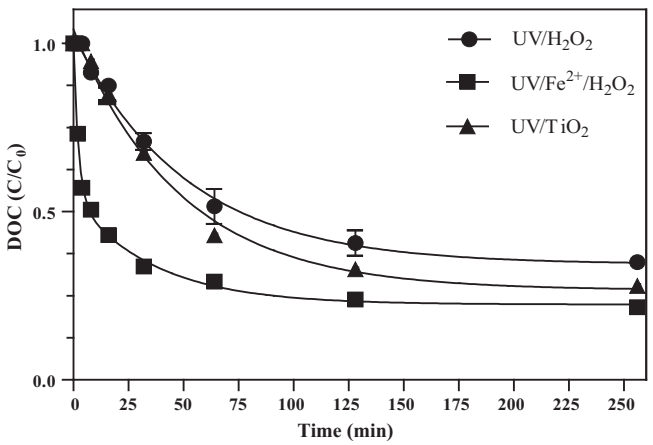

Fig. 1. Kinetics of the mineralization of MTX under the three different treatment reactions. Values are the means ± SD from two independent experiments. Initial pH values of the UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 were 6.4, 5 and 6.4, respectively.

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 ·OH and Fe^{2+} (Augugliaro et al., 2006). The fast oxidation is typical for Fenton reactions being indicative of a direct and fast ·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

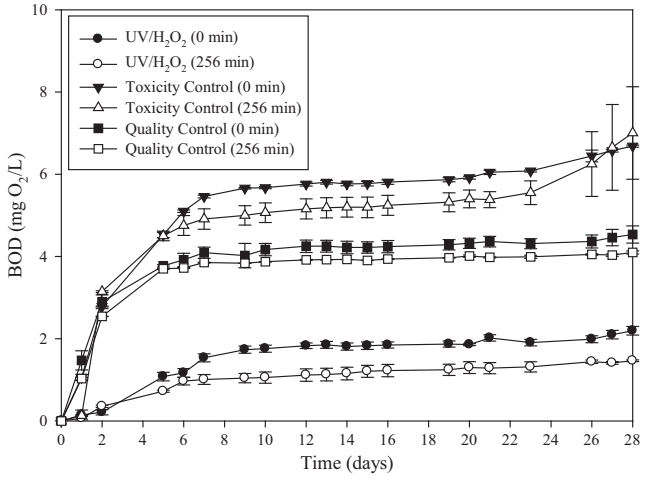

Fig. 2. Degradation in Closed Bottle Test of methotrexate (0 min) and of the photodegradation mixture (256 min of irradiation).

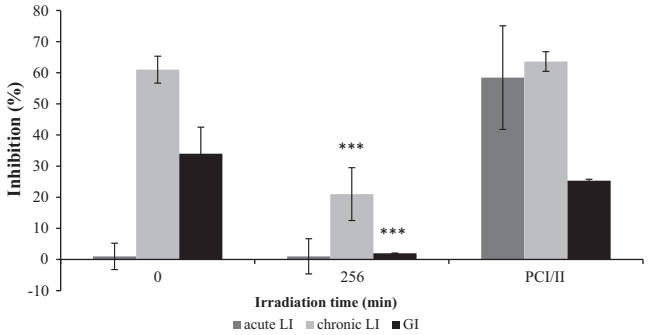

Fig. 3. Toxicity tests with *V. fischeri* before (0 min) and after (256 min) the UV/H_2O_2 treatment ($n = 2$) considering three endpoints: acute Luminescence inhibition after 30 min (acute LI), chronic Luminescence inhibition after 24 h (chronic LI) and Growth inhibition after 14 h (GI) Positive control 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

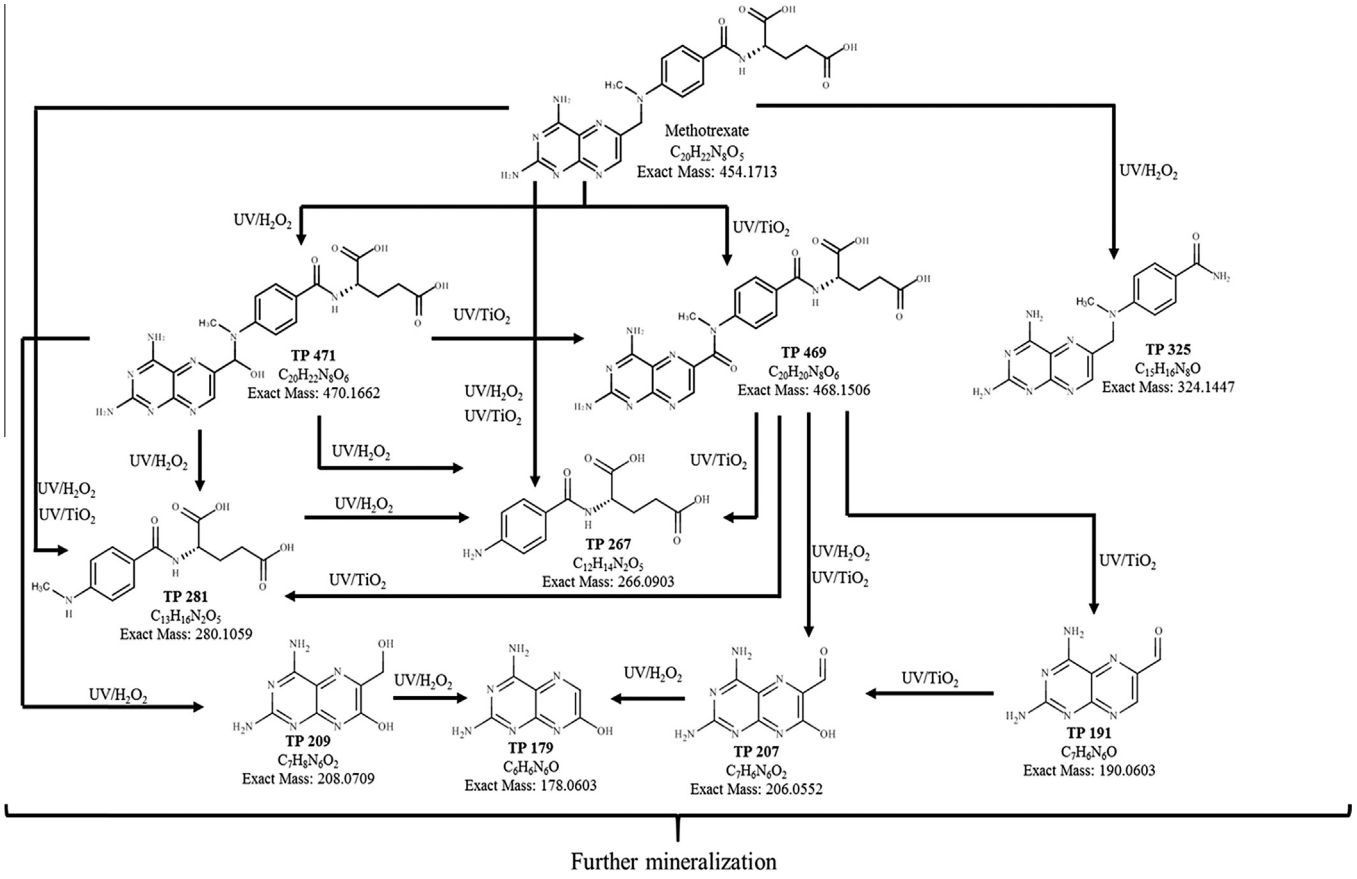

Fig. 4. Proposed degradation pathway of MTX by means of the UV/H_2O_2 and UV/TiO_2 processes.

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-diaminopteridine-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

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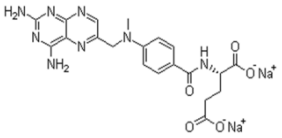
Table S1 – Structure and main physico-chemical properties of methotrexate.

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).

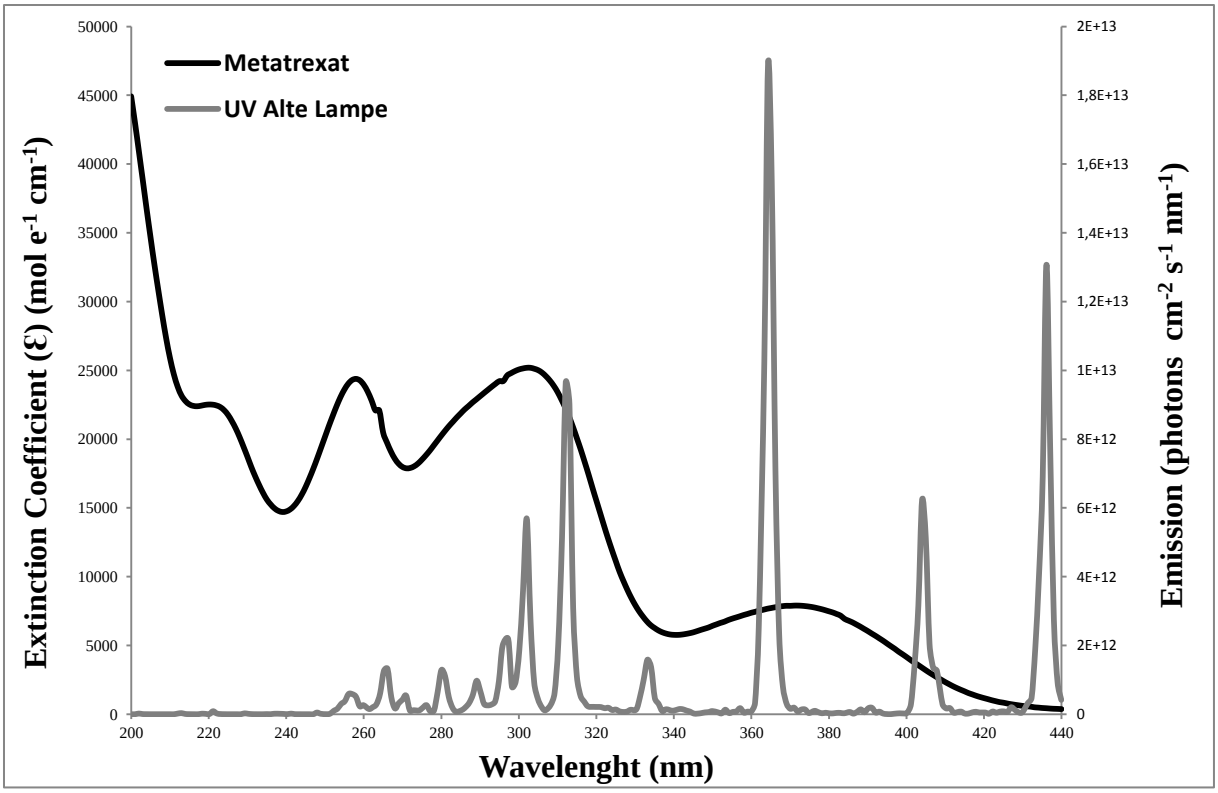


Fig. S1 – Intensity of light emitted by the used UV lamp and molar extinction coefficient of MTX for the range of 200 nm to 440 nm. (Concentration of 10 mg/L)

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:

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

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 = 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:

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

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

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

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 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		+		+

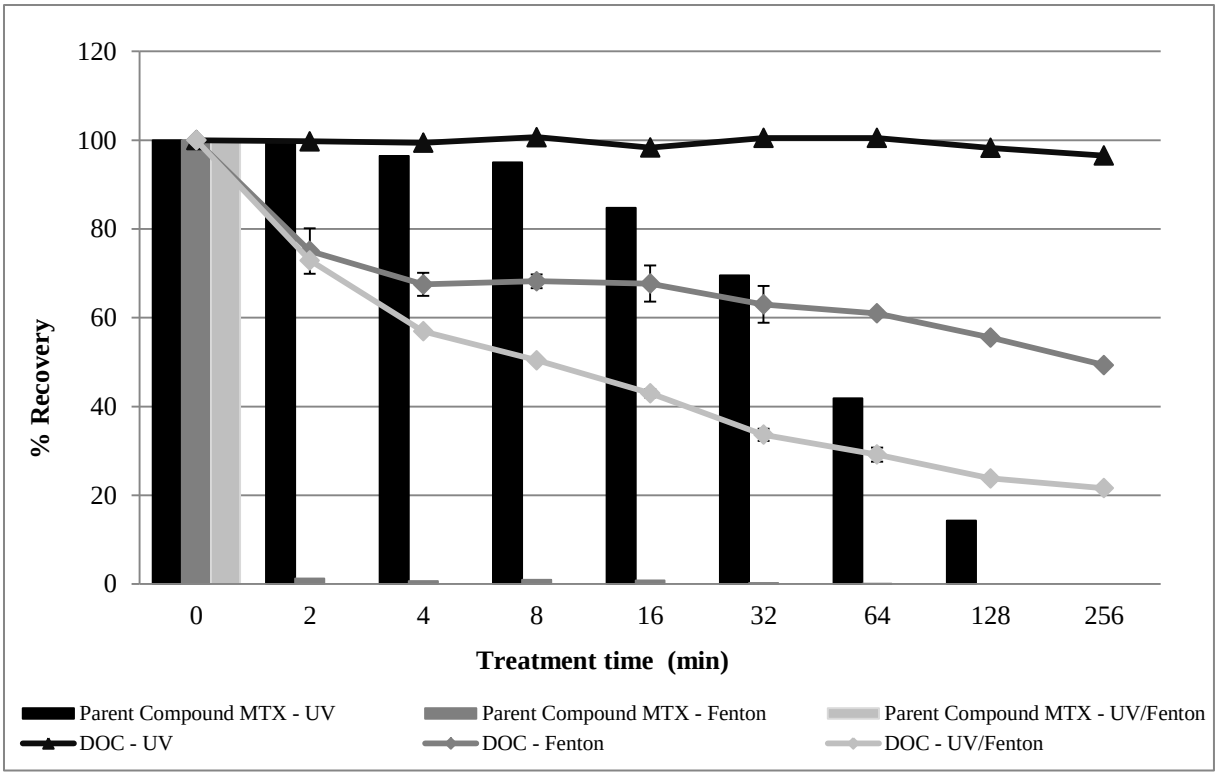


Fig. S2 – Degradation and mineralization rates of MTX and its photo-transformations products through UV, Fenton and UV/Fenton treatments.

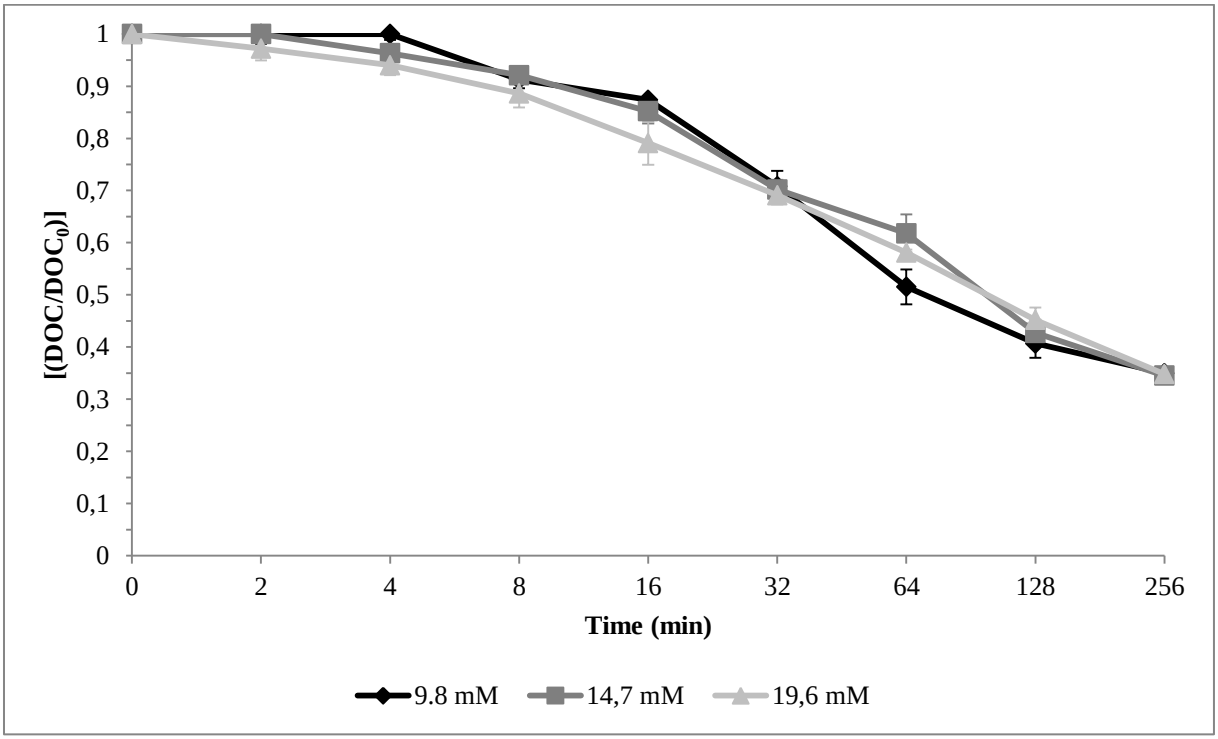


Fig. S3 - Effect of the H_2O_2 concentration on the mineralization degrees of MTX

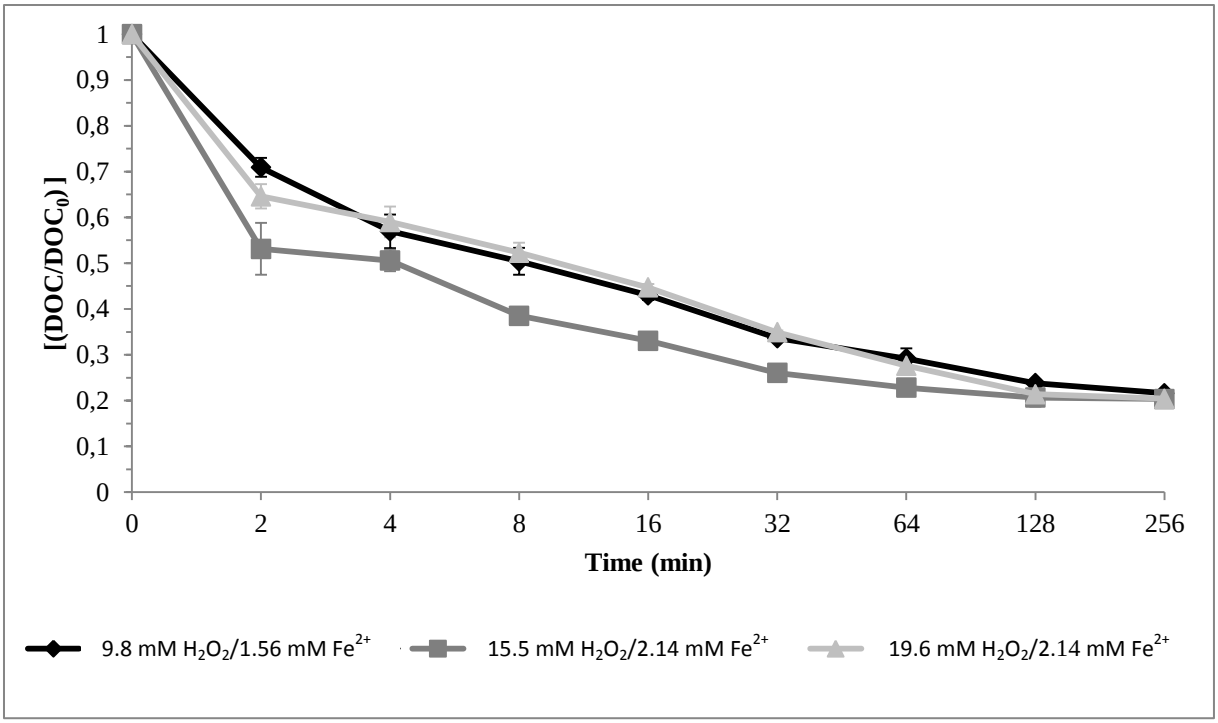


Fig S4 - Mineralization of MTX under UV/Fe^{2+}/H_2O_2, with different concentrations of H_2O_2 and Fe^{2+}

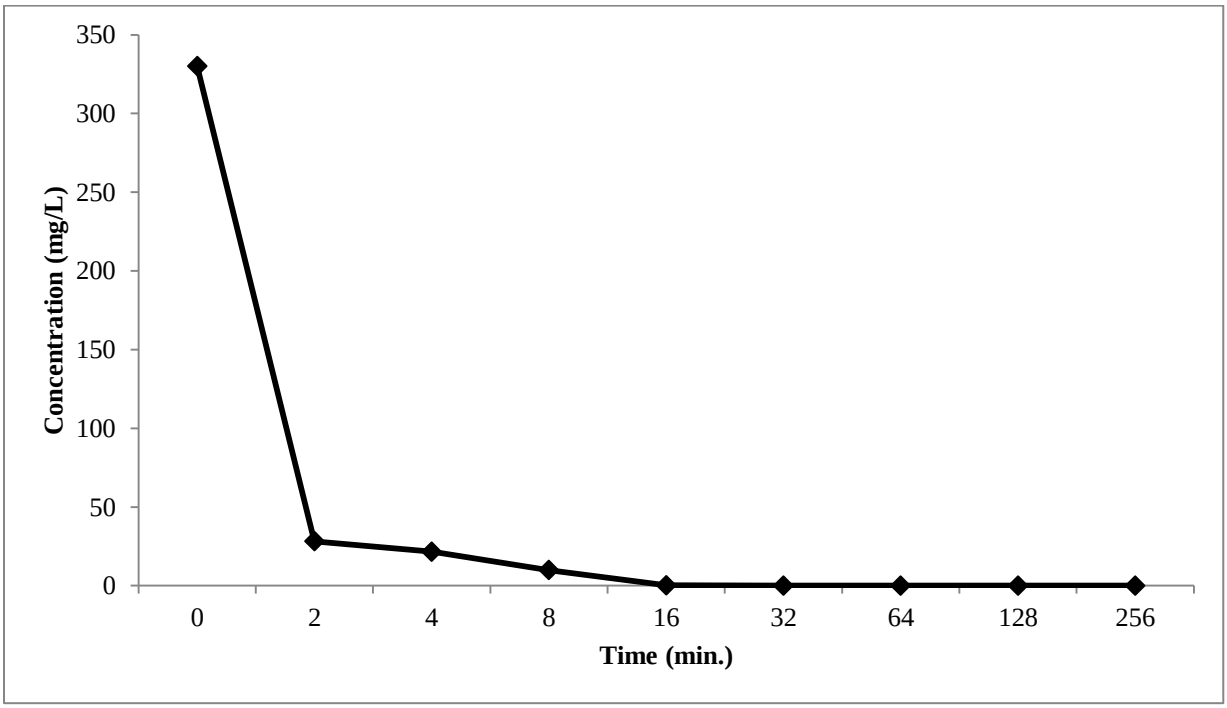


Fig. S5 – Consumption of H_2O_2 during the photo-Fenton treatment.

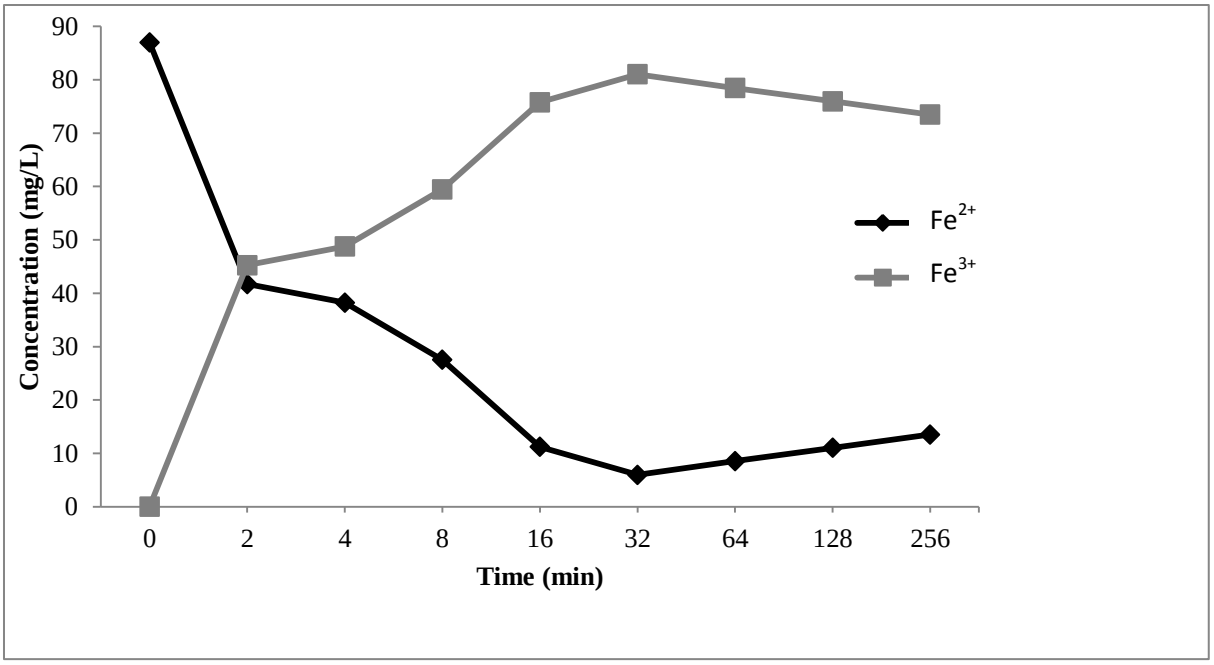


Fig. S6 – Consumption of ferric and ferrous ions during the UV/Fe^{2+}/H_2O_2

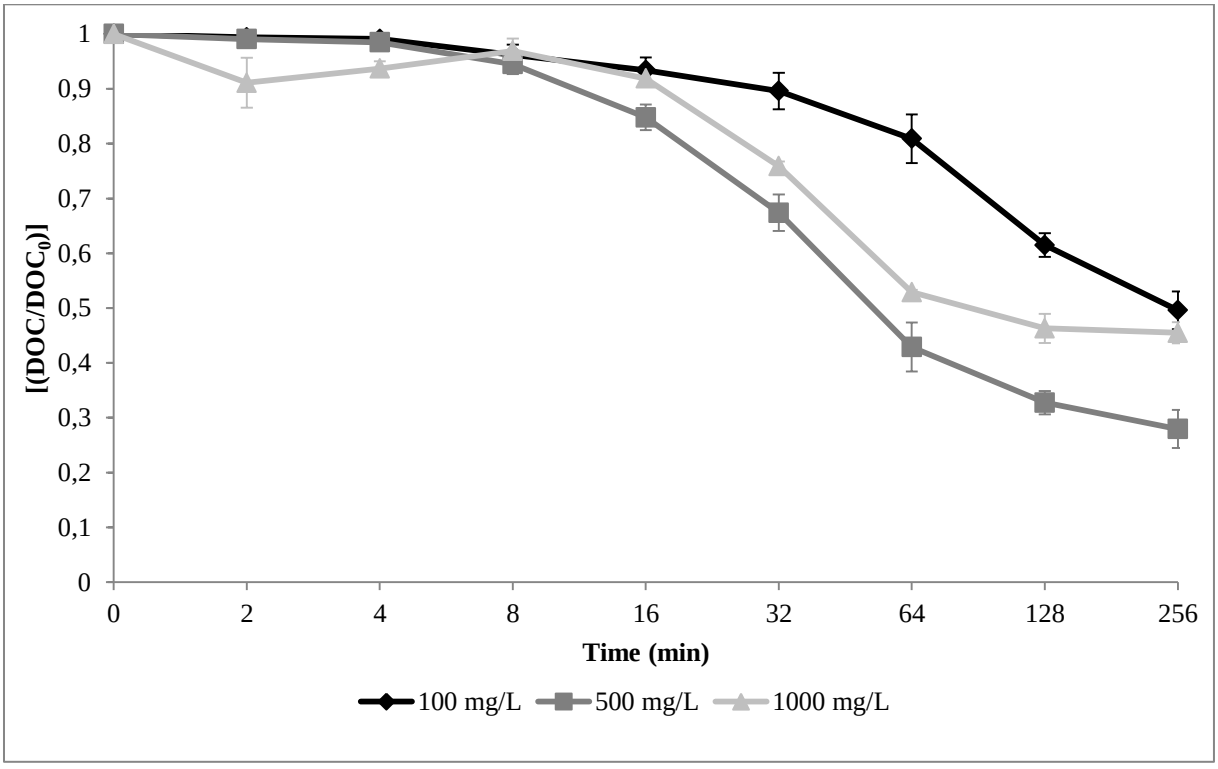


Fig. S7- Effect of catalyst loading on the mineralization degrees of MTX

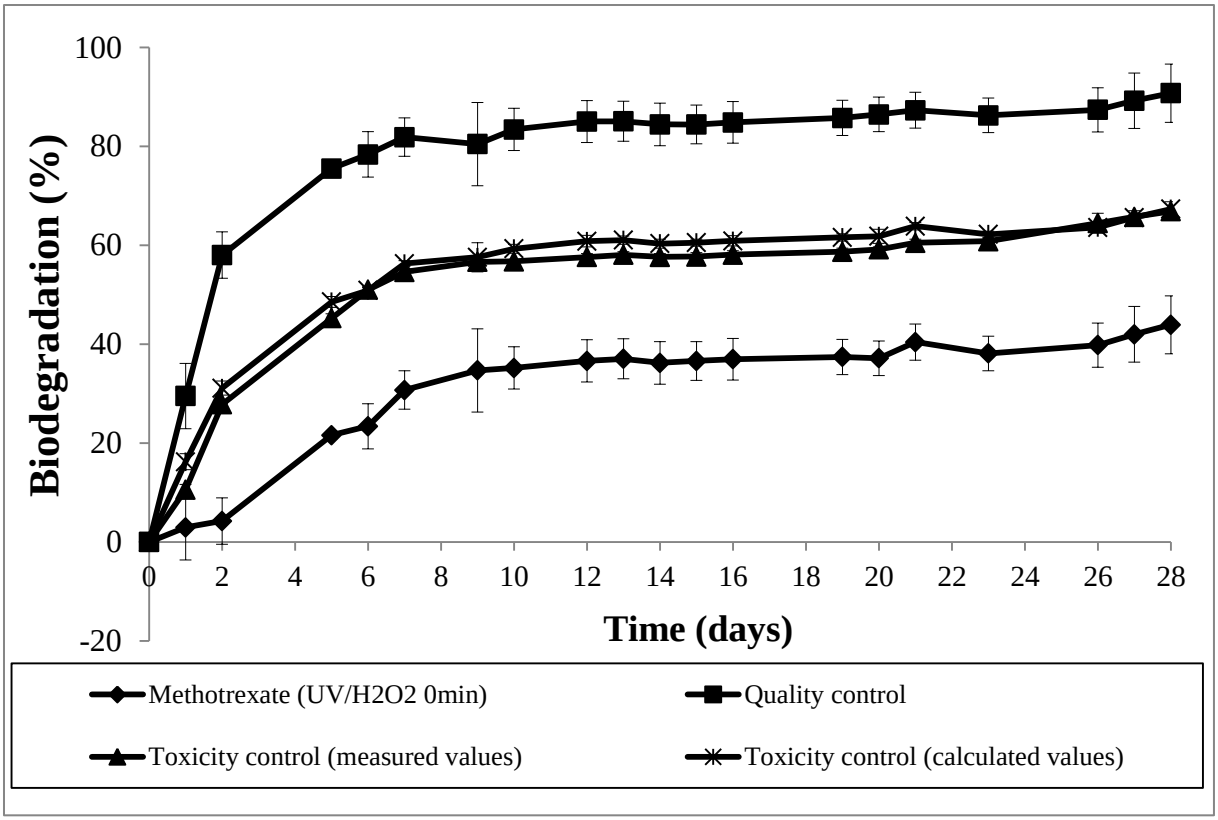


Fig S8– Closed Bottle Test (CBT) of MTX (n=2)

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

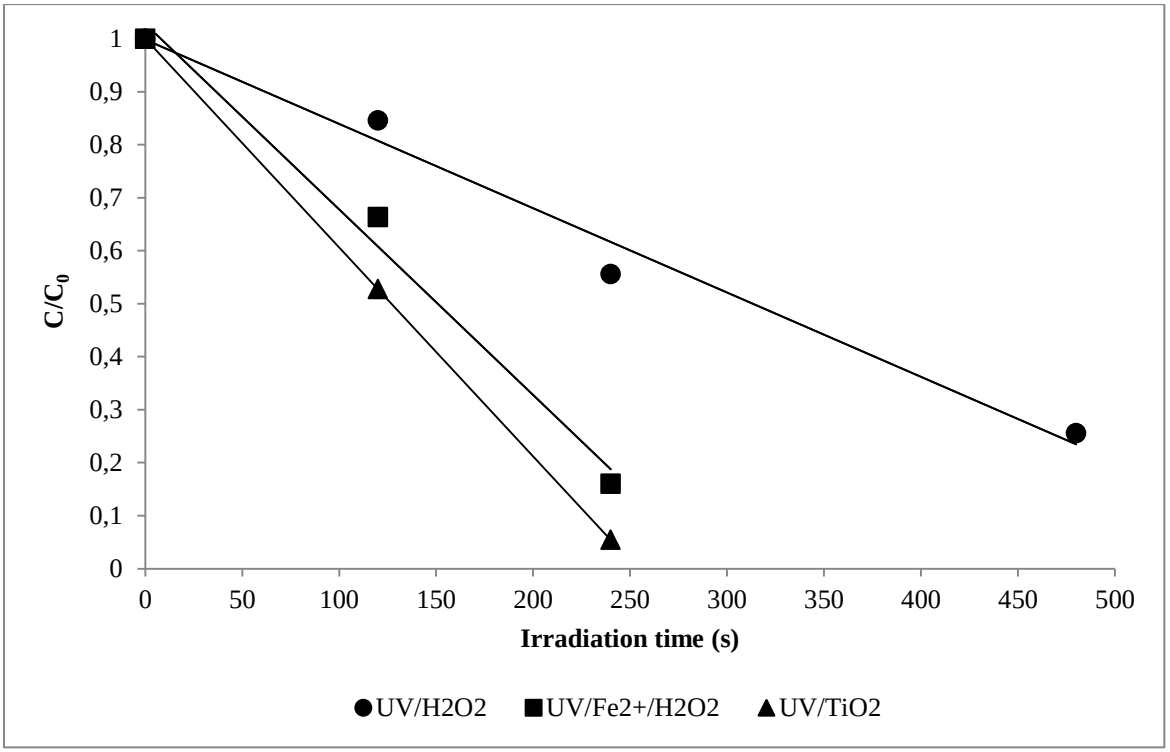


Fig. S9 – Kinetics of the primary elimination of MTX by UV/H_2O_2, $UV/ Fe^{2+}/H_2O_2$ and UV/ TiO_2.

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

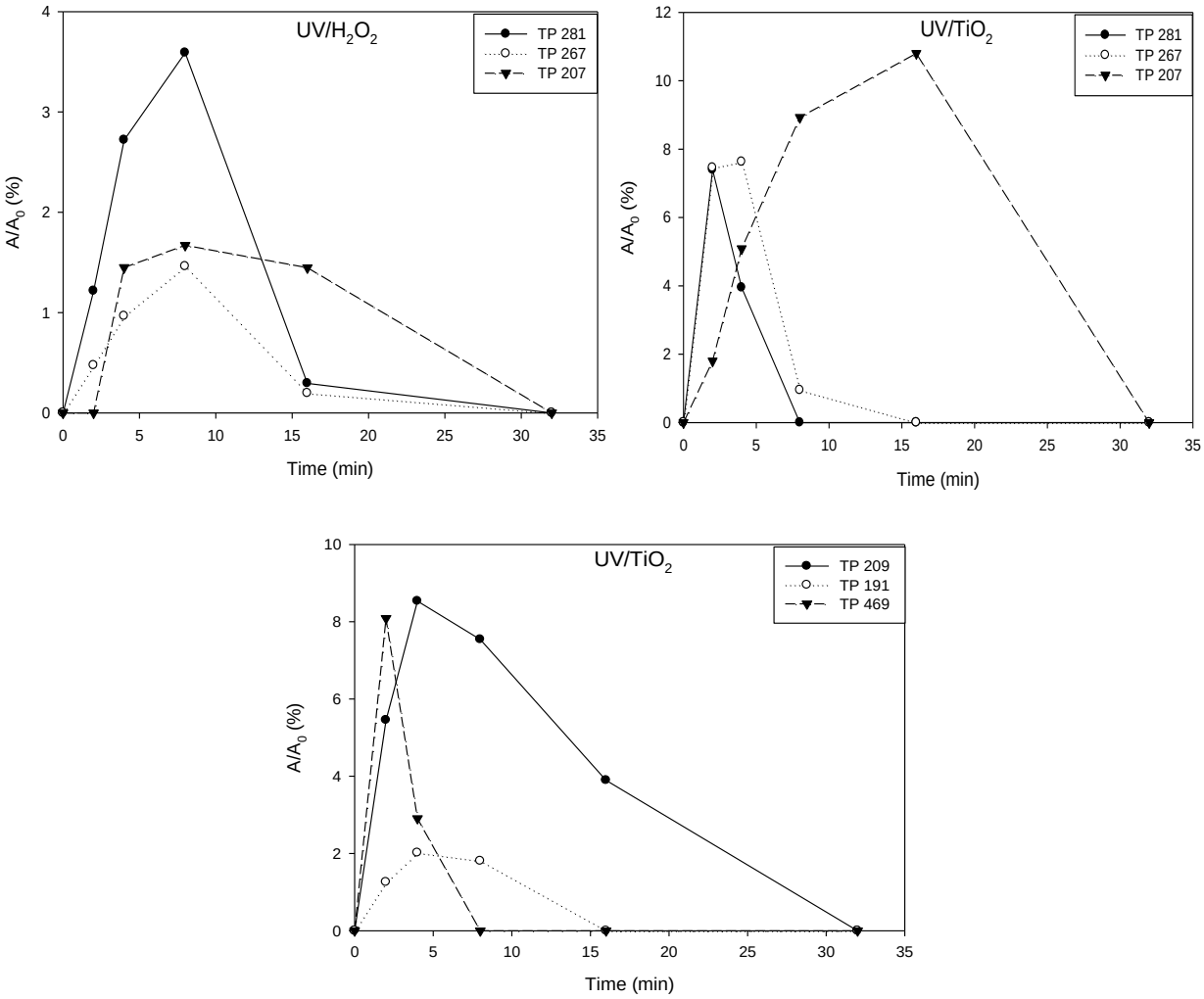
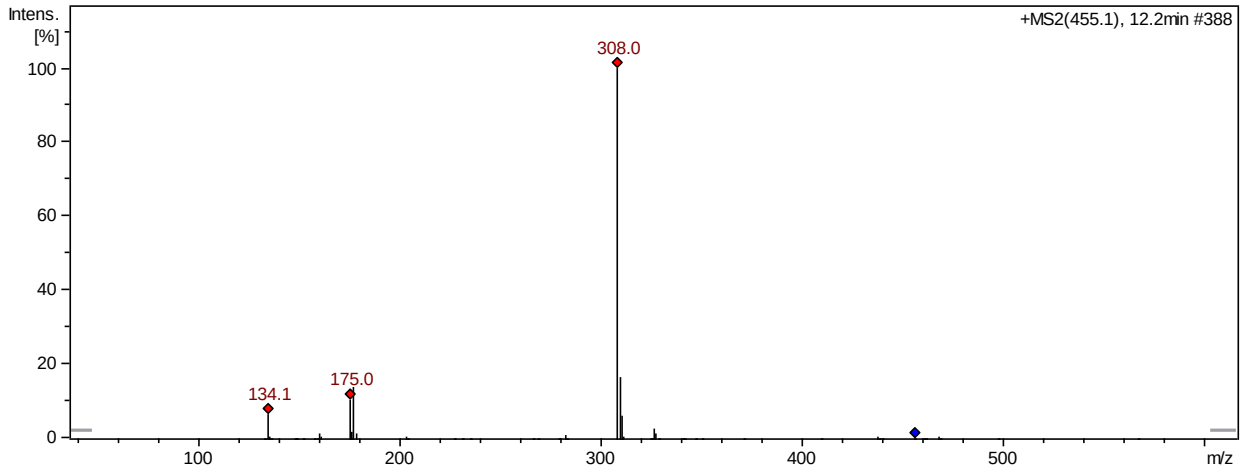
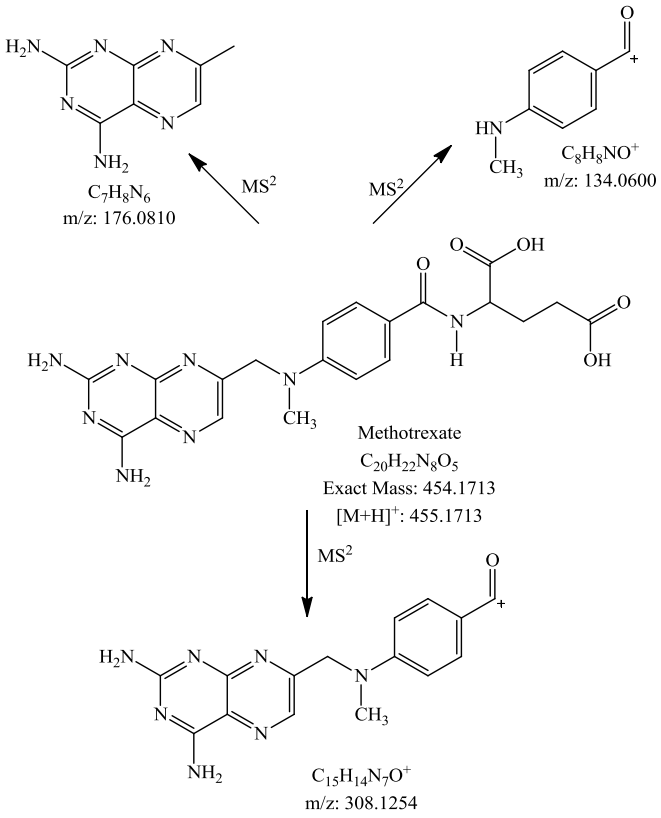


Fig. S10 - Profile of the peak area ratio (A/A_0) of the identified TPs formed during the UV/H_2O_2 and UV/TiO_2 processes.

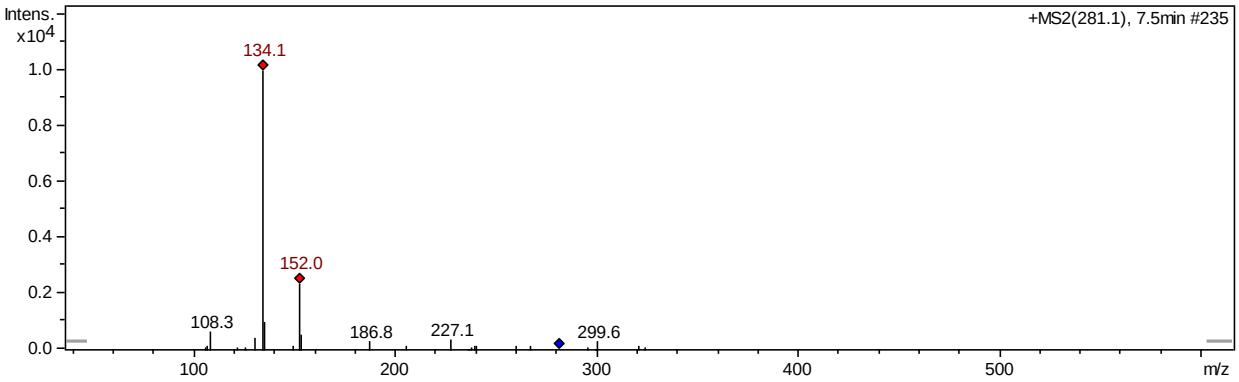
Text S4 – Identification and elucidation of the TPs from MTX by means of LC-MS/MS.

MTX





TP 281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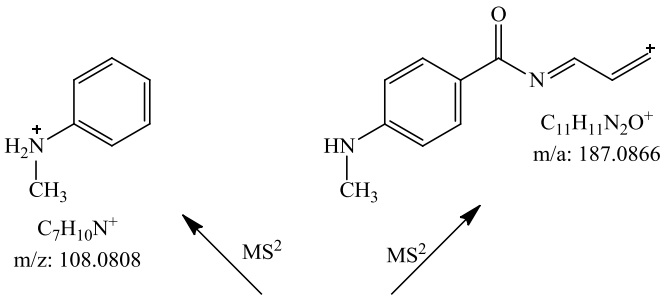
x10^4
+MS2(281.1), 7.5min #235
134.1
152.0
108.3
186.8
227.1
299.6
m/z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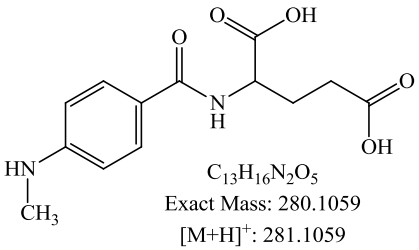
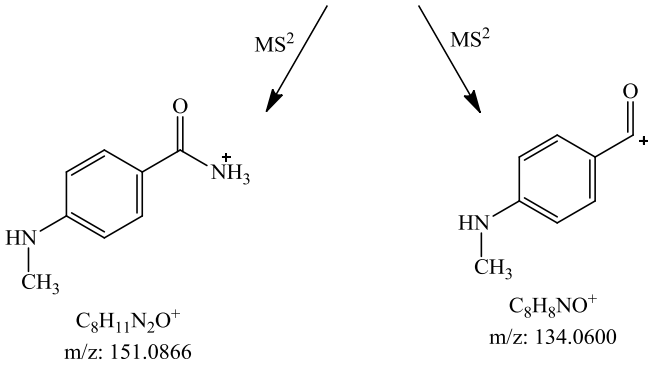
C7H10N+
m/z: 108.0808
MS2
MS2


C13H16N2O5
Exact Mass: 280.1059
[M+H]+: 281.1059


MS2
MS2
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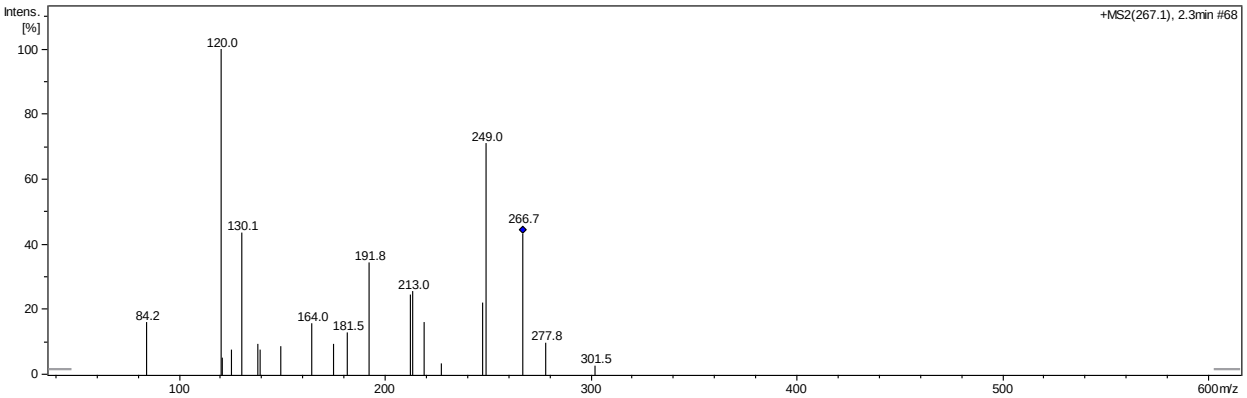
m/z: 151.0866
C8H8NO+
m/z: 134.0600

TP 267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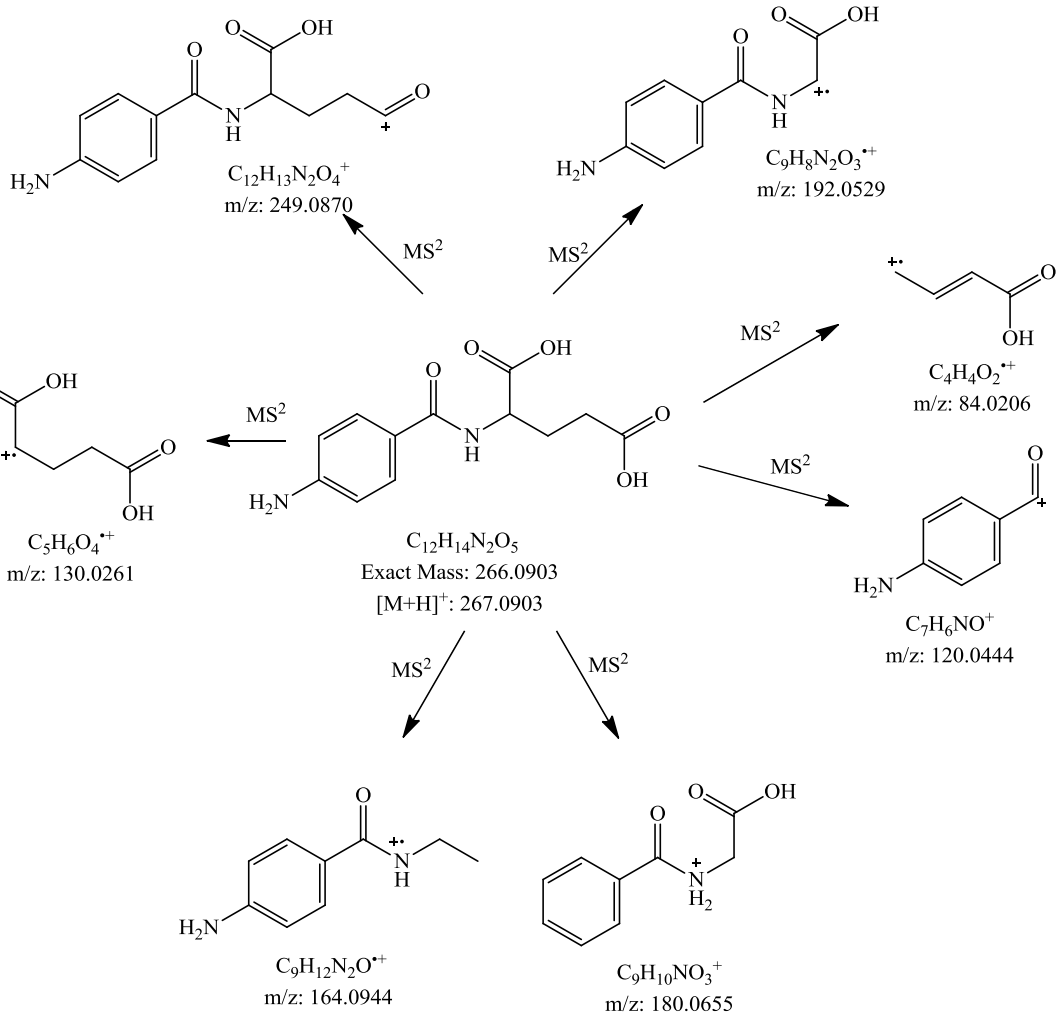
[%]
+MS2(267.1), 2.3min #68
120.0
249.0
130.1
266.7
191.8
213.0
84.2
164.0
181.5
277.8
301.5
100
80
60
40
20
0
100
200
300
400
500
600 m/z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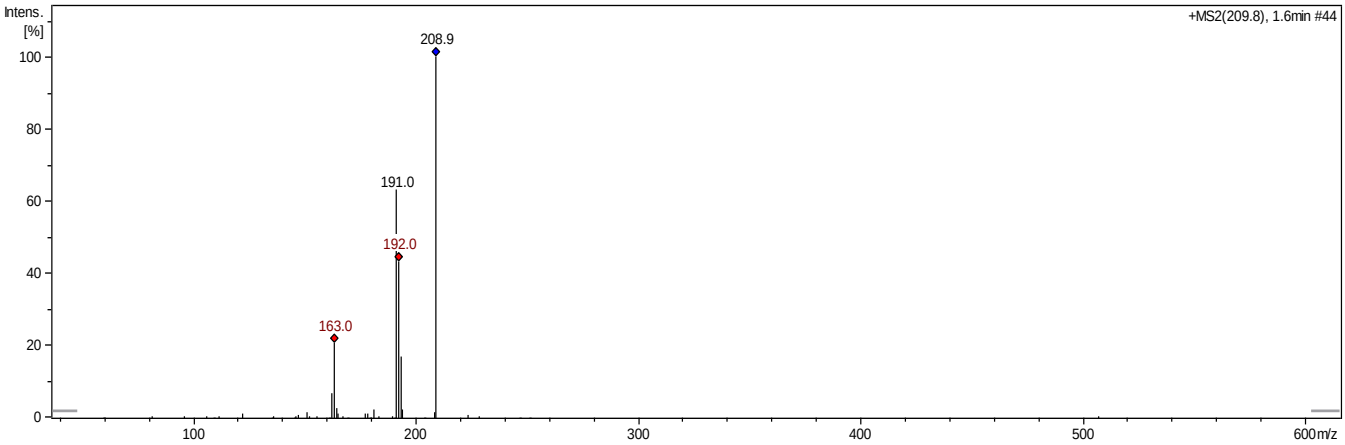
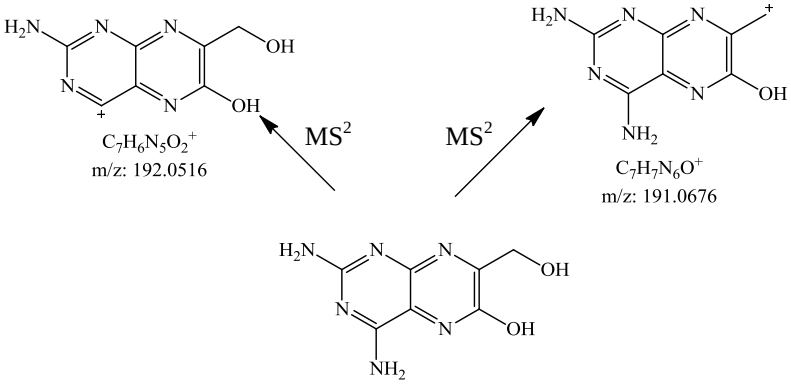
m/z: 249.0870
C9H8N2O3•+
m/z: 192.0529
MS2
MS2
C4H4O2•+
m/z: 84.0206
C5H6O4•+
m/z: 130.0261
C12H14N2O5
Exact Mass: 266.0903
[M+H]+: 267.0903
C7H6NO+
m/z: 120.0444
C9H12N2O•+
m/z: 164.0944
C9H10NO3+
m/z: 180.0655

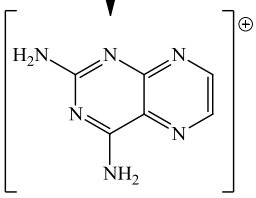
TP 209





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

MS²



$C_6H_7N_6^+$
m/z: 163.0727

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Paper 3

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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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.

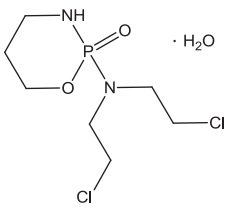
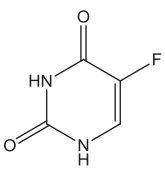
Recently, some researchers have tried to understand the environmental fate of CP and 5-FU with the help of photodegradation, biodegradation and toxicity studies (e.g. Kosjek et al., 2013; Lin et al., 2013; Lin and Lin, 2014). However, to the best of our knowledge, there have been no studies comparing 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 $C_7H_{15}Cl_2N_2O_2P \cdot H_2O$ 6055-19-2		27–272 (Taiwan, 2005) 55 (UK, 2001) 305.73 (France, 2008) 260–288 (Germany, 2010)	 20 >25 11–20 20 15–25 10–40 5–25	Lin et al. (2013) Rowney et al. (2009) Besse et al. (2012) Mompelat et al. (2009) Kümmerer and Al-Ahmad (2010) Kovalova et al. (2009) Zhang et al. (2013) Gómez-Canela et al. (2013)
5-Fluorouracil $C_4H_3FN_2O_2$ 51-21-8		119 (Austria, 1997) 74–297 (Taiwan, 2005) 5593.37 (UK, 2001) 1733.2 (France, 2008)	2–35 7–11 20 5–15 15	Mahnik et al. (2007) Lin et al. (2013) Rowney et al. (2009) Besse et al. (2012) Kovalova et al. (2009) Zhang et al. (2013)

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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):

$$ln\frac{C}{C_0} = -kt \quad (1)$$

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)):

$$t_{1/2} = \ln 2/K \quad (2)$$

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

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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

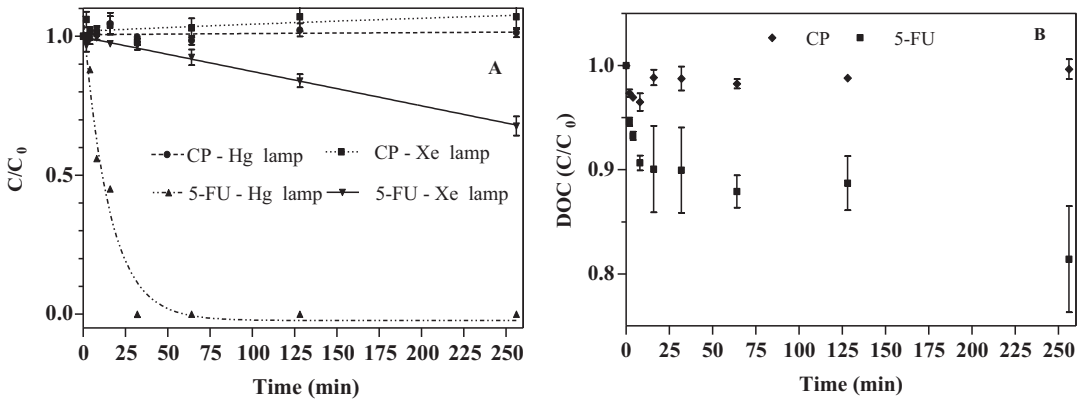

Fig. 1. **A** – Primary elimination of the parent compounds (20 mg/L) using the Hg and Xe Lamps during 256 min (n = 2). **B** – Mineralization degrees of the target compounds during 256 min using Hg Lamp. (n = 2). In the case of 5-FU there seem to be a change in kinetics between 128 und 256 min.

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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

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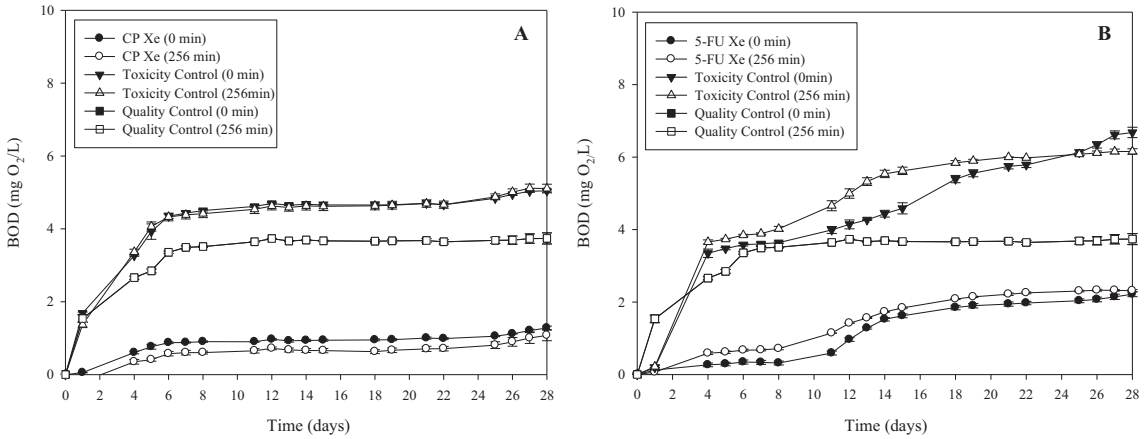

Fig. 2. Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Xe lamp) **B** – 5-fluorouracil (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Xe lamp) (n = 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.

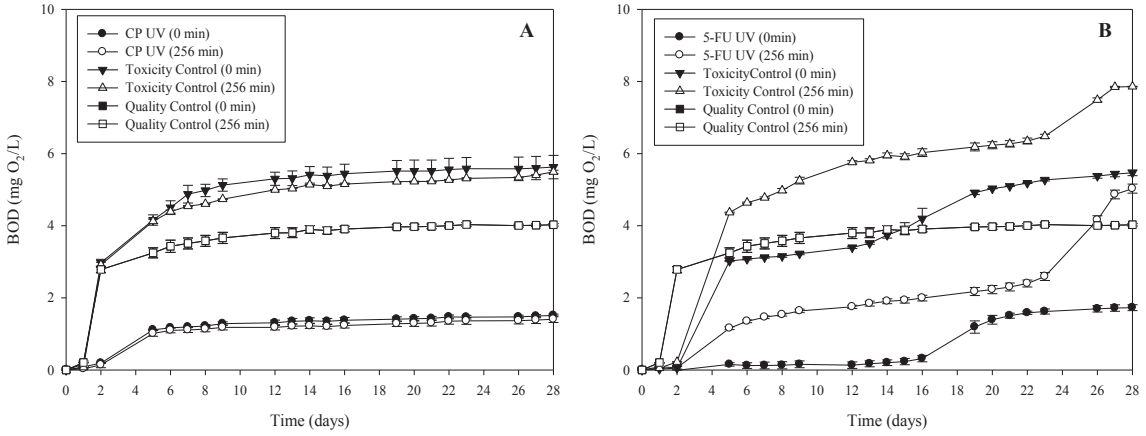

Fig. 3. Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Hg lamp) **B** – 5-fluorouracil (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Hg lamp) (n = 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.

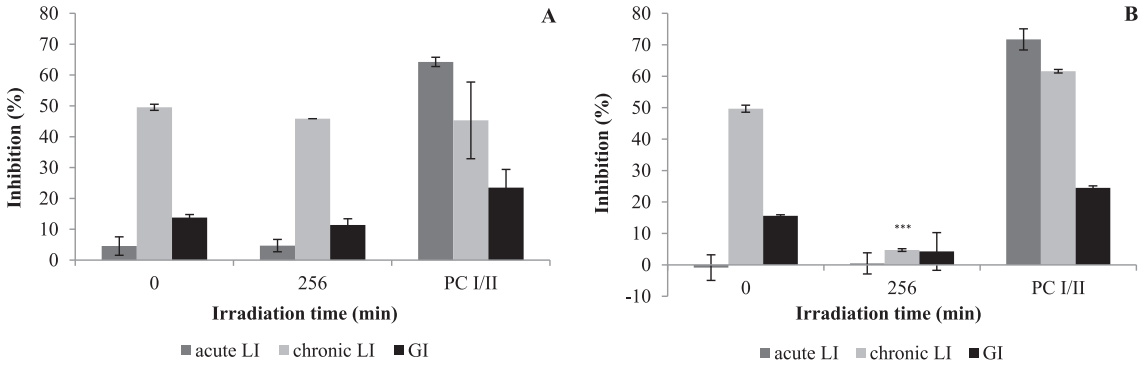

Fig. 4. Toxicity tests with *V. fischeri* considering three endpoints before (0 min) and after (256 min) the treatments with both lamps (n = 2) **A** = Xe Lamp and **B** = Hg. Photolysis was conducted with an initial 5-fluorouracil concentration of 20 mg/L. Considering the EC_{50} for the CI (0.45 mg/L) 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 ($P < 0.0001$).

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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

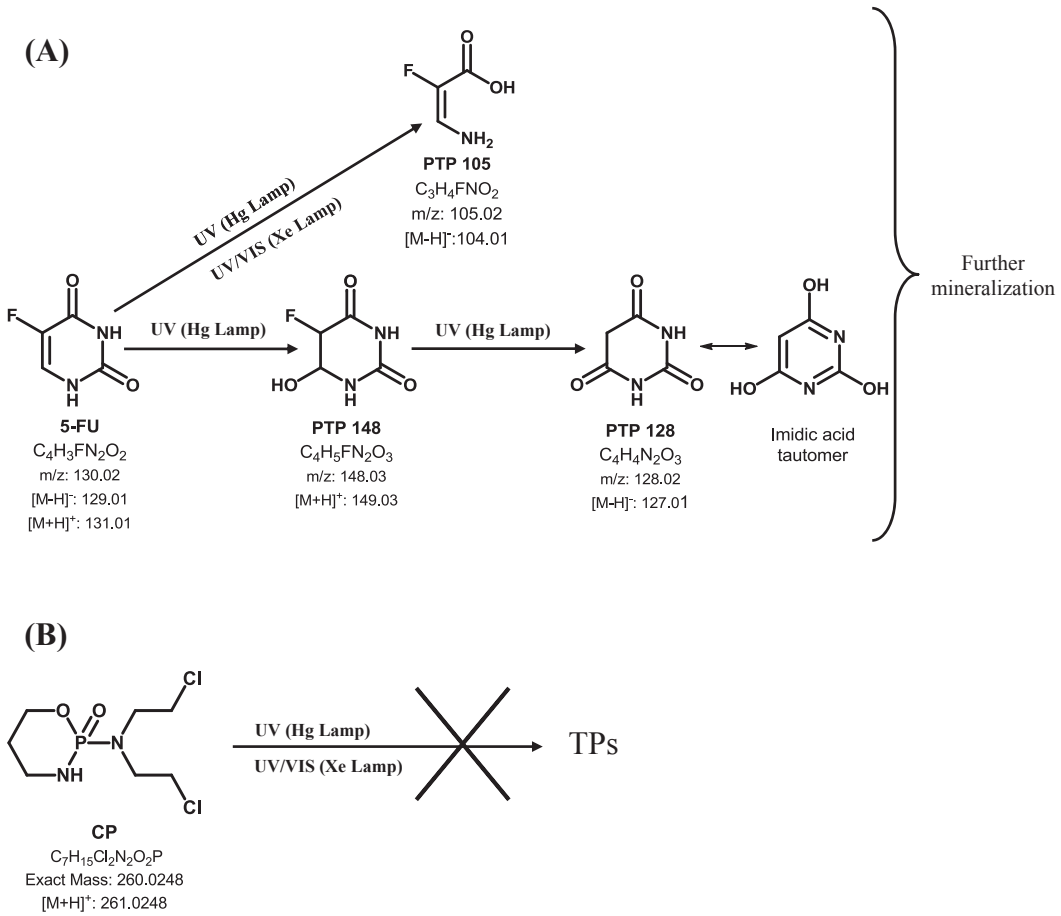

Fig. 5. Degradation pathway of 5-FU (A) and CP (B) by UV (Hg lamp) and Xe photolysis.

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).

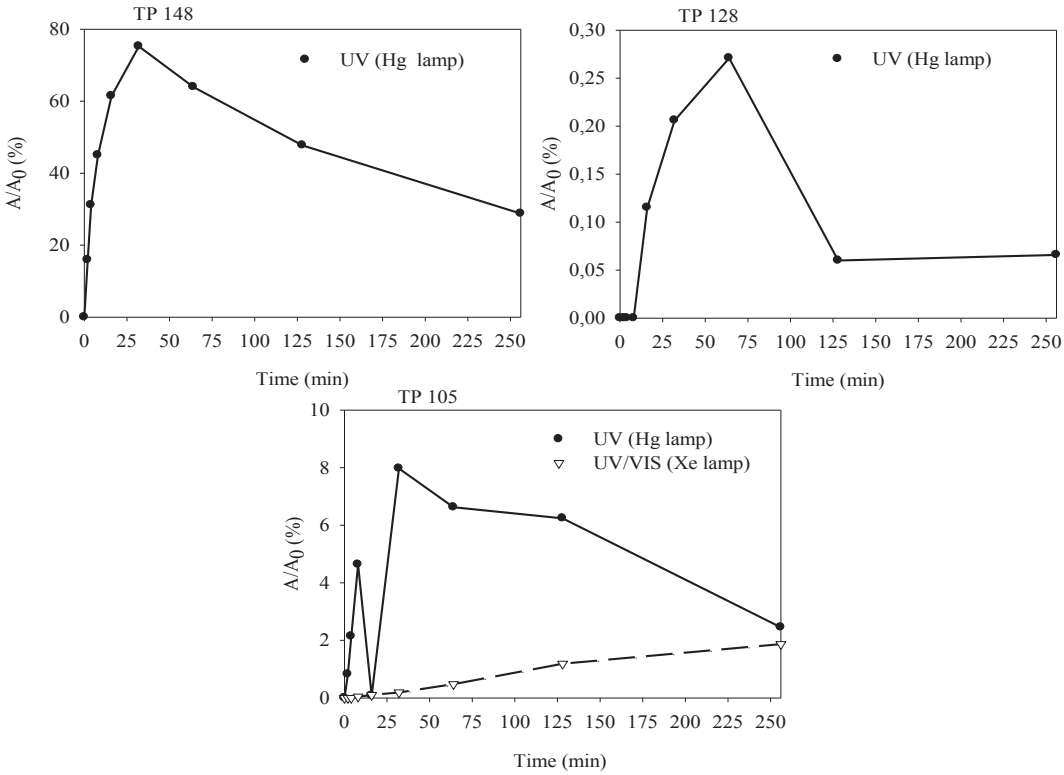

Fig. 6. Profile of the peak area ratio (A/A_0) of the identified PTPs from 5-FU during direct UV photolysis (Hg medium pressure Lamp) and simulated sunlight irradiation (Xe Lamp).

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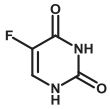
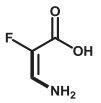
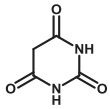
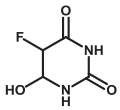
Table 4
In silico toxicity predictions by different models of CASE Ultra for 5-FU and the TPs formed during photodegradation experiments.

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.

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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 by UV and simulated sunlight treatments: assessment of the enhancement of the biodegradability and toxicity

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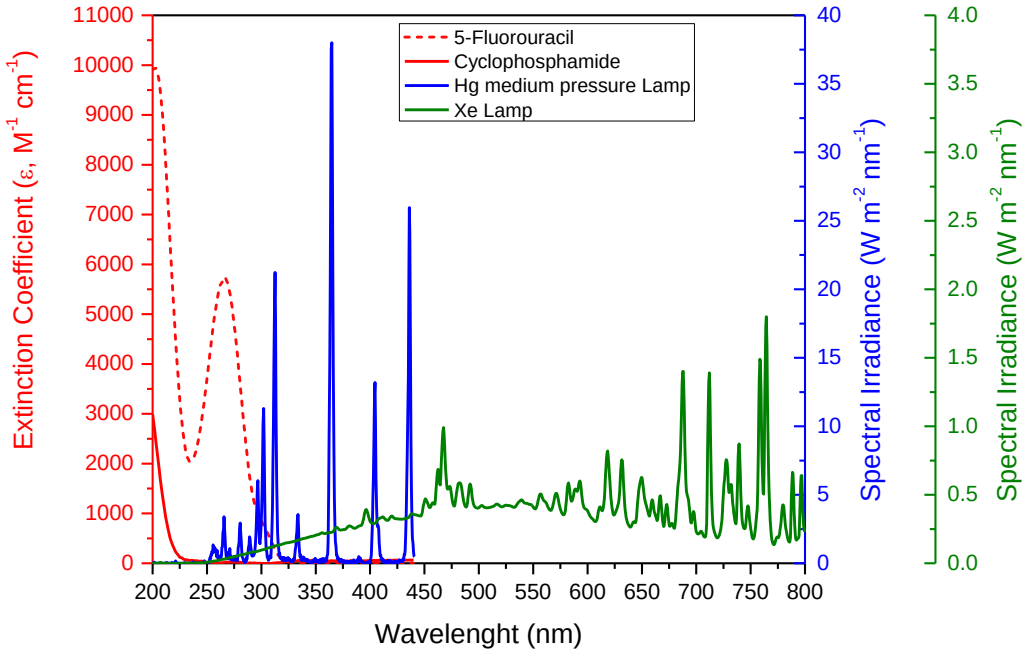


Fig. S1 – Intensity of light emitted by the used Hg and Xe lamps and molar extinction coefficient of 5-FU for the range of 200 nm to 800 nm. (Concentration of 10 mg/L)

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.

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

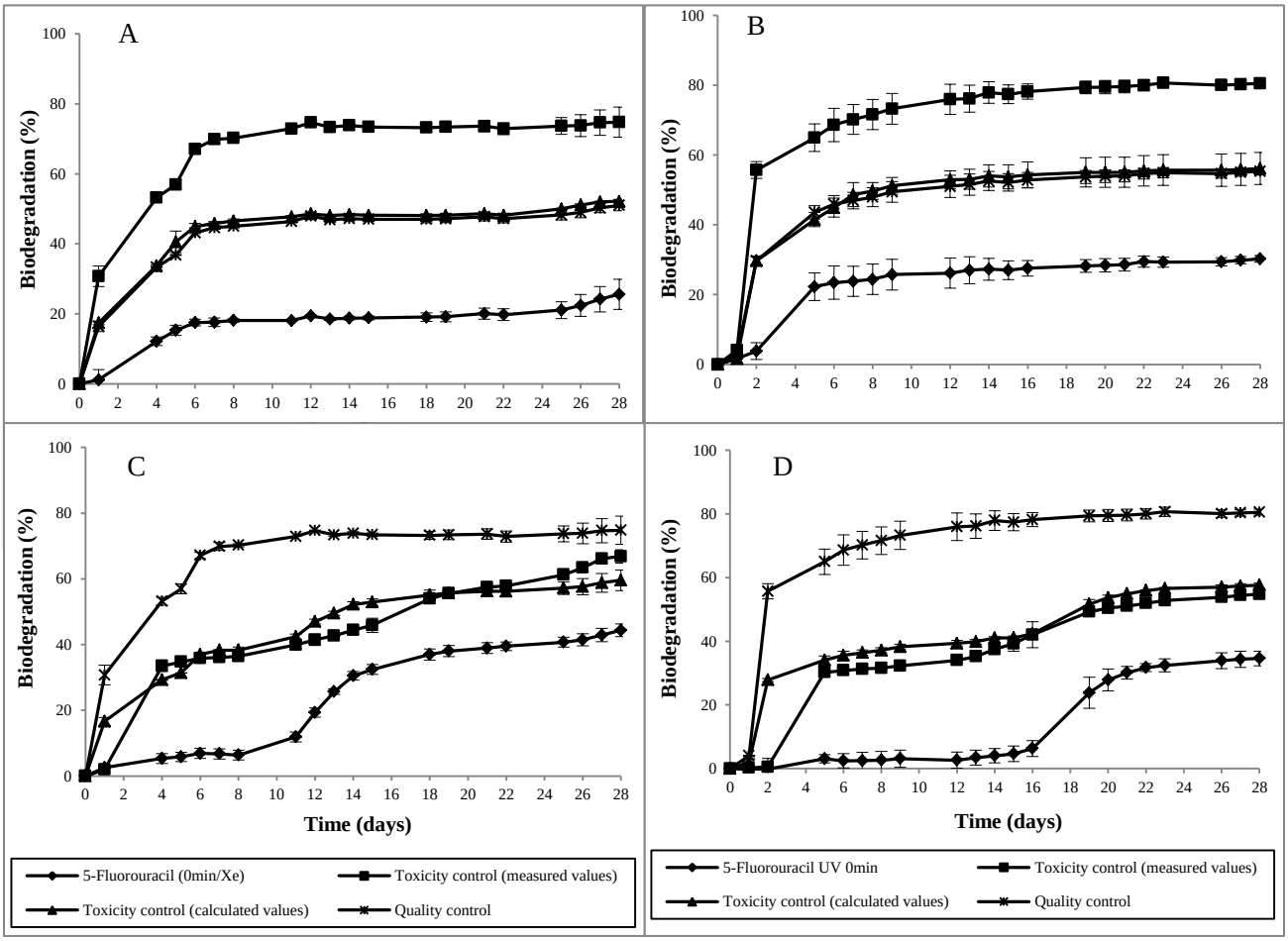
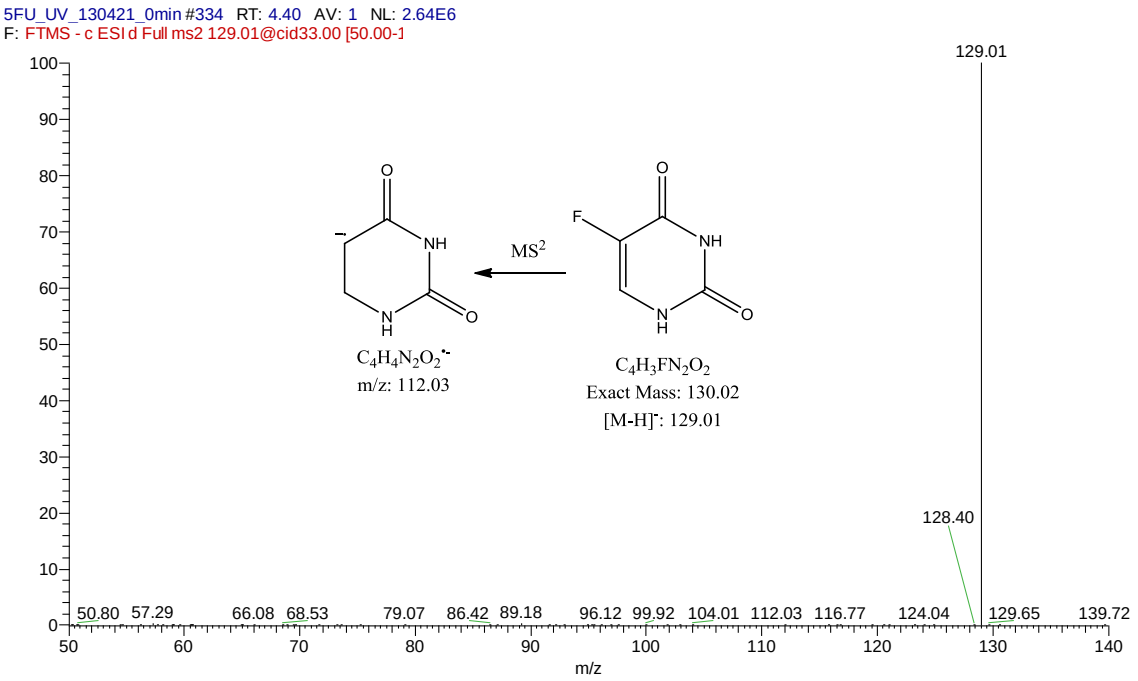


Fig. S2 – Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min Xe Lamp) and **B** – cyclophosphamide (4.5 mg/L; irradiation time 0 min Hg Lamp) **C –** 5-fluorouracil (4.5 mg/L; irradiation time 0 min Xe lamp) and **D** – 5-fluorouracil (4.5 mg/L; irradiation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

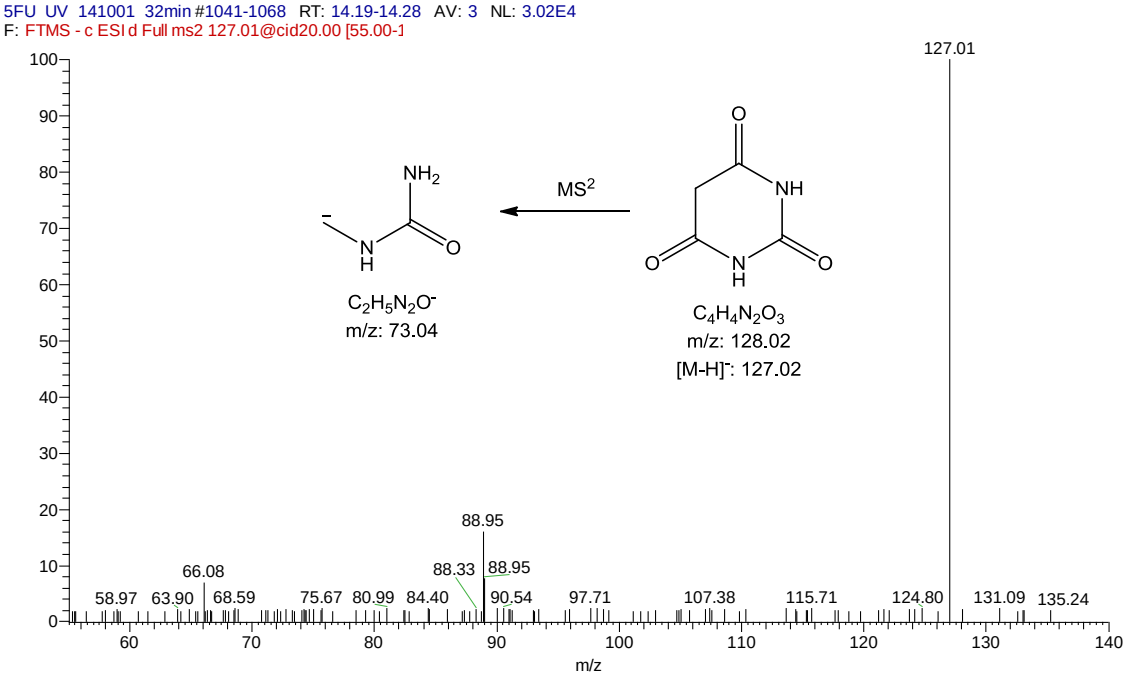
Text S3 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-HRMS.

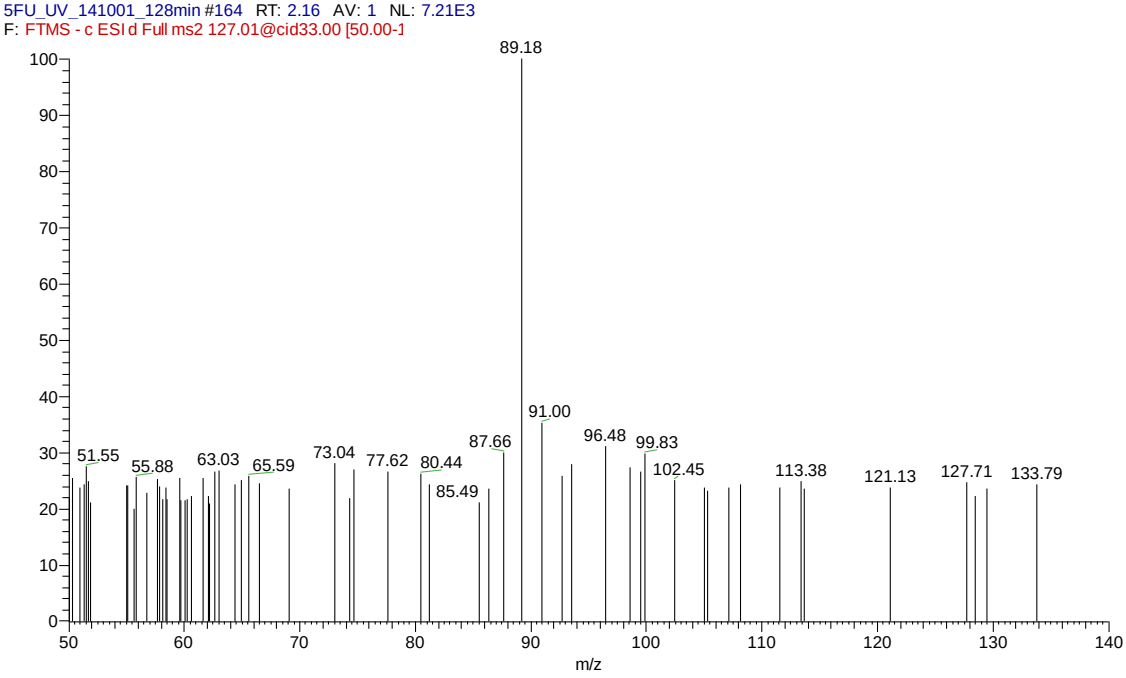
5-FU



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

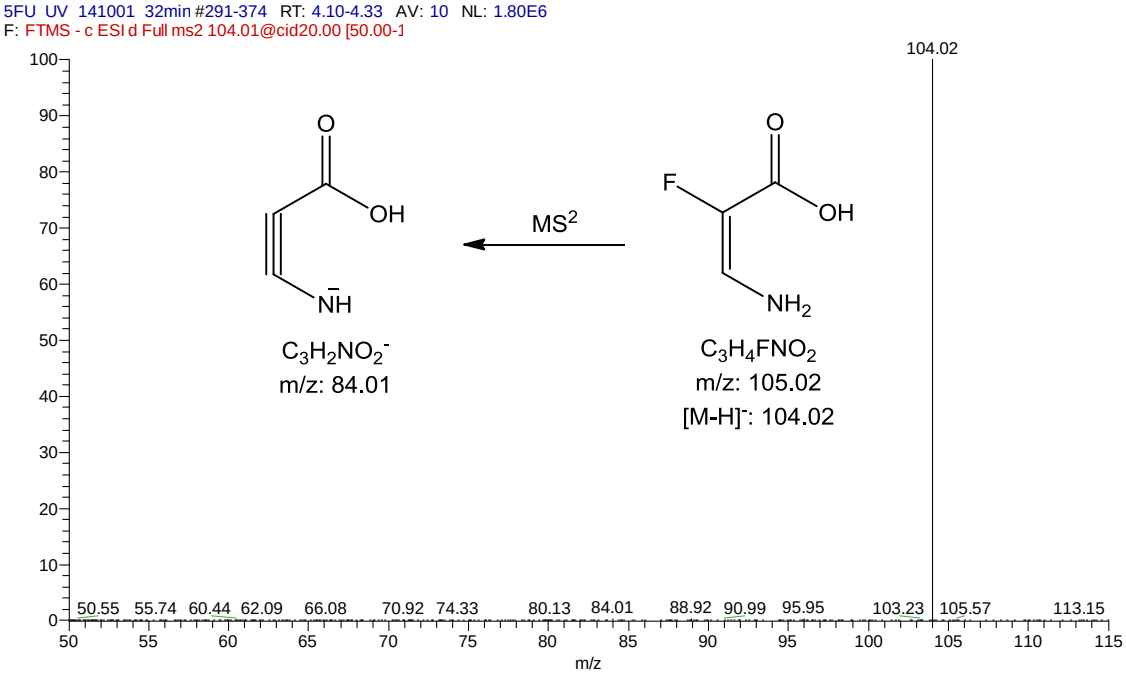
PTP 128



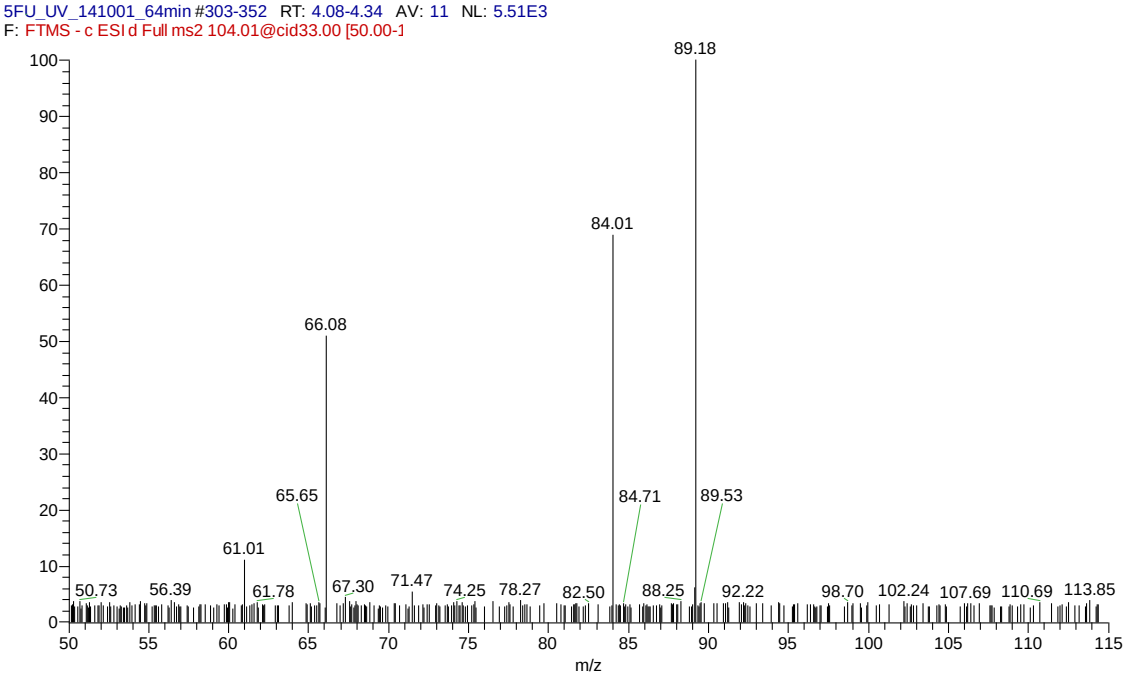


m/z [M-H]-	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
127.01527	$C_4H_3O_3N_2$	30205.1	100	4.5	0.355	Even
73.04181	C_2H_5ON2	20209	28.03	1.5	1.074	Even

TP 105

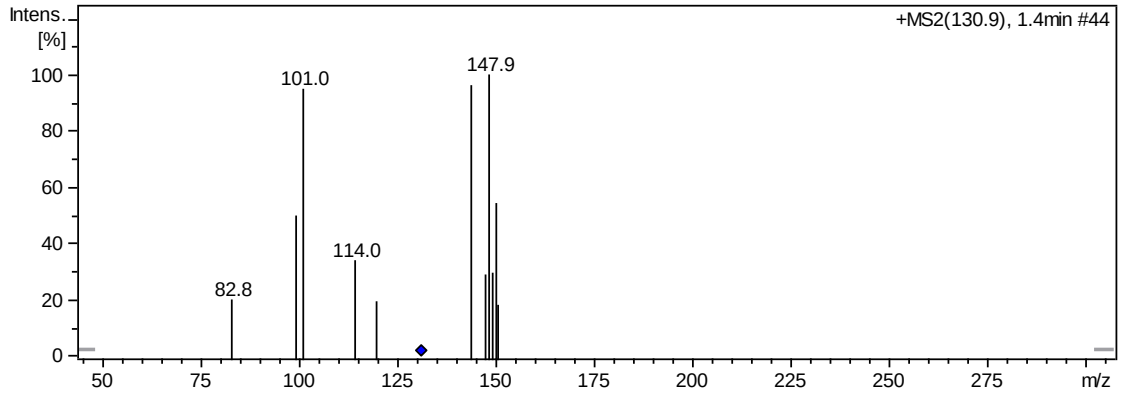


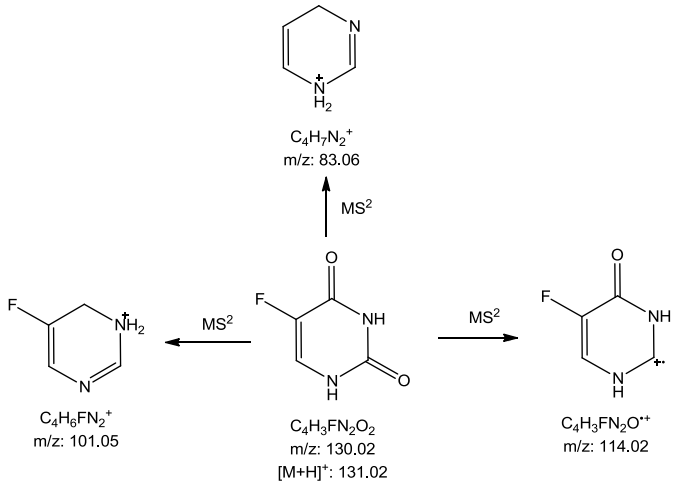
m/z [M-H]⁻	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
104.01528	$C_3H_3O_2NF$	314564.1	100	2.5	-0.050	Even
84.00905	$C_3H_2O_2N$	447.4	0.14	3.5	-0.052	Even



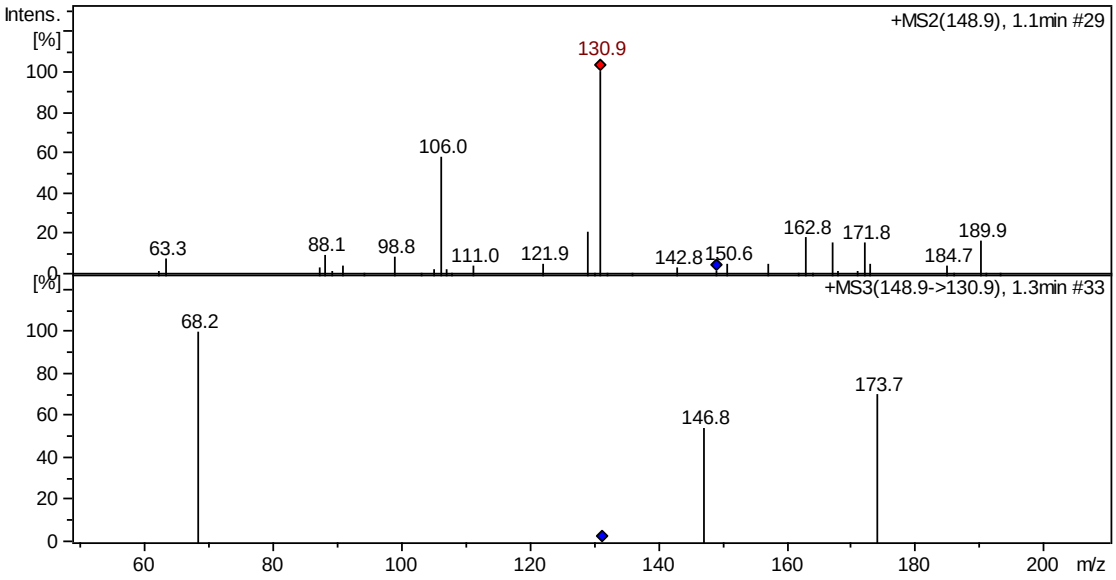
Text S4 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-IT-MS/MS.

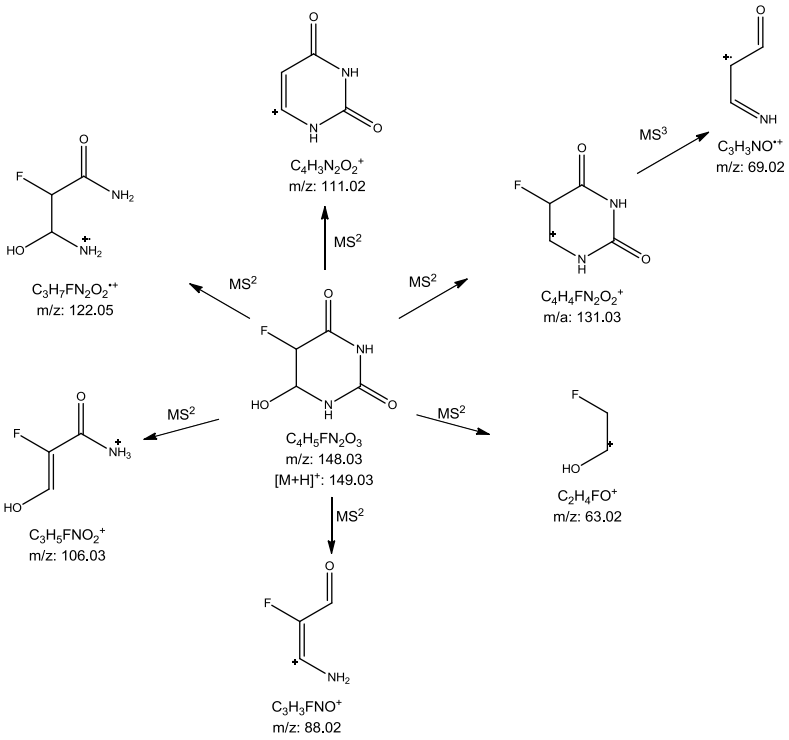
5-FU





TP 148


Intens.
[%]
+MS2(148.9), 1.1min #29
130.9
106.0
63.3
88.1
98.8
111.0
121.9
142.8
150.6
162.8
171.8
184.7
189.9
+MS3(148.9->130.9), 1.3min #33
68.2
146.8
173.7
m/z


C4H3N2O2+
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MS2
C3H7FN2O2•+
m/z: 122.05
C4H4FN2O2+
m/a: 131.03
MS3
C3H3NO•+
m/z: 69.02
C4H5FN2O3
m/z: 148.03
[M+H]+: 149.03
C3H5FNO2+
m/z: 106.03
C2H4FO+
m/z: 63.02
C3H3FNO+
m/z: 88.02

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Mahmoud, W.M., Kümmerer, K., 2012. Captopril and its dimer captopril disulfide: photodegradation, aerobic biodegradation and identification of transformation products by HPLC-UV and LC-ion trap-MS(n). Chemosphere, 88, 1170-7.

OECD, 1992. Organisation for Economic Co-operation and Development. OECD guideline for testing of chemicals 301D: ready biodegradability. Closed Bottle test. OECD Publishing.

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.

Paper 4

Evaluation of the toxic effects of four anticancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation.

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Evaluation of the toxic effects of four anti-cancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation

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HIGHLIGHTS

- Toxicological assays using seeds of *L. sativa* and *A. cepa* were carried out with CP, MTX, 5-FU and IM.
- The results indicated MTX as the most phytotoxic compound, followed by 5-FU, CP and IM.
- CP, MTX and 5-FU presented cytotoxic activity whereas CP, 5-FU and IM genotoxic potential.
- The four compounds showed significant formation of micronuclei.

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ABSTRACT

Anti-cancer drugs are compounds that are of high environmental relevance because of their lack of specific mode of action. They can be extremely harmful to living organisms even at low concentrations. The present study evaluated the toxic effects of four frequently used anti-cancer drugs against plant seedlings, namely Cyclophosphamide (CP), Methotrexate (MTX), 5-Fluorouracil (5-FU) and Imatinib (IM). The phytotoxicity experiments were performed with *Lactuca sativa* seedlings whereas cytotoxicity, genotoxicity and mutagenicity investigations were performed with the well-established *Allium cepa* assays. MTX was the most phytotoxic compound, followed by 5-FU, CP and IM. Significant differences in the Mitotic Indexes (MI) were observed in three of the studied compounds (MTX, 5-FU and CP), indicating potential cytotoxic activity of these substances. Chromosome aberrations were registered in cells that were exposed to 5-FU, CP and IM. All the four compounds caused the formation of micronucleated cells indicating mutagenic potential. Besides, the assays performed with MTX samples presented a high number of cell apoptosis (cell death). Although it is unlikely that the pharmaceuticals concentrations measured in the environment could cause lethal effects in plants, the obtained results indicate that these compounds may affect the growth and normal development of these plants. So, both tests can constitute important tools for a fast screening of environmental contamination e.g. in the context of the reuse of treated wastewater and biosolids of agricultural purpose.

1. Introduction

Nowadays around 4000 active pharmaceutical compounds, used as human and veterinary drugs, are available on the European market (Mompelat et al., 2009) among them about 100 anti-cancer drugs (Kümmerer et al., 2014). Due to an increased prevalence of cancer worldwide (Stewart and Wild, 2014), the consumption of anti-cancer drugs is expected to increase over the next years (Besse et al., 2012). Consequently their presence in the surface and wastewater is also expected to rise (Kümmerer et al., 2014; Zhang et al., 2013).

The first results confirming the presence of anti-cancer drugs in aquatic systems were published still in the 1980s (Aherne et al., 1985) followed by investigations in the 1990s (Aherne et al., 1990) and now in the 21st century (Besse et al., 2012).

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0045-6535/

Nevertheless, there is still a lack of knowledge concerning the environmental fate of the drugs itself and its metabolites after excretion and possible risks connected to their presence in the aquatic environment (Kümmerer et al., 2014).

Although the measured low environmental concentrations compared to other groups of pharmaceuticals (Kosjek and Heath, 2011), the anti-cancer drugs require a special attention because they are mostly are non-selective in their modes of action, affecting both cancerous and non-cancerous cells and often causing severe systemic side effects (Allwood et al., 2002). Some of them interfere directly with the DNA and are recognized, even at low concentrations, as potentially fetotoxic, genotoxic, mutagenic and teratogenic substances in non-target organisms (Allwood et al., 2002).

Despite the benefits arising from the reuse of treated wastewater to irrigate agricultural crops and application of biosolids for agricultural purposes, some studies have demonstrated that plant uptake from water or soil spiked with pharmaceutical chemicals is associated with recycling of the waste products from WWTPs (Tanoue et al., 2012). Albeit the low measured concentrations of human pharmaceuticals in soils, due to the persistent nature of many pharmaceuticals and the repeated application of contaminated biosolids or irrigation water, there is a potential for accumulation to occur in soil systems (Schmidt and Redshaw, 2015). So, there is a growing concern that residual pharmaceutical chemicals have the potential to be taken up by edible plants and then enter the food supply (Tanoue et al., 2012).

Bioassays involving higher plants have been extensively used in environmental monitoring studies over the last years. Among them the assay with *Lactuca sativa* (lettuce) is one of the most common. Besides being quite simple and not expensive, this test has been accepted and validated for use in toxicity studies (Sobrero and Ronco, 2004) e.g. by the United States Environmental Protection Agency (U.S. EPA) and the Organization for Economic Co-operation and Development (OECD, 2003; U.S. EPA, 1996). Although *L. sativa* cannot be considered a representative species of the aquatic environment, the results generated in this assay can be used to evaluate the possible toxic effects of contaminants on plants that live near polluted water bodies (Sobrero and Ronco, 2004) or as a result of irrigation with reused water if contaminants are not fully removed in predicting treatment. Furthermore, it is a representative of main dicotyledon commercial crops and considered a standard species due to moderate sensitivity and high frequency of use in phytotoxicological tests (D'Abrosca et al., 2008; Hillis et al., 2011).

The use of assays with higher plants to evaluate the genotoxic effects of environmental pollutants has increased during the last years. According to Fiskesjö (1985) the facilities to store and handle plants, the good chromosomes conditions, the low costs and a good correlation with other systems are some reasons that make them very useful. Furthermore these tests have a great capacity to detect mutagenic effects in different environments and allow the evaluation of distinct genetic endpoints, which range from point mutations to chromosome aberrations (CA) in cells of different organs and tissues, such as leaves, roots and pollen (Leme and Marin-Morales, 2009).

Because of its high sensibility in detecting environmental chemicals and some structural advantages such as the large size and small number of their chromosomes ($2n = 16$), *Allium cepa* root-tip cells have been widely used in assays to evaluate chromosomes damages and disturbances in the mitotic cycle (Leme and Marin-Morales, 2009). Furthermore, the test involving this species is considered an easy handling assay and has advantages over other bioassays that require previous treatments of tested samples, as well as the addition of exogenous metabolic system, as in the Ames test. Besides all the advantages above mentioned, the *A. cepa* test has shown a good correlation when compared with other test systems, e.g. mammals and being even more sensitive than the Ames and the Microscreen test (Rank and Nielsen, 1994).

As already mentioned above, the bioassay with *A. cepa* allows the assessment of different endpoints. In this test, the screening of the cytotoxic potential is determined by the alterations of the Mitotic Index (MI), characterized by the total number of dividing cells in cell cycle. A significant reduction of the MI in comparison to the negative control may be due to a chemical action in the growth and development of exposed organisms. On the other hand, MIs higher than the negative control are results of an increase in cell division, which can be harmful to the cells, leading to a disordered cell proliferation and even to the formation of tumor tissues (Leme and Marin-Morales, 2009).

The evaluation of the chromosome aberrations (CA) has been used in the *A. cepa* test as a parameter to detect potentially genotoxic agents. Structural chromosomal alterations may be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and replication of altered DNA. For the assessment of the potential genotoxic effects several types of CA found in the different phases of the cell cycle (prophase, metaphase, anaphase and telophase) are considered. Chromosome bridges and breaks, chromosome losses, fragments, delays, adherence, viscosity, multipolarity and C-metaphases are among the most common CA used to evaluate the genotoxicity in the *A. cepa* test.

Furthermore, the *A. cepa* test also enables the evaluation of the mutagenicity through the formation micronucleus (MN). For many authors, MN is the most effective and simplest endpoint to analyze the mutagenic effect promoted by chemicals. MN results from damages wrongly repaired (or not repaired) in the parental cells being easily measured in daughter cells as a structure similar to the main nucleus, but in a reduced size (Fenech, 2000). Therefore, MN arise from the development of some chromosome aberrations (CA), such as, chromosome breaks and losses or may still derive from other processes as polyploidization, in which they originate from the elimination of exceeding DNA of the main nucleus in an attempt to restore the normal conditions of ploidy (Fernandes et al., 2007; Leme and Marin-Morales, 2009).

Therefore, the present study aimed to assess the phytotoxicity, cytotoxicity, genotoxicity and mutagenicity of four anti-cancer drugs commonly used in anti-cancer treatments (Cyclophosphamide (CP), Methotrexate (MTX), 5-Fluorouracil (5-FU) and Imatinib (IM)) in two plant bioassays. Besides been among the most consumed anti-cancer drugs worldwide, the selected compounds are known not to be fully removed by biological and oxidative treatment (Besse et al., 2012; Booker et al., 2014; Steger-Hartmann et al., 1997; Yu et al., 2006).

2. Materials and methods

Since cytostatic drugs are potential cytotoxic, genotoxic, mutagenic and teratogenic compounds, their handling requires strict safety precautions (Allwood et al., 2002; Eitel et al., 1999). All stock solutions were prepared under a biological safety cabinet with laminar airflow. All the waste generated during the experiments was disposed and treated as hazardous and the instruments and glassware used were carefully cleaned before and after usage applying appropriate safety measures.

2.1. Test compounds, chemicals and seedlings

Cyclophosphamide monohydrate (CAS nr. 6055-19-2, 99%), methotrexate (CAS nr. 59-05-2, 98%) and imatinib mesylate (CAS nr. 220127-57-1, 99%) were kindly provided by Blausiegel Industry and Commerce Inc. (Cotia, SP, Brazil) whereas 5-fluorouracil (CAS nr. 51-21-8, 99%) was obtained from Sigma–Aldrich Biochemie

GmbH (Hamburg, Germany). Table S1 provides further information about the studied compounds. Stock solutions of the four compounds were prepared in distilled water: CP (100 mg/L), MTX (50 mg/L), 5-FU (10 mg/L) and IM (50 mg/L). The lettuce and onion seeds were acquired from ISLA Seeds Ltda (Porto Alegre, Brazil).

2.2. Phytotoxic assays with Lactuca sativa

The phytotoxicity of the four anticancer drugs was evaluated by adapting the method of Sobrero and Ronco (2004). The assay consists in the exposure of lettuce seeds plated on Petri dishes with a diameter of 100 mm lined with 90 mm Whatman n° 3 filter paper. Before exposure of the seeds, the filter paper was soaked in 4 mL of samples at five different concentrations at a ratio of ½, ranging between 6.25% and 100%. Distilled water was used as negative control and the experiments were carried out in triplicate. Twenty seeds per dish were exposed, totaling 60 individuals per concentration in each test. Three independent assays were performed amounting to 180 seeds per concentration at the end of the experiments. After exposure, the seeds were incubated in the dark at a controlled temperature of 20 ± 1 °C for 120 h. At the end of the test plants were frozen at −20 °C in order to stop the growing process as well as to facilitate the handling and the measuring of the roots.

2.3. Plant seedling assays with Allium cepa

The cytogenetic evaluation was carried out using the methodological adaptations proposed in Fiskesjö (1985, 1995) for the mitotic index; Grant (1982), for chromosome aberrations and Ma et al. (1995) for the analysis of micronuclei. Seeds of onion without any chemical treatment were germinated in Petri dishes and incubated at 20 ± 0.5 °C; each dish was covered with filter paper and individually poured with a different compound sample. Distilled water was used as negative control and potassium dichromate (10 mg/L) as positive control. After 7 days the roots were collected and fixed in alcohol–acetic acid (3:1 v/v) for 24 h at room temperature (25 ± 2 °C). For slide preparation, the material fixed in Carnoy's solution was submitted to acid cell lysis with hydrochloric acid (HCl) 1 mol/L at 60 °C for 11 min and later exposed to Schiff reactive staining for 20 min in the dark as described by Feulgen and Rossenbeck apud Mello and Vidal (Mello and Vidal, 1978). Thereafter, the meristematic regions were removed, carefully squashed into two drops of acetic acid (45%) and covered with coverslips sealed with colorless nail polish. The observations were made under a light microscope, using the count of 5000–6000 cells/group of five slides.

The analysis of the Mitotic Index (MI) was used as indicator of the presence of cytotoxic substances that could inhibit cell division. Chromosome aberrations (CA) found in the different phases of the cell cycle (metaphase, anaphase and telophase) were considered to assess the genotoxicity and their quantification was restricted to the presence of changes such as losses, fragments, delays, bridges, adhesions, viscosity, among others. The mutagenicity was determined by the presence of micronucleated cells (MNC).

2.4. Data analysis

In the experiments performed with *L. sativa*, the percentages of relative Seed Germination (SG), Root Elongation (RE) and germination index (GI) were calculated according to standard methods using Eqs. (1)–(3) (US Department of Agriculture and US Composting Council, 2001):

$$SG = \frac{\text{seeds germinated}}{\text{seed germinated in control}} \times 100 \quad (1)$$

$$RE = \frac{\text{mean root length}}{\text{mean root length in control}} \times 100 \quad (2)$$

$$GI = \frac{(SG) \times (RE)}{100} \quad (3)$$

The results were analyzed with one-way ANOVA followed by Dunnett's multiple comparison test at a significance level of 0.05 using the software Prism5 (Graphpad Inc., CA, USA). The IC_x values were calculated using the ICPIN program (Norberg-King, 1993) which calculates the IC values by linear interpolation and 95% confidence intervals by the bootstrap method.

MI was determined by the cell division rate using the following equation:

$$MI = \frac{\text{number of cells in mitosis (from prophase to anaphase)}}{\text{total number of cells}} \times 100 \quad (4)$$

CA and MCN were determined by their frequency in the total number of cells counted and compared to the negative control. The data obtained in the *A. cepa assays* were analyzed by each index and the differences were calculated by the chi-square test (X^2), in a 2 × 2 contingency table, at a significance level of 0.05 also using Prism5.

3. Results and discussion

3.1. Lactuca sativa phytotoxic experiments

The assay with *L. sativa* can be considered a rapid, low-cost and efficient screening test to prioritize studies requiring more intensive testing of novel compounds, such as pharmaceuticals and in this way evaluate the potential reuse of treated wastewater or biosolids for agricultural purposes. Furthermore, studies addressing range-finding tests are often necessary to establish concentration ranges to be used in definitive tests, such as soil column growth studies (Hillis et al., 2011).

As one can see in Table 1 none of the compounds, even at the highest concentrations, affected significantly the seed germination index. Nevertheless, it must be pointed out that although MTX has not affected the germination of the seeds, the presence of dun spots on the seedlings root was a clear indication of its toxic effects, leading to the necrosis of the tissues and consequently affecting the other two parameters (RE and GI).

By taking into account the mean root length statistically significant differences ($p < 0.001$) were observed for MTX at all the tested concentrations, altering the post-germinative development of the seedlings. Significant root elongation inhibition ($p < 0.05$) was also observed for the seeds exposed to IM at the lowest concentration (3.13 mg/L). Although root growth inhibitions and stimulations have been observed for the other two compounds, none of them was statistically significant.

The germination indexes MTX showed significant inhibition ($p < 0.001$) at all the tested concentrations. Furthermore IM also inhibited significantly ($p < 0.05$) the germination index of *L. sativa* at a concentration of 3.13 mg/L. No significant inhibitions were registered for 5-FU and CP even at the highest concentrations.

Table 2 lists the IC_x values of the impact of the four anticancer drugs on root growth of the lettuce seeds. Again MTX was the most toxic of the compound with IC_x values ranging from 0.44 to 2.2 mg/L. 5-FU was the second most toxic (IC_{10} = 2.16 mg/L)

Table 1
Seed germination tests as indicators of phytotoxicity of the four anticancer drugs against *L. sativa*. Values reported are the means ± standard deviation of three independent assays. Statistically significant differences (* and ***) compared to the negative control were identified by one way ANOVA following Dunnett's multiple comparison test ($P < 0.05$ and $P < 0.0001$, respectively). CP = Cyclophosphamide, MTX = Methotrexate, IM = Imatinib, 5-FU = 5-Fluorouracil; SD = Seed Germination, RE = Root Elongation, GI = Growth Inhibition.

Compound	Concentration range (mg/L)	SG ± SD (%)	RE ± SD (%)	GI ± SD (%)
CP	6.25	98 ± 4.1	85.7 ± 64	83.8 ± 2.8
	12.5	95.1 ± 13.4	93.9 ± 7.7	88.2 ± 5.3
	25	84.6 ± 11.1	70.5 ± 8.8	60.6 ± 15.3
	50	78.6 ± 23.5	81.6 ± 18.4	68.5 ± 33.6
	100	89.8 ± 8.1	68.4 ± 7.5	62 ± 12.2
MTX	3.13	108.3 ± 2.7	28.8 ± 2.6***	32 ± 4.1***
	6.25	89.6 ± 20	23.2 ± 21.3***	21.8 ± 9.3***
	12.5	79.2 ± 29.1	20 ± 34.4***	19.6 ± 13.1***
	25	91.7 ± 8.5	23.2 ± 9.8***	21.8 ± 4.9***
	50	77.1 ± 20.2	15.2 ± 19.5***	13.4 ± 6.3***
IM	3.13	82.1 ± 3	65.1 ± 5.8*	53.8 ± 0.6*
	6.25	103.2 ± 7.4	113 ± 3.9	116.9 ± 0.7
	12.5	94.9 ± 13.6	100 ± 0.8	94.7 ± 0.28
	25	91.7 ± 12.5	83 ± 0.1	76 ± 0.5
	50	86.3 ± 3.3	73.9 ± 0.6	63.8 ± 0.1
5-FU	0.625	105.3 ± 5.3	116.1 ± 1.4	122.2 ± 4.7
	1.25	97.3 ± 4.8	102.4 ± 4.5	99.8 ± 9.4
	2.5	92.9 ± 15.6	93.9 ± 7.8	88.5 ± 21.9
	5	94 ± 16.6	97.9 ± 8.1	93.4 ± 23.9
	10	107.6 ± 9.4	89.1 ± 6.6	96.4 ± 15.5

Table 2
Concentration ranges, IC_{10}, IC_{25} and EC_{50} values of *L. sativa* exposed to Cyclophosphamide (CP), 5-Fluorouracil (5-FU), Methotrexate (MTX) and Imatinib mesylate (IM) for the root growth endpoint. n.d. = not determinable. (95% confidence intervals).

Test compound	Concentration range (mg/L)	Inhibition concentrations		
		IC_{10} (mg/L)	IC_{25} (mg/L)	IC_{50} (mg/L)
CP	6.25–100	12.91 (3.59–21.31)	56.73 (n.d.)	n.d.
MTX	3.13–50	0.44 (0.42–0.48)	1.11 (1.03–1.18)	2.20 (2.08–2.43)
IM	3.13–50	16.01 (2.0–23.75)	46.69 (36.65–48.7)	n.d.
5-FU	0.63–10	2.16 (1.08–9.17)	n.d.	n.d.

followed by IM (IC_{25} = 46.69). CP was the least toxic compound with IC_{25} values >50 mg/L.

Khan (1966) investigated the inhibition of the germination and seedling growth of two varieties of lettuce by different anti-cancer drugs out of the group of antimetabolites. Even at higher concentrations (130 mg/L) no inhibition of the seed germination was observed for 5-FU. Nevertheless, the results indicated a strong decrease of the mean length for both varieties (80–90% inhibition) after 72 h exposure when using the same concentration (130 mg/L).

D'Abrosca et al. (2008) studied the phytotoxicity of five pharmaceutical pollutants detected in surface water. The results indicated lettuce as most sensitive test organism for the germination and root elongation endpoints. An extensive research addressing the impact of the application of biosolids and wastewater to agricultural land in lettuce plants, found out that hormones that are prevalent in irrigation water are absorbed by lettuce plants and can be found at high concentrations, whereas the effect of biosolids was negligible when compared to that of the irrigation water (Shargil et al., 2015).

Hillis et al. (2011) evaluated the toxic effects of ten antibiotics over a wide concentration range (1–10,000 μg/L) against three plant species: *L. sativa*, *Medicago sativa* (alfafa) and *Daucus carota* (carrot). The results of this study revealed high differences of the phytotoxic responses, with EC_{25} values ranging from 3.9 to 10,000 μg/L. and *D. carota* as the most sensitive plant species. The authors also verified no significant decrease of the seed germinations up to the highest antibiotic concentrations (10,000 μg/L) which suggests that germination, as well as in our study, is not a useful end point for testing the toxicity of the compounds.

More recently Pichler et al. (2014) investigated the impact of different concentrations of IM on the root growth of onions. Significant inhibition effects were observed with concentrations of 59 mg/L, whereas the calculated EC_{10} and EC_{50} values were of 11 and 66 mg/L, respectively. Mišík et al. (2014) obtained EC_{50} values for the inhibition of root growth of *A. cepa* after an exposure of 72 h to 5-FU of around 95 mg/L. In the same study, the authors verified no significant growth inhibition of the roots exposed to CP even at the highest concentrations (279 mg/L).

The results of the studies mentioned above clearly demonstrate that pharmaceutical compounds can have phytotoxic effects on different plant species. However, the wide range of responses obtained show that the extrapolation of toxic effects between plant species cannot be done simply from one species to others. Since the toxic effects will depend on the type compound, its concentration and the plant species, with distinct metabolism and uptake characteristics (Carvalho et al., 2014), a battery of different plant assays should be performed for testing environmental contaminants and screening of treated waste water intended for irrigation.

3.2. Allium cepa assays

The cytogenetic analysis for each drug trial was individually recorded and compared with the negative control (NC) to determine the cytotoxic, genotoxic and mutagenic effects through changes in meristematic cells using the test system with *A. cepa*.

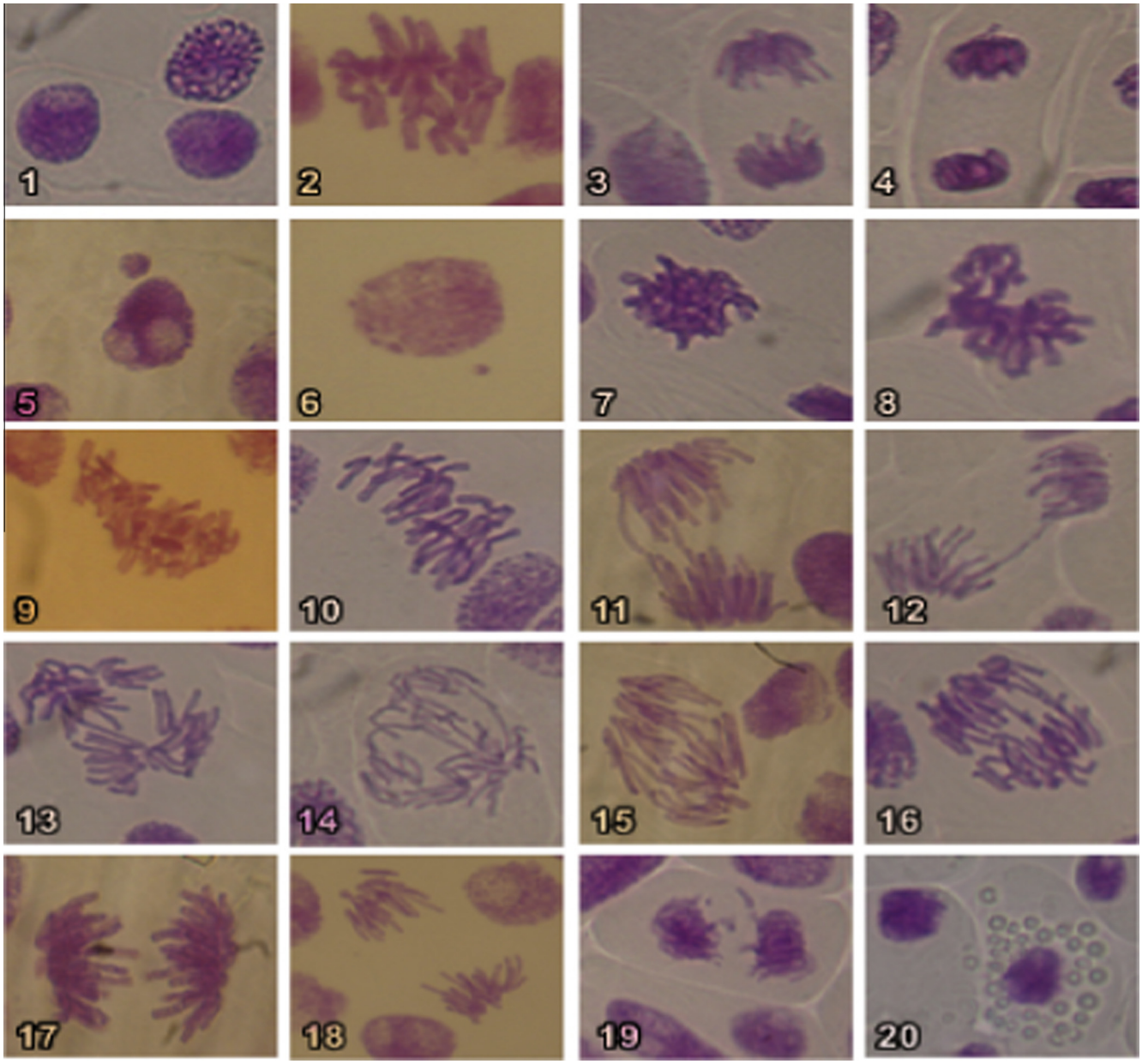

Fig. 1. Schematic representation of some meristematic cells of *A. cepa* exposed to the negative control (NC), the positive control (PC), and the four investigated anti-cancer drugs. These images represent the occurrence of several types of results reported in Table 3. Magnification for all images of 400×. 1. Two interphases and a normal prophase; 2. Core with normal metaphase; 3. Normal anaphase; 4. Normal telophase; 5. Interphase with a large micronucleus; 6. Interphase with a small micronucleus; 7. Metaphase with viscosity I; 8. Metaphase with viscosity II; 9. C-metaphase I; 10. C-metaphase II; 11. Anaphase with chromosome bridges I; 12. Anaphase with chromosome bridges II; 13. Multipolar anaphase with bridges I; 14. Multipolar anaphase with bridges II; 15. Anaphase with adhesion I; 16. Anaphase with adhesion II; 17. Anaphase with chromosome delay; 18. Anaphase with fragment; 19. Telophase with chromosome bridge broken; 20. Cell apoptosis.

Fig. 1 presents some of the variations registered in our analysis. Response variations involving the MI occurred both through stimulation of the cell division, as by the potential inhibition of meristematic dividing cells. On the one hand MI values significantly lower than the negative control indicate changes resulting from the chemical action on the growth and development of the exposed organisms. On the other hand values significantly higher than the negative control are result of an increase in cell division, that can be harmful to the development of the organism, leading to an uncontrolled proliferation and even to the formation of tumor tissue (Leme and Marin-Morales, 2009).

In our studies, significant differences were observed in three of the four tested compounds (Table 3). Cells exposed to 5-FU (10 mg/L) exhibited stimulation of the cell divisions, with a significant increase of 26.5% when compared to NC ($p < 0.0001$) whereas the cells exposed to CP (20 mg/L) showed an inhibition of the cell divisions, with a significant reduction of 13.9% in comparison to the NC ($p < 0.02$). However, the most significant results were observed for MTX (5 mg/L). Individuals exposed to MTX showed a very significant reduction (291.4%) of the mitotic activity when compared to the NC ($p < 0.0001$). IM (10 mg/L) resulted in no significant reductions of mitotic indexes (<0.1%) in relation to the NC.

In the literature, some authors tried to establish concentration ranges for the determination of cytotoxic conditions (sub-lethal and lethal levels) related to the responses presented by the meristematic cells of *A. cepa*. Antosiewicz (1990) determined that a decrease of about 22% in relation to the negative control is

Table 3
Results of cytogenetic evaluation with the *Allium cepa* test.

	NC	PC	5-FU 10	CP 20	IM 10	MTX 5
Interphase	5313	4870	5157	5055	5185	5891
Prophase	316	518	431	236	313	69
Metaphase	34	80	76	60	51	25
Anaphase	21	20	38	34	17	9
Telophase	205	267	247	135	168	48
Total	5889	5755	5949	5520	5734	6042
In division	576	885	792	465	549	151
MI	9.78 ± 5.06	15.38 ± 9.92	13.30 ± 1.76	8.42 ± 5.16	9.57 ± 2.83	2.50 ± 3.16
X^2 dif.	0.00	64.12, 1**	28.35, 1**	5.124, 1*	0.09632, 1	243.5, 1**
p value	0.00	<0.0001	<0.0001	0. 02	0.76	<0.0001
Dif.% CN	0.00	36.4	26.49	-13.90	-2.16	-291.37
CA	0.66 ± 0.42	1.36 ± 0.77	1.73 ± 0.49	1.36 ± 0.99	1.05 ± 0.20	0.66 ± 0.84
X^2 dif.	0.00	13.09, 1*	26.98, 1**	13.00, 1*	4.550, 1*	0.01242, 1
P value	0.00	0.0003	<0.0001	0.0003	0.03	0.91
Dif.% CN	0.00	51.14	61.75	51.25	36.71	0.00
MCN	0.05 ± 0.07	1.01 ± 0.45	2.32 ± 2.53	0.31 ± 0.78	0.28 ± 0.23	2.75 ± 1.61
X^2 dif.	0.00	48.79, 1**	124.5, 1**	9.302, 1*	7.889, 1*	149.1, 1**
p value	0.00	<0.0001	<0.0001	0.0023	0.005	<0.0001
Dif.% CN	0.00	94.94	97.80	83.45	81.74	98.14
APOP	0.00	0.00	1.09 ± 2.87	0.00	0.00	2.18 ± 3.97

NC = Negative Control; PC = Positive Control; 5-FU 10 = 5-Fluorouracil 10 mg/L; CP 20 = Cyclophosphamide 20 mg/L; IM 10 = Imatinib 10 mg/L; MTX 5 = Methotrexate 5 mg/L; MI = Mitotic Index; CA = Frequency of Chromosomic Aberrations; MCN = frequency of micronucleated cells; APOP = frequency of cells in apoptosis; Dif.% NC = Percentage reduction or increase compared to the NC; X^2 dif = value of chi-square test, *p* value = probability value.
* *p* (<0.05), according to the Chi-square test.
** *p* (<0.0001), according to the Chi-square test.

indicative of sub-lethal effects to the organism, establishing thus, a probable chronic relationship. According to Sharma (1983) and Panda and Sahu (1985), an inhibition of 50% of the exposed cells can be attributed to a possible lethal effect to the test organism.

In the present work, the cells exposed to CP (20 mg/L), a well-known alkylating agent with cytotoxic properties, was closest to the threshold (22%) of the sub-lethal effects established in the literature. On the other side, the registered inhibition of the cells exposed to MTX (5 mg/L) was almost six times higher than the limit established for the incidence of lethal effects on test organism. So, it is noteworthy to mention that a high incidence of apoptotic cells (cell death process) was recorded for cells exposed to samples of MTX (5 mg/L) and 5-FU (10 mg/L).

The incidence of cells with chromosomic aberrations is a potential indicative of genotoxicity (Table 3). In the analyzed samples, the genotoxicity values occurred homogeneously in three of four drugs evaluated. 5-FU (10 mg/L), a widely used antimetabolite classified as cytotoxic, exhibited the highest incidence of aberrations, with a significant increase of 61.8% when compared to the NC ($p < 0.0001$). This incidence value is more than 10% higher the value registered on the positive control (PC). CP (20 mg/L) presented the second highest incidence of aberrant cells with a significant increase (51.3%) of the cells whereas IM (10 mg/L) showed significant 36.7% ($p < 0.03$). No incidence of aberrant cells was registered in the individuals exposed to samples of MTX (5 mg/L) when compared to NC (Table 3). Nevertheless, these results can be strongly related to the sharp decrease of mitotic activity.

The mutagenicity of the samples was evaluated by the incidence of micronucleated cells (MCN). All the four compounds presented significant increases in the incidence. These increases were more pronounced in the samples of MTX and 5-FU, with incidences of micronucleated cells of, respectively, 98.1% and 97.8% ($p < 0.0001$). Samples of CP and IM exhibited slightly lower incidence of micronucleated cells, with values of respectively, 83.5% and 81.7% in relation to the NC.

The obtained results show a higher sensitivity of the *A. cepa* test to detect genotoxic and mutagenic activities of the anticancer drugs in comparison with more common and standardized tests such as the Ames and Umu C Test. Screening assays performed in our laboratories with concentrations up to 20 mg/L for the four compounds (CP, 5-FU, MTX and IM) indicated negative mutagenicity in the Ames test using the bacterial strains *Salmonella typhimurium* TA 98 and TA 100 with and without metabolic activator. Moreover CP, 5-FU, MTX showed no genotoxic activity in the Umu C Test (IM was not tested).

Over the last decades some studies have been conducted to evaluate the cytotoxic, genotoxic and mutagenic effects of some anticancer drugs in different bioassays. Zounkova et al. (2007) performed genotoxicity assays with 5 cytotoxic compounds using *Escherichia coli* SOS chromotest (with and without the use of metabolic activator) and *Saccharomyces cerevisiae* (eukaryotic yeast GreenScreen Assay (GSA)). The results indicated 5-FU as the most genotoxic compound for *S. cerevisiae* (Minimal Genotoxic Concentration – MGC = 0.02 mg/L) and very toxic in the assays involving *E. coli* without metabolic activation (MGC = 1.4 mg/L). CP showed no significant genotoxic effect for *E. coli* but a low genotoxicity was detected (MGC = 470 mg/L) in the yeast cells. The high genotoxic responses of 5-FU reported in this study seem to be consistent with the data found in our assays with *A. cepa*.

Yasunaga et al. (2006) investigated the genotoxicity of 18 anticancer drugs with the Umu C test. The results indicated positive responses of 5-FU (500–1000 mg/L) without metabolic activation and for CP (with 500–5000 mg/L) and MTX (with 2000–5000 mg/L) with S9 mix. However, it must be pointed out that these positive responses were registered at very high concentrations, some order of magnitudes higher than the ones used in our assays, which may indicate a greater sensitivity of the test with *A. cepa* for detecting genotoxic effects. Santos and Pacheco (1995) tested CP as standard compound at a range between 6.25 and 100 mg/L to assess the mutagenic activity of compounds in assays with eel (*Anguila anguilla*) gills. The authors verified a dose response effect for CP, with significant mutagenic activity starting from 12.5 mg/L.

Grisolia and Cordeiro (2000) tested the MN formation after intra-abdominal injection on three introduced species of fish and verified that CP was the most effective compound among

four compounds, though the other three, including 5-FU, also produced micronuclei. The results of our studies also indicated the incidence of micronucleus for these two compounds; however differently from the results obtained by Grisolia and Cordeiro (2000), in our assays 5-FU produced a higher number of micronucleus than CP.

More recently Parrella et al. (2014) investigated the cytotoxicity of six anticancer drugs (including 5-FU and IM) with on MCF-7 and MDA-MB-231 breast cancer cell lines after two incubation times (48 and 72 h). The calculated IC_{50} values for 5-FU were 96 and 42 mg/L (after 48 and 72 h, respectively) for MCF-7 while for MDA-MB-231 cells the authors reported values of 108 and 9.5 mg/L (48 and 72 h, respectively). IM was a little more cytotoxic with an IC_{50} of 37 and 34 mg/L (48 and 72 h, respectively) for MCF-7 cells and 35.4 and 18.6 (48 and 72 h, respectively) using MDA-MB-231 cells. Pichler et al. (2014) showed that IM induced the formation of micronuclei in *Tradescantia* cells at doses of 5.9 mg/L and at 0.6 mg/L in *A. cepa* root cells. The authors also verified a significant inhibition of the cell division of *A. cepa* at 5.9 mg/L after 24 h exposition and of 0.3 mg/L after 72 h exposition. These results are in contradiction with the ones obtained in our assays, since we observed no significant inhibition of the cell divisions at a concentration of 10 mg/L.

Mišík et al., 2014 found significant formation of MN of CP at around 0.84 mg/L, whereas no significant differences of the Mitotic Index (MI) were observed even at the highest tested concentrations (8.4 mg/L) when compared to the negative control. On the other hand the authors verified a significant MN of the cells exposed to 5-FU at a concentration of 13 mg/L and a significant inhibition of the MI at 6.5 mg/L.

From the above mentioned studies, it became evident that the four studied compounds can exert cytotoxic, genotoxic and mutagenic activities against different organisms. Most of the (few) monitoring studies in general involve bacterial and eukaryotic yeast assays and in comparison to these tests, the assay with *L. sativa* presents some advantages. While the more "standardized" tests (Ames, Umu C Test, GreenScreen Assay) are, in general costly and require more sophisticated apparatus, the *A. cepa* test is considered as a low cost and easy handling assay, which enables the evaluation of three different effects (cytotoxicity, genotoxicity and mutagenicity). Other point refers to the high sensitivity of the *A. cepa* test in detecting possible toxic effects, whereas in more traditional tests like the Ames, anti-cancer drugs often require relatively higher concentrations to be detected. Furthermore, the *A. cepa* test has shown a good correlation when compared with other test systems, e.g. mammals (Leme and Marin-Morales, 2009). Rank and Nielsen (1994) showed a correlation of 82% of the *A. cepa* test in relation to the carcinogenicity test in rodents and verified a higher sensitivity in comparison to the Ames and the Microscreen tests. According to the authors the *Allium* test can be recommended for the screening of the genotoxicity of wastewaters because it has a high sensitivity, is cheap, rapid, easy to handle, and because it can be used on wastewater without pretreatment of the samples. Therefore, this test may constitute an important tool for a fast screening of environmental contamination screening e.g. in the context of the reuse of treated wastewater and biosolids of agricultural purpose.

From the environmental point of view, the obtained results show that the concentrations needed to induce acute effects on *L. sativa* or genotoxic effects on *A. cepa* are in general 6–7 orders of magnitude higher than the currently measured environmental concentrations (Table S2). However when considering the lowest predicted no effect concentration, which is derived from the EC_{50} divided by the assessment factor of 1000 (EMA, 2006), the risk of possible negative impacts of the investigated compounds on the studied plants cannot be completely ruled out specially at some point sources such as hospital effluents or manufacturing plants where relatively high levels may occur. For instance, the calculated lowest predicted no effect concentration for MTX was 2.2 μg/L and concentrations close or even higher than this value were already measured in some hospital effluents (Table S2).

Furthermore, in order to improve the efficiency of the chemotherapeutic treatment many of the anticancer drugs are administrated as cocktails, i.e. in combination with other anticancer drugs. So the excreted residues of these compounds can occur in the aquatic environment as mixtures and due to potential synergistic effects may pose a higher threat for non-target organisms than that posed by the individual compounds. In a study performed with 4 anticancer drugs, Brezovšek et al. (2014) verified a strong synergistic effect of 5-FU and IM for the alga *Pseudokirchneriella subcapitata*. Finally, with the increasing of the population age and a higher prevalence of cancer cases, the consumption of anticancer drugs will increase and consequently their presence in the aquatic is also expected to rise.

4. Conclusions

In the present study four anticancer drugs were submitted to phytotoxic, cytotoxic, genotoxic and mutagenic analysis. On the one hand, the results obtained in our experiments with *L. sativa* confirm that the seed germination is not the most appropriate endpoint to indicate a possible phytotoxic effect of a compound. On the other hand, the measurement of growth inhibition appears to be a useful tool to evaluate the sub-lethal effects of different pollutants at concentrations levels that are not sufficient to inhibit germination (lethal effects) but may affect and/or retard the processes involving the root development.

Taking into account that the evaluation of the genotoxicity and mutagenicity are considered key parameters for a toxicological assessment and the high costs associated to "more standardized toxicity tests", such as Ames and Umu C test, the *Allium* test might be considered a promising screening tool for a first toxicity assessment of water reuse to be used for irrigation purposes since it is inexpensive and rapid, easy to handle and the samples can be tested without any pretreatment. Furthermore, the obtained results confirmed that the *A. cepa* test has a higher sensitivity to detect mutagenic and genotoxic activities of anticancer drugs when compared to the Ames and Umu C tests. MTX, 5-FU, and CP showed potential cytotoxic effects, whereas IM, 5-FU and CP presented CA, i.e. a genotoxic potential. All the compounds induced the formation of MNC, which are indicative of mutagenic activity. Besides, an incidence of apoptotic cells was observed for cells exposed to samples of 5-FU and MTX.

Although it is unlikely that the pharmaceuticals concentrations measured in the environment could cause lethal effects in plants, the results obtained in this study indicate that these compounds may affect the growth and normal development of these plants. Furthermore, as long as knowledge on plant up-take, persistence and accumulation of these compounds in aquatic and terrestrial plants is not known these tests can serve as screening tests for a first assessment of waste water to be used for irrigation purposes.

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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.2015.05.019.

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Supplementary Material

Evaluation of the toxic effects of four anticancer drugs in plant bioassays and its potency for screening in the context of waste water reuse for irrigation

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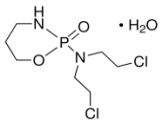
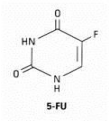
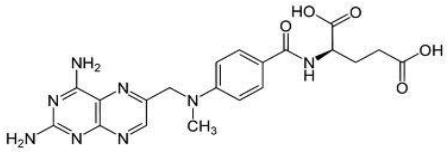
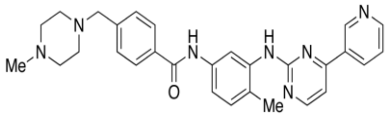
Table S1. Anticancer drugs (commercial names) Chemical Abstract Services numbers (CAS no.), chemical structures, and chemical formulas

Substance (commercial name and producer)	CAS No.	Structure	Chemical Formula	Kow	Water Solubility
Cyclophosphamide Monohydrate	6055-19-2		$C_7H_{15}Cl_2N_2O_2P \cdot H_2O$	0.63 (1)	40 g/L (1)
5-Fluorouracil	51-21-8		$C_4H_3FN_2O_2$	−0.69 (2)	11.1 g/L (3)
Methotrexate	59-05-2		$C_{20}H_{22}N_8O_5$	-1.28 (2)	2.6 g/L (1)
Imatinib Mesylate	220127-57-1		. $MeSO_3H$ $C_{30}H_{35}N_7O_4S$	2.89 (3)	200 g/L (4)

(1) Kosjek and Heath, 2011; (2) (Sanderson and Thomsen, 2009)(3) Zhang et al., 2013; (4) Santa Cruz Biotechnology

Table S2 Concentrations of anticancer drugs in different environmental samples (expressed in ng/L). WWTP: wastewater treatment plant; nd: not detected;

Compound	Sample	Concentration	References
Cyclophosphamide	Hospital Effluent	19 – 4,486	Steger-Hartmann et al. (1997)
	Hospital Effluent	5,730	Gómez-Canela et al. (2013)
	Surface Waters	n.d. – 64.8	Moldovan (2006)
	WWTP effluent	300	Catastini (2008)
5-Fluorouracil	Hospital Effluent	20,000 – 122,000	Mahnik et al. (2004)
	Hospital Effluent	8,600 – 123,500	Mahnik et al. (2007)
	Manufacturing	9×10^8	Anheden et al. (1996)
	Surface Water	<34	Zhang et al. (2013)
	Municipal	4.7 – 14	Kosjek et al. (2013)
Methotrexate	Hospital Effluent	1,000	Aherne et al. (1985)
	Hospital Effluent	4 – 4,689	Yin et al. (2010)
	WWTP effluent	2.1 – 20	Negreira et al. (2013)
	Surface Waters	12.6	Castiglioni et al. (2005)
Imatinib	PEC	5	Besse et al. (2012)

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Zhang, J., Chang, V.W.C., Giannis, A., Wang, J.-Y., 2013. Removal of cytostatic drugs from aquatic environment: A review. Science of The Total Environment, 445–446, 281-298.

Paper 5

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; $HO^{\bullet}$, 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).

$$H_2O_2 + HO^{\bullet} \rightarrow H_2O + HO_2^{\bullet} \quad (2)$$

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.

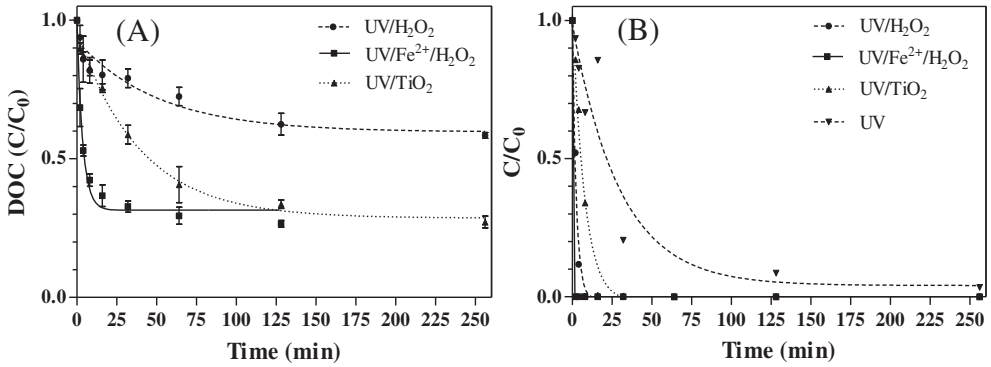


Fig. 1. A — kinetics of the mineralization of 5-FU under the three different treatment reactions. B — kinetics of the primary elimination of 5-FU. (n = 3).

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

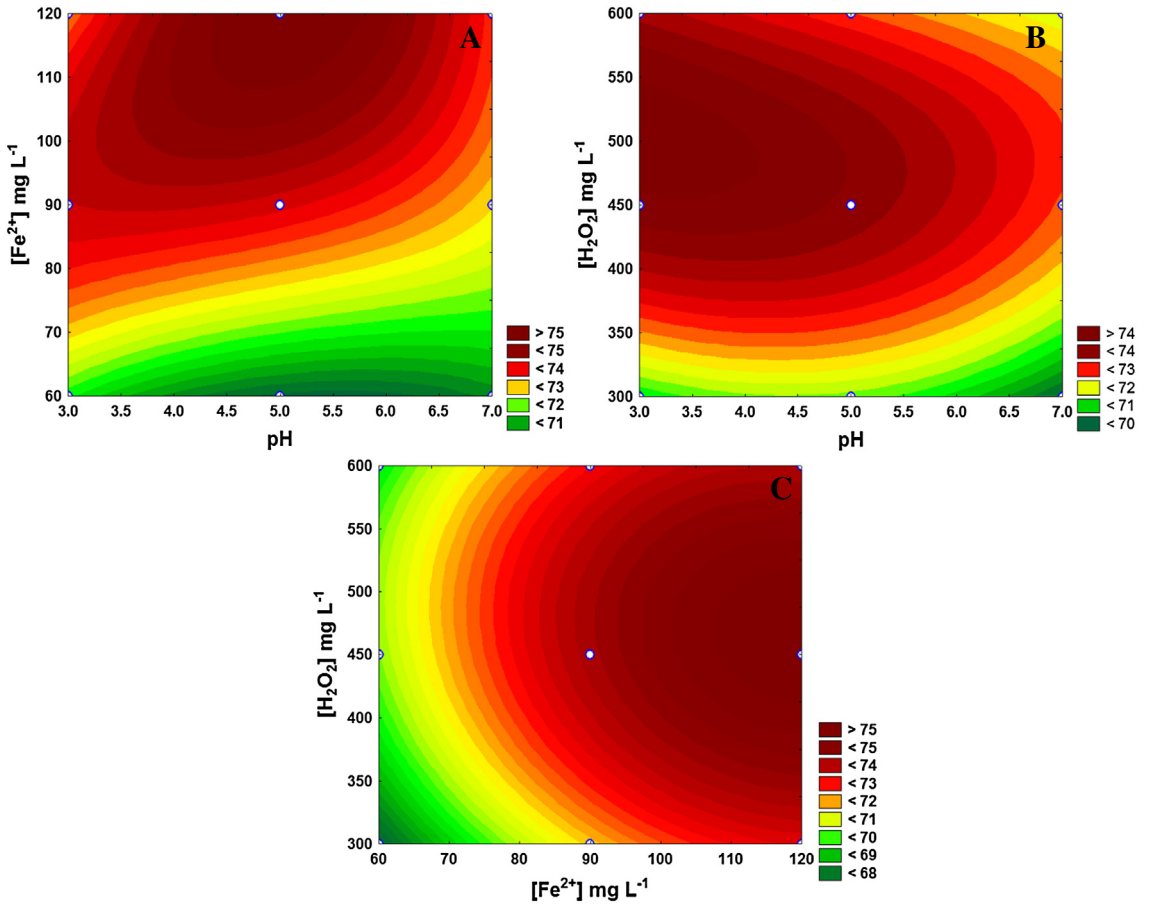


Fig. 2. Contour plot of the response surface of 5-FU mineralization by means of photo-Fenton process. (A) Interaction between pH and $[Fe^{2+}]$; (B) interaction between pH and $[H_2O_2]$ and (C) interaction between $[Fe^{2+}]$ and $[H_2O_2]$.

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).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^- \quad (4)$$

$$HO\bullet + Fe^{2+} \rightarrow HO^- + Fe^{3+} \quad (5)$$

$$Fe^{3+} + H_2O_2 \leftrightarrows [Fe\text{–}OOH]^{2+} + H^+ \quad (6)$$

$$Fe\text{–}OOH^{2+} \rightarrow HOO\bullet + Fe^{2+} \quad (7)$$

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.

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

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

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.

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.

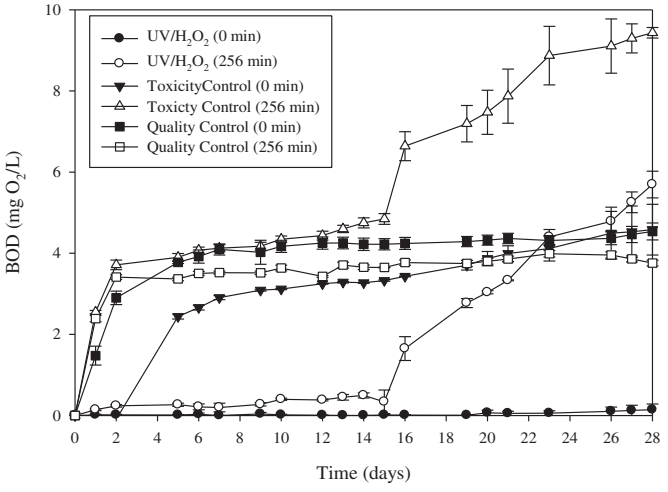


Fig. 3. Aerobic biodegradation (CBT) of 5-FU (0 min) and the photolytic mixture (256 min) formed after the of UV/H_2O_2 process (n = 2).

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

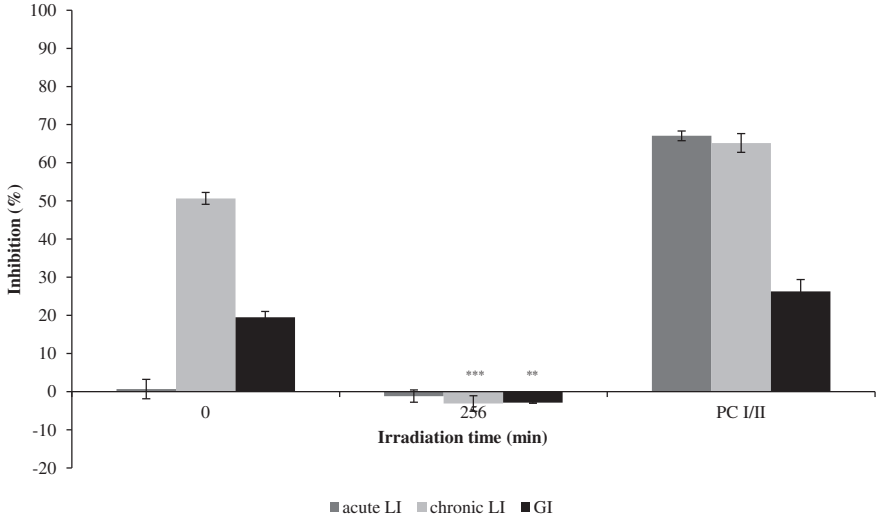


Fig. 4. Ecotoxicological assay tests with *V. fischeri* before (0 min) and after (256 min) the UV/H_2O_2 process (n = 2) considering three endpoints: acute luminescence inhibition after 30 min (acute LI), chronic luminescence inhibition after 24 h (chronic LI) and growth inhibition after 14 h (GI) Positive 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(−) MS^2 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(+) 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 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

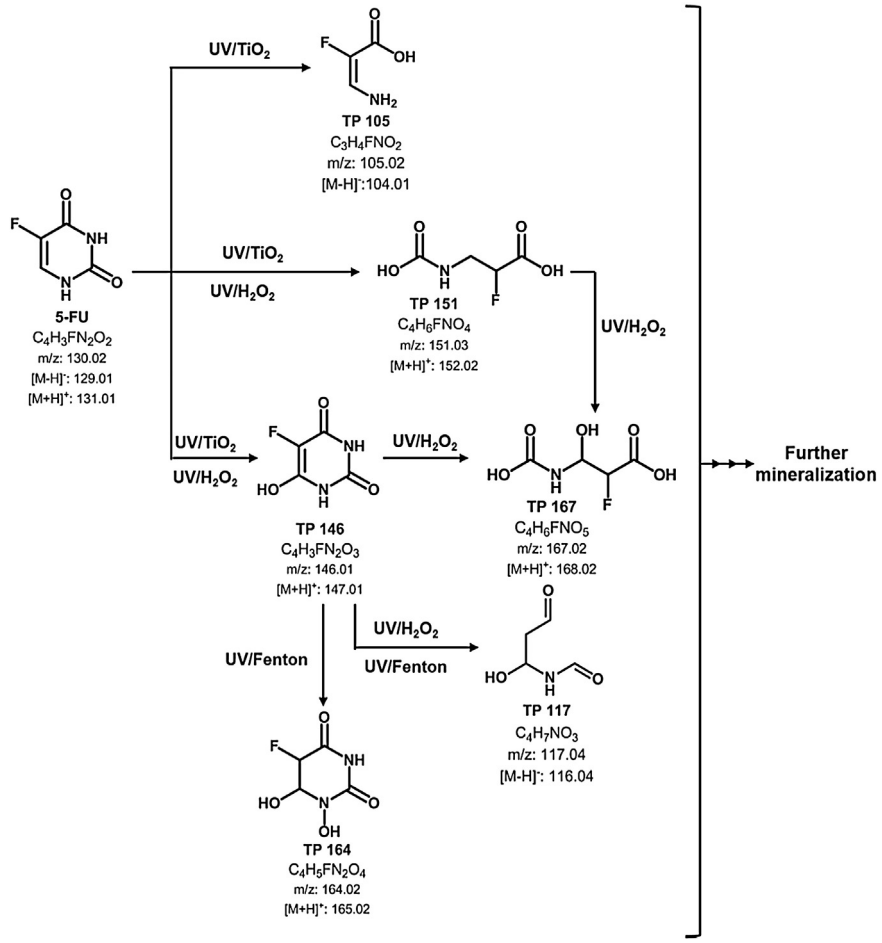


Fig. 5. Proposed degradation pathway of 5-FU by means of the different photocatalytic processes based on HO• generation (UV/TiO_2, UV/H_2O_2 and UV/Fenton).

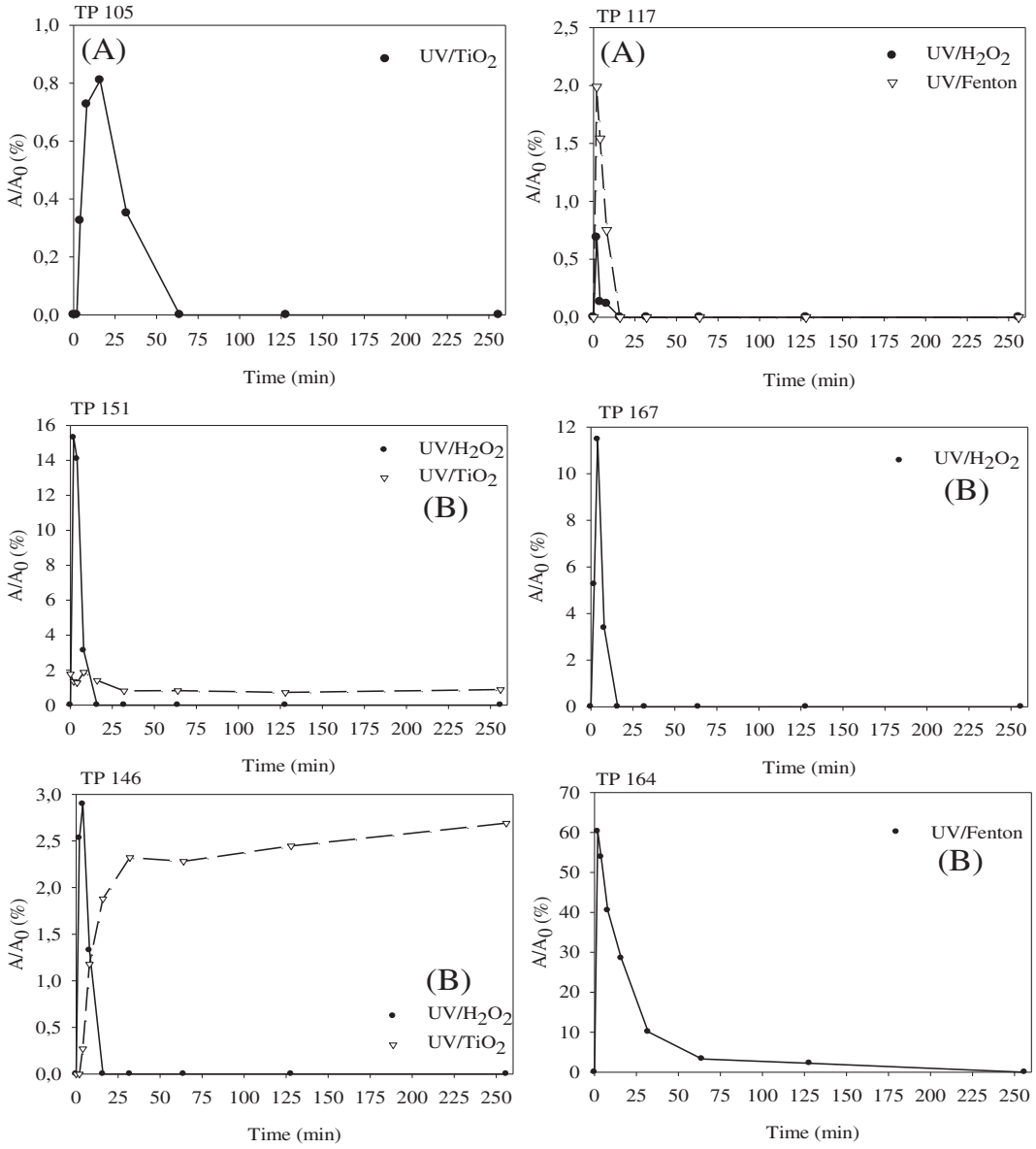


Fig. 6. Profile of the peak area ratio (A/A_0) of the TPs identified in negative ion mode by means of (A) LC–HRMS and positive ion mode by (B) LC–IT-MS during the different photocatalytic process.

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.

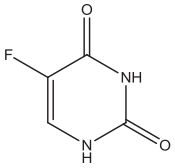
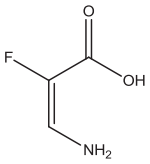
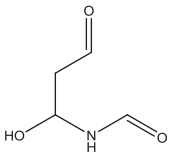
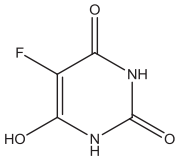
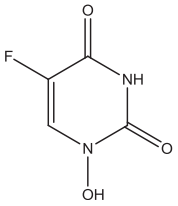
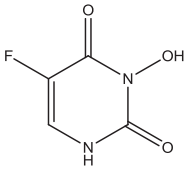
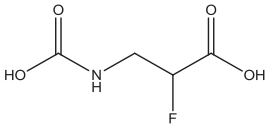
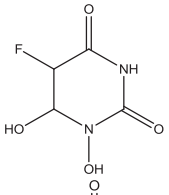
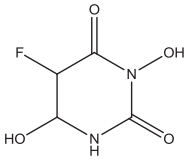
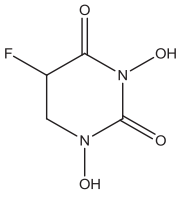
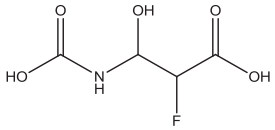
Name, MS (m/z), Rt (min)	Structures	CASE Ultra QSAR model				
		A	B	C	D	E
5-FU, (130.09); (4.45)		+	IN	+	+	−
TP 105, (104.01); (4.23)		OD	OD	+	OD	OD
TP 117, (116.03); (9.02)		−	OD	−	OD	−
TP 146 I, (147.0); (2.0)		OD	OD	+	−	−
TP 146 II, (147.0); (2.0)		IN	+	+	+	IN
TP 146 III, (147.0); (2.0)		IN	IN	+	IN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

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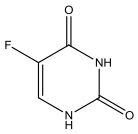
Table S1 – Structure and main physico-chemical properties of 5-fluorouracil

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

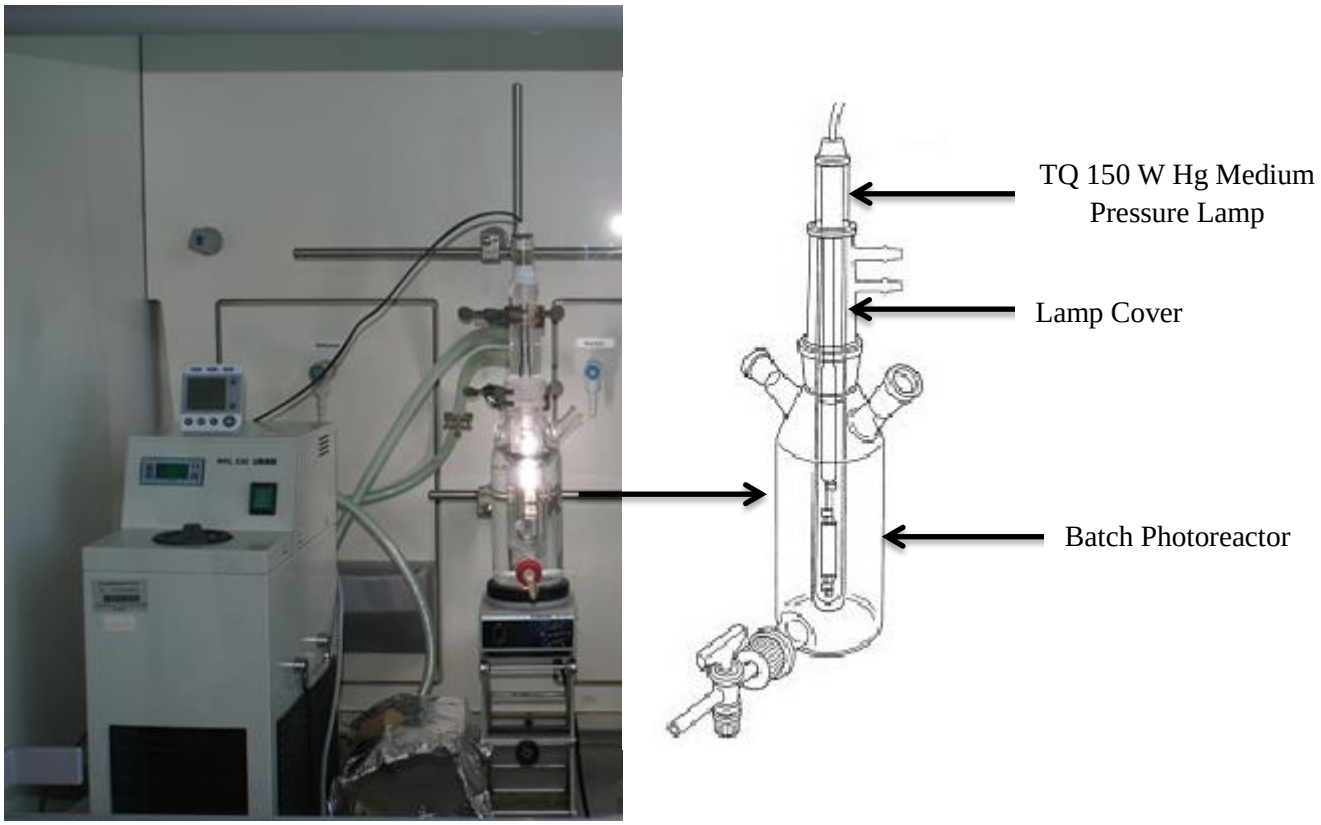


Fig. S1 Schematic of the lab scale plant used to perform the photodegradation experiments.

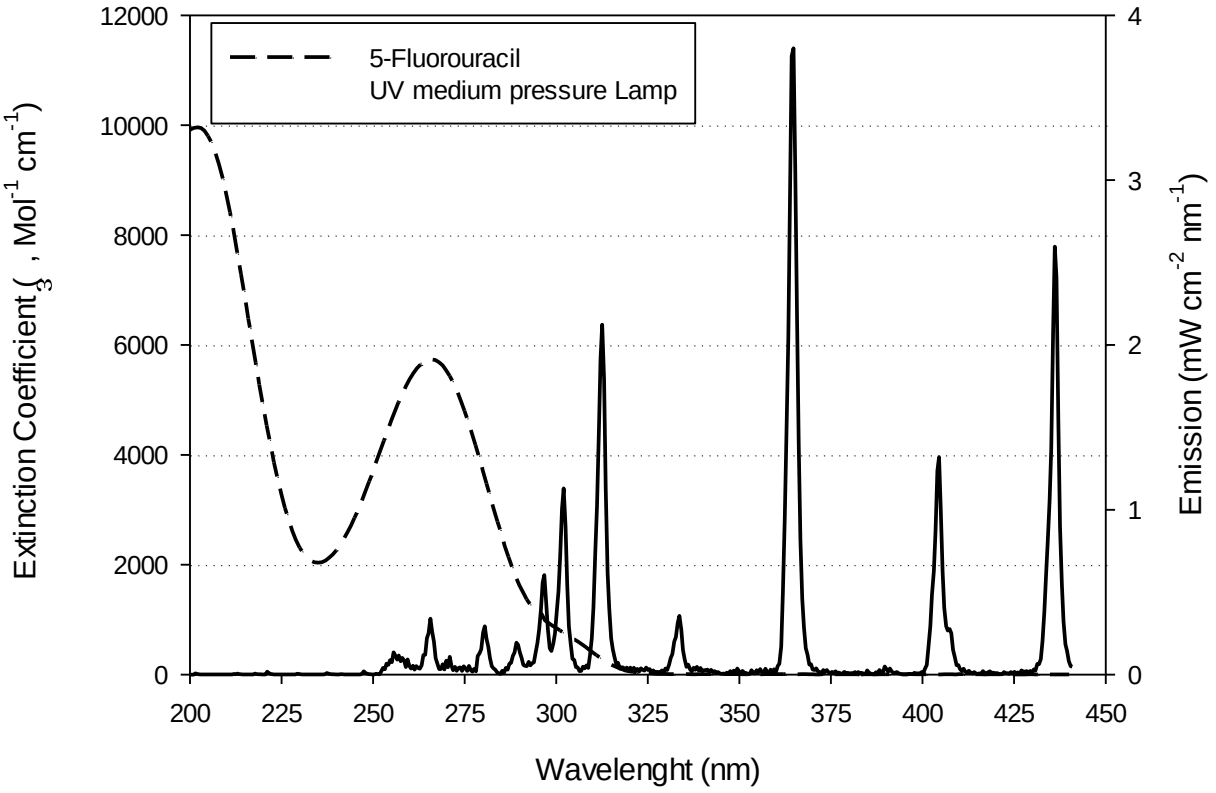


Fig. S2 – UV-VIS absorption spectrum of the 5-FU compounds at pH 7 in the wavelength range of $200 < \lambda < 440$. Concentration 10 mg/L in ultrapure water.

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 octopole two	10.71 V 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.

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

Fig. S3

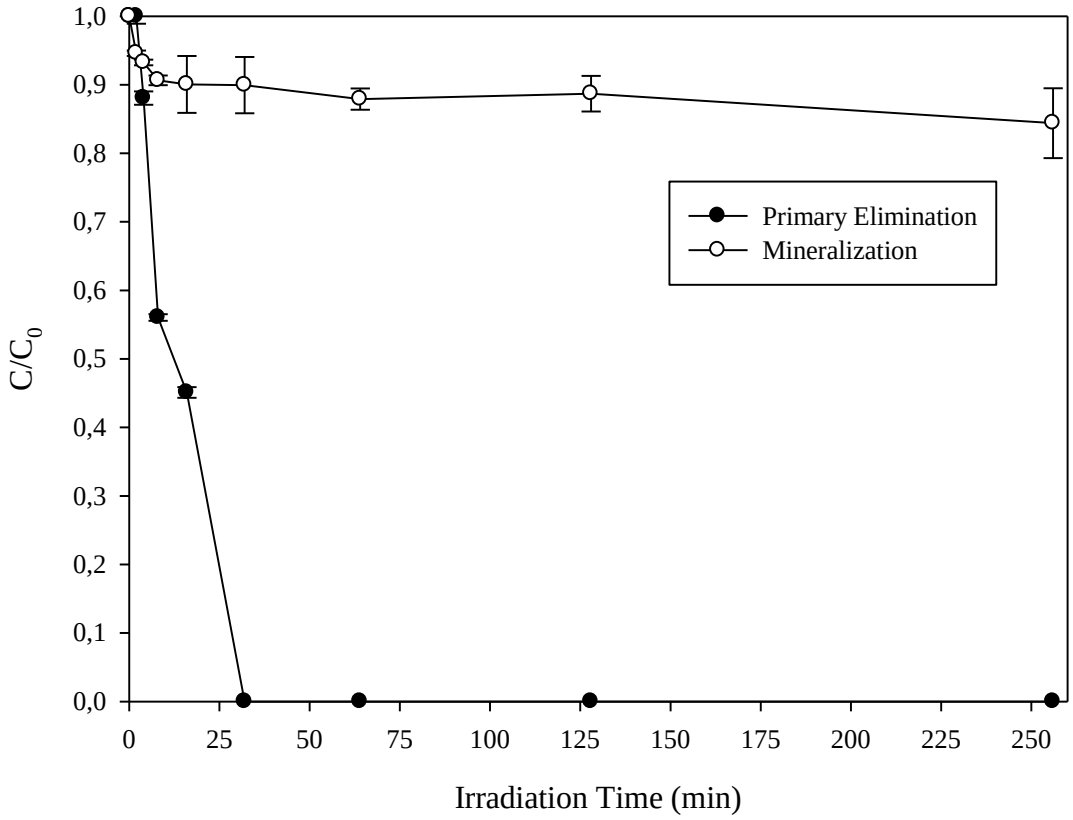


Fig. S3 – Primary Elimination and Mineralization of 5-FU under UV radiation using Hg lamp.

Fig. S4

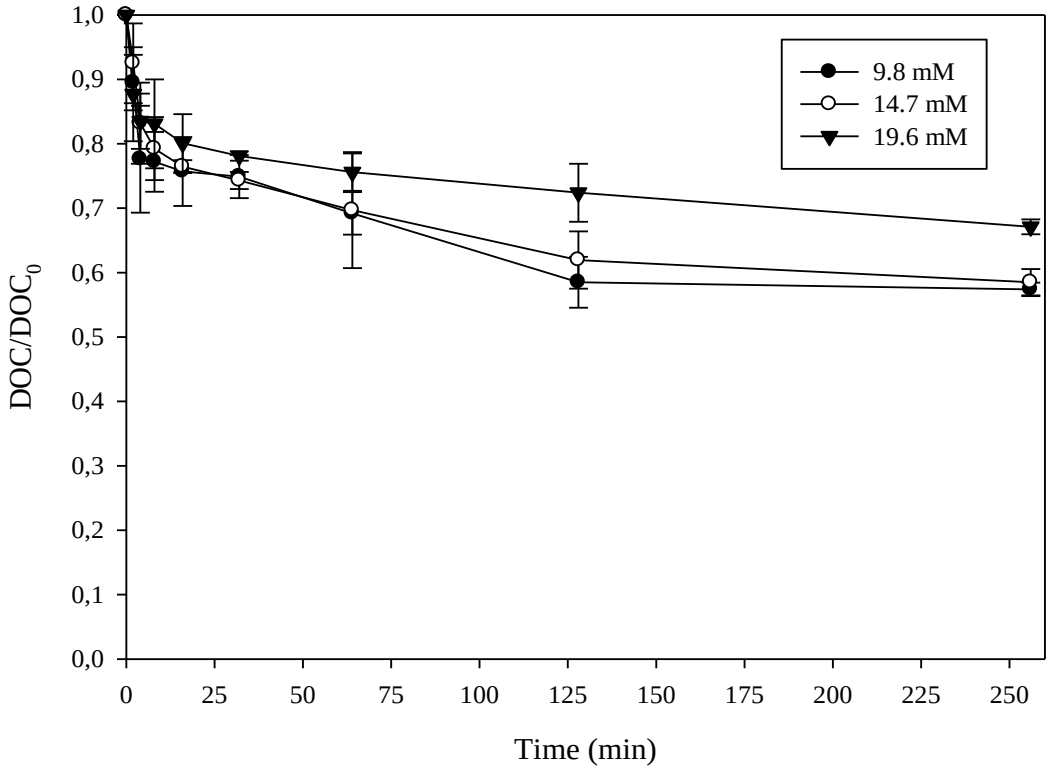


Fig. S4 – Effect of the H_2O_2 concentration on the mineralization rates of 5-Fluorouracil. (Concentration of 5-FU 20 mg/L)

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

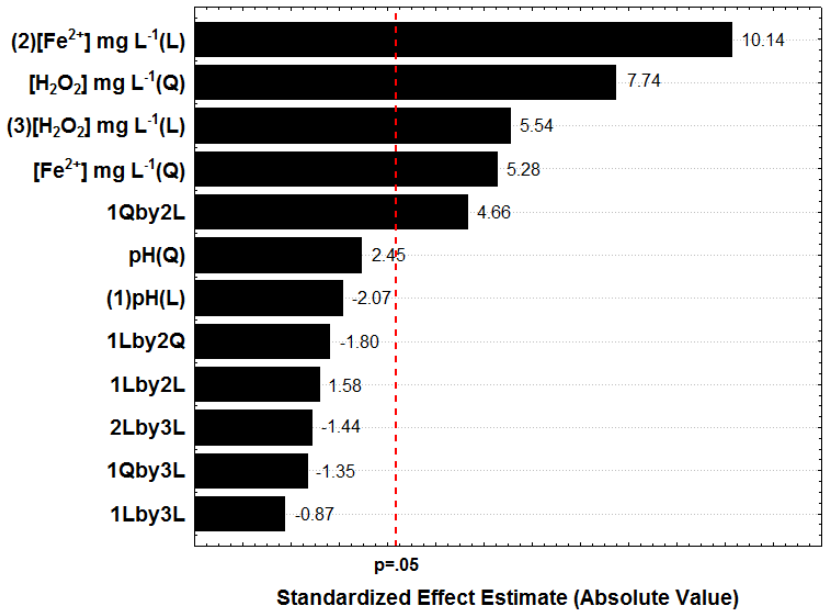


Fig. S5 – Pareto chart of effects of BBD applied for photo-Fenton treatment with a significance above 95% (p = 0.05).

Fig. S6

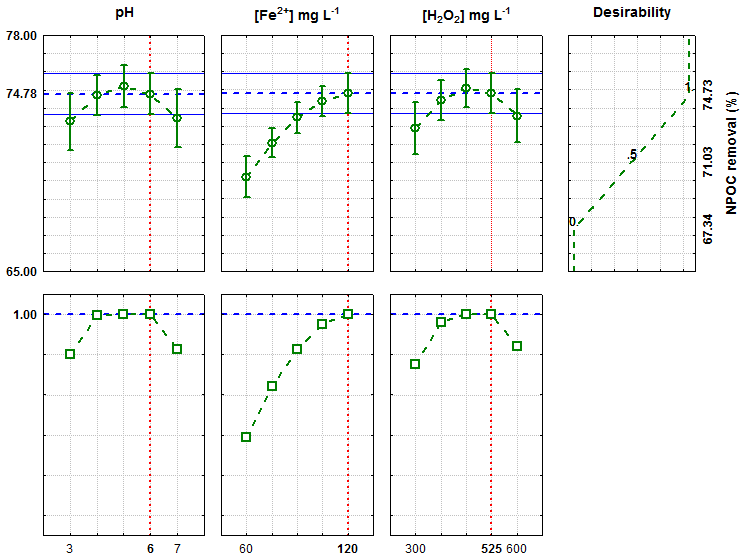


Fig. S6 – Desirability profile of the variables pH, [Fe^{2+}] and [$H2O_2$] of photo-Fenton process considering the optimized results of NPOC removal by means of BBD.

Fig. S7

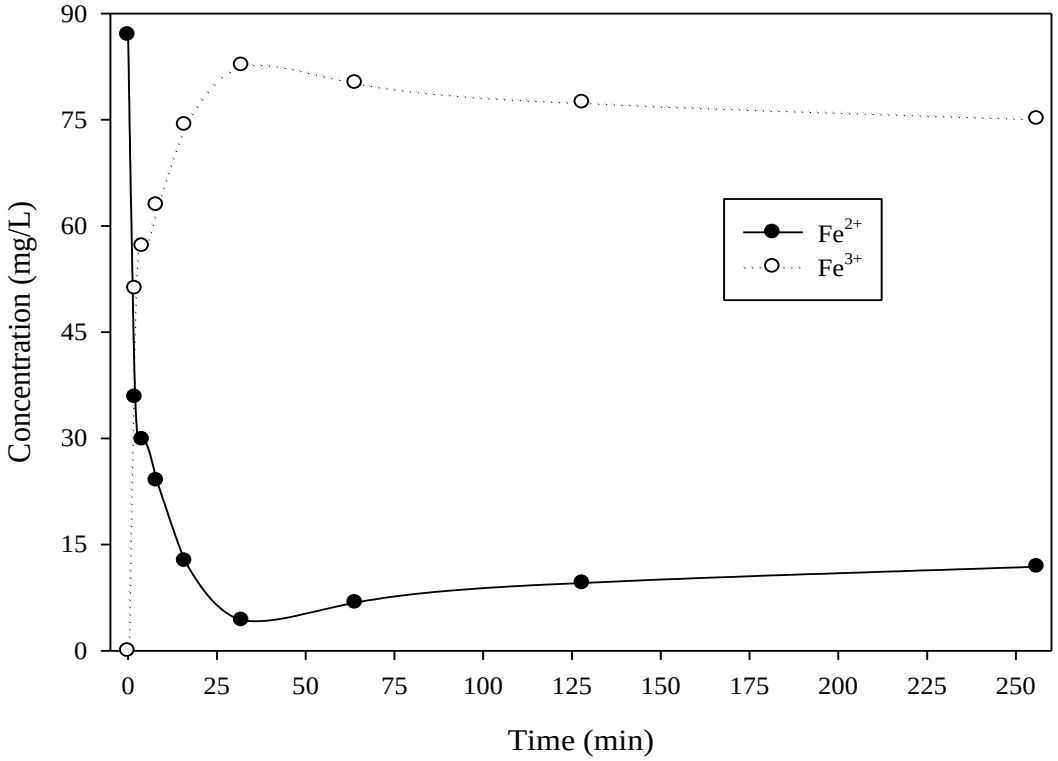


Fig. S7 – Consumption of ferric and ferrous ions during the UV/Fe^{2+}/H_2O_2.

Fig. S8

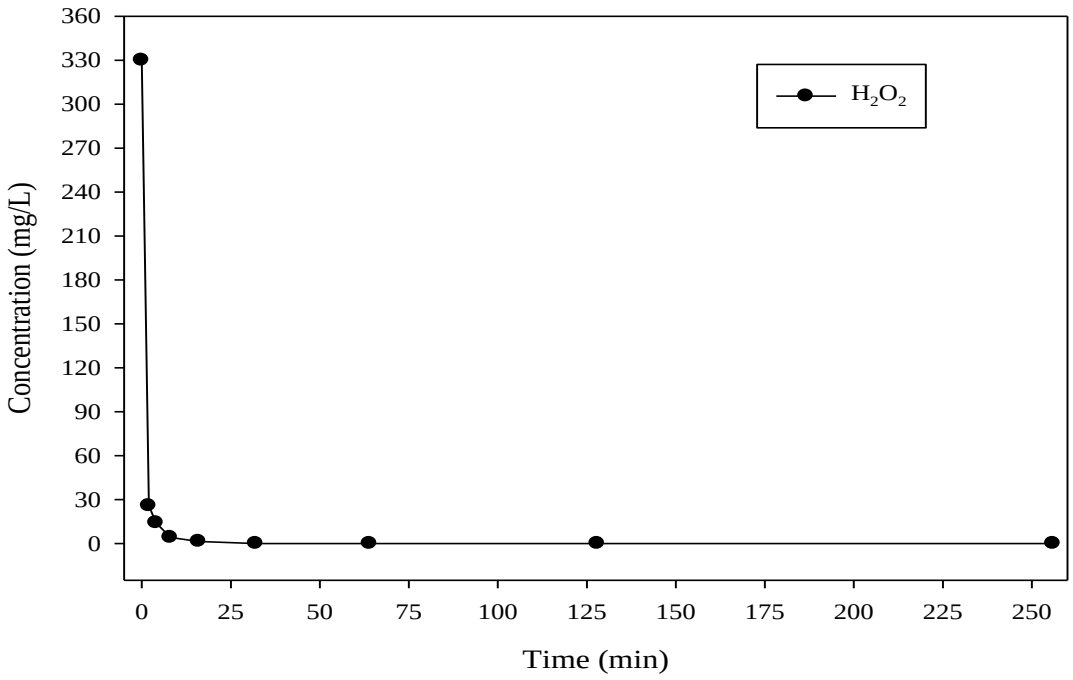


Fig. S8 – Consumption of H_2O_2 during the photo-Fenton treatment

Fig. S9

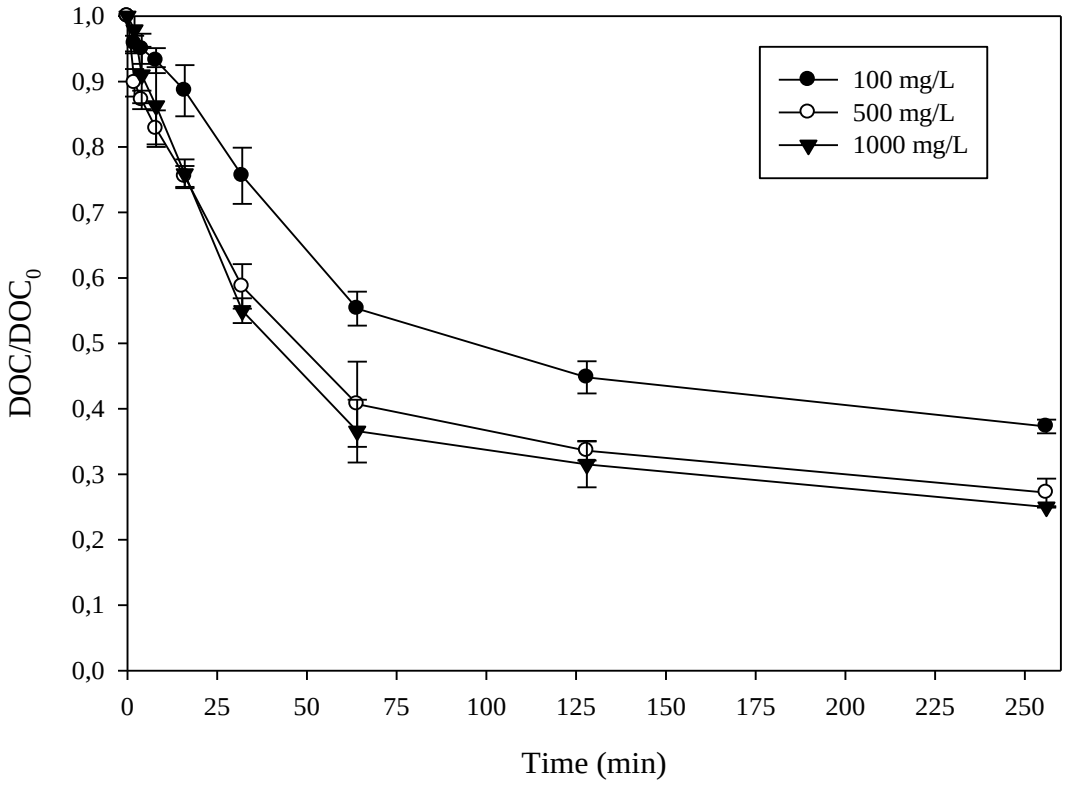
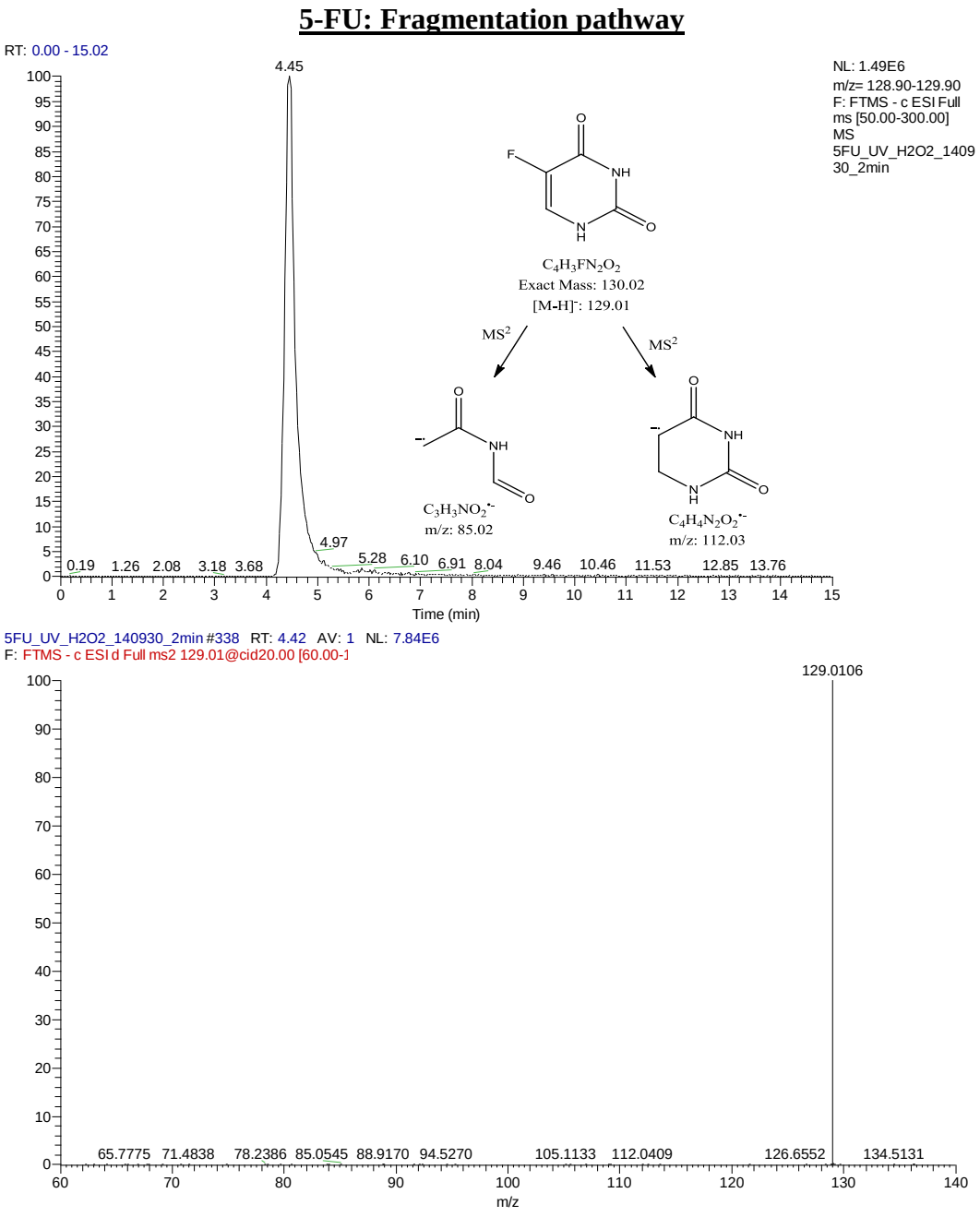


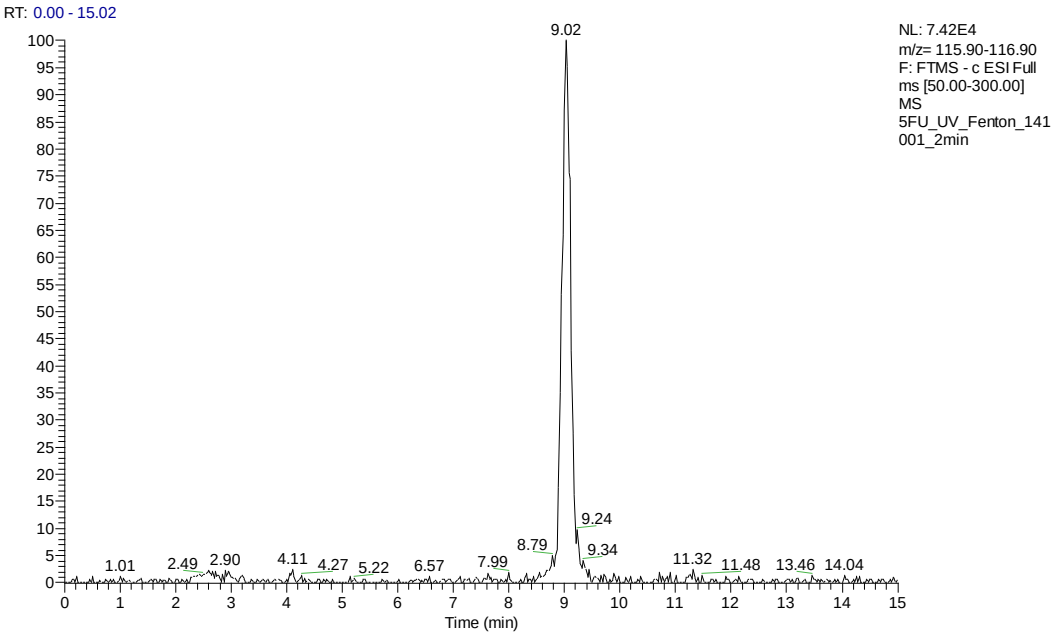
Fig. S9 – Effect of catalyst loading on the mineralization rates of 5-Fluorouracil. (Concentration of 5-FU 20 mg/L), n = 3

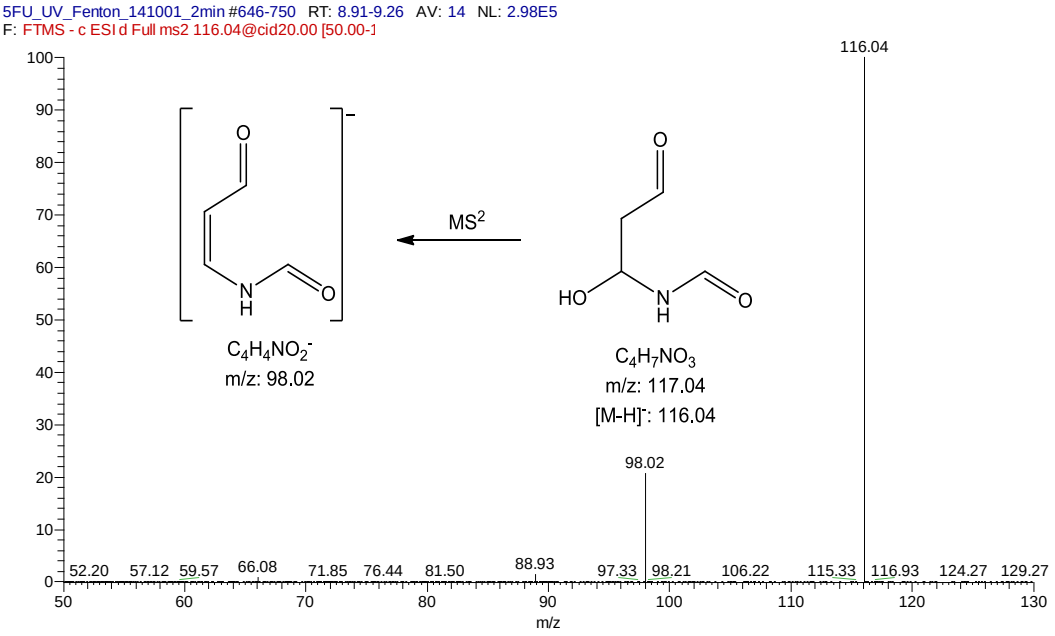
Text S4 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-HRMS.



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

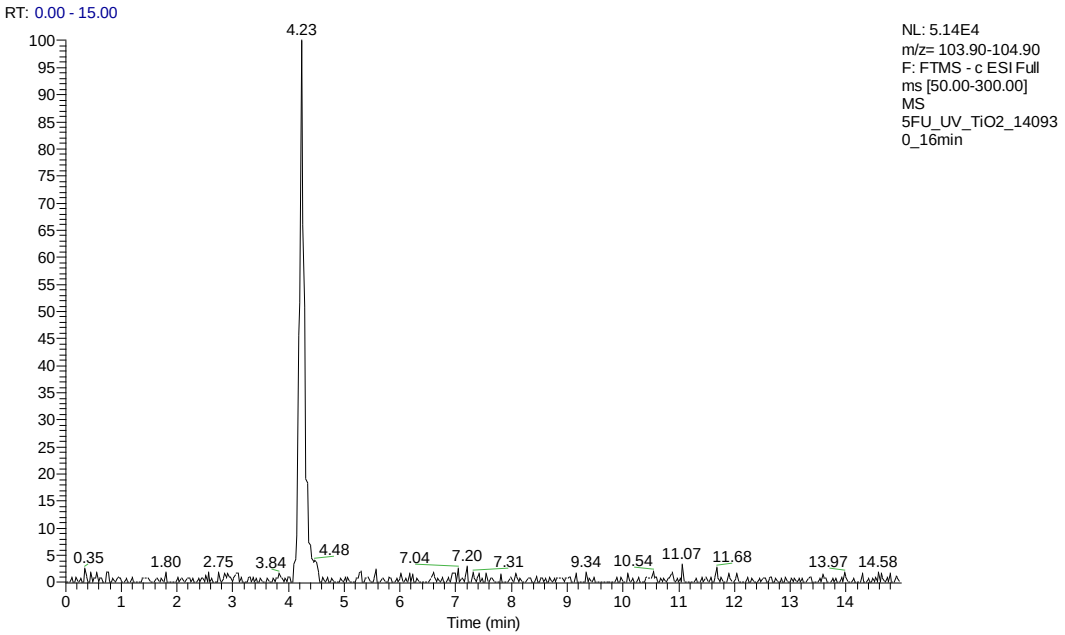
TP 117: Fragmentation pathway

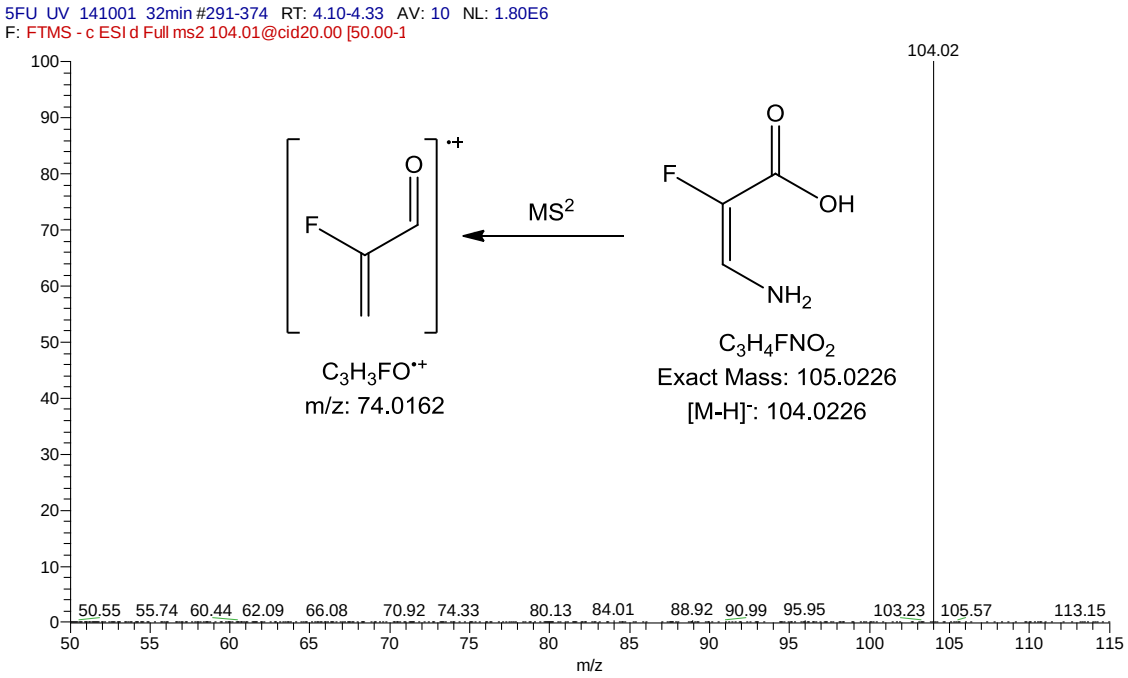




m/z [M-H]⁻	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
116.03437	$C_4H_6O_3N$	298101.5	100	2.5	-0.946	Even
98.02458	$C_4H_4O_2N$	61540.4	20.64	3.5	-1.315	Even

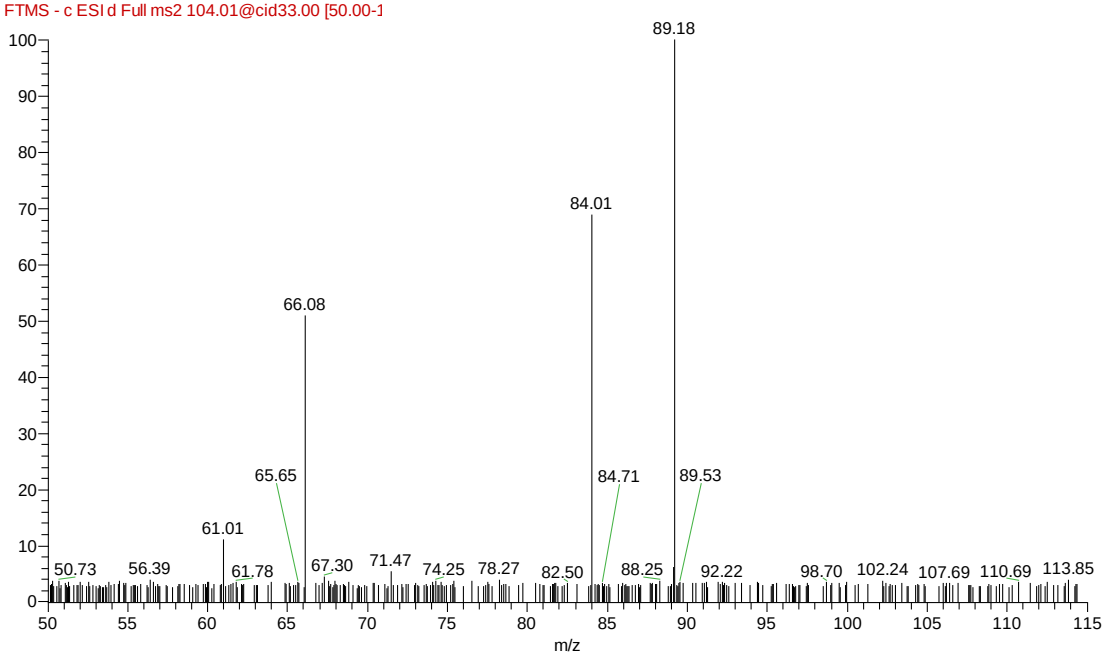
TP 105: Fragmentation pathway





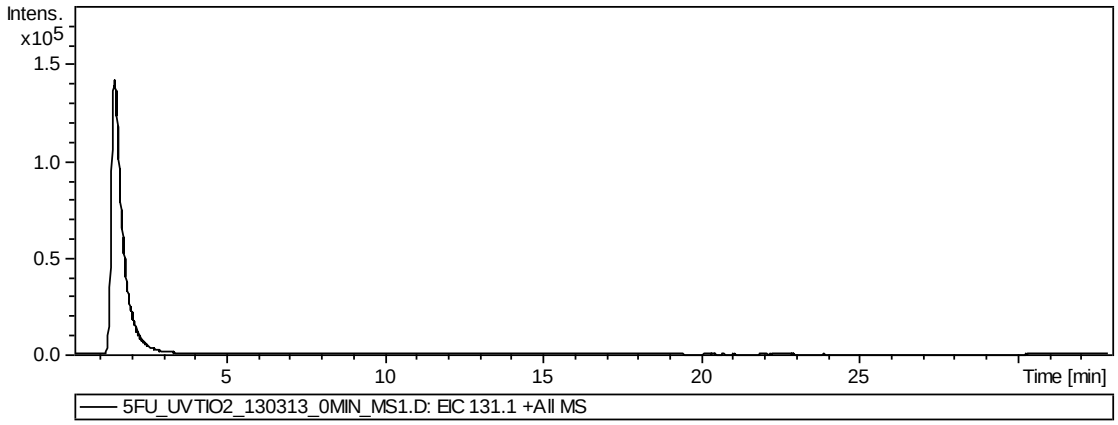
m/z [M-H]⁻	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N-rule
104.01528	$C_3H_3O_2NF$	568393.5	100	2.5	-0.050	Even
74.01685	C_3H_3OF	1982	0.35	2.0	-0.491	Odd

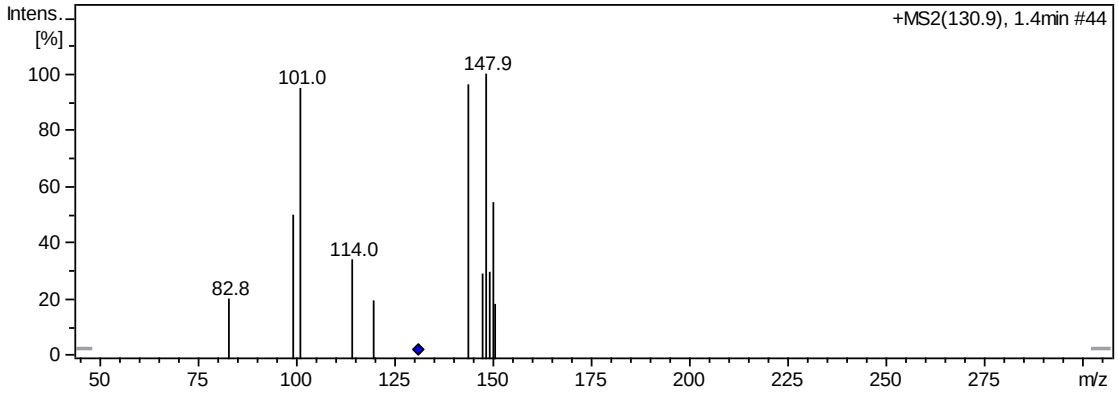
5FU_UV_141001_64min #303-352 RT: 4.08-4.34 AV: 11 NL: 5.51E3
F: FTMS - c ESI d Full ms2 104.01@cid33.00 [50.00-1

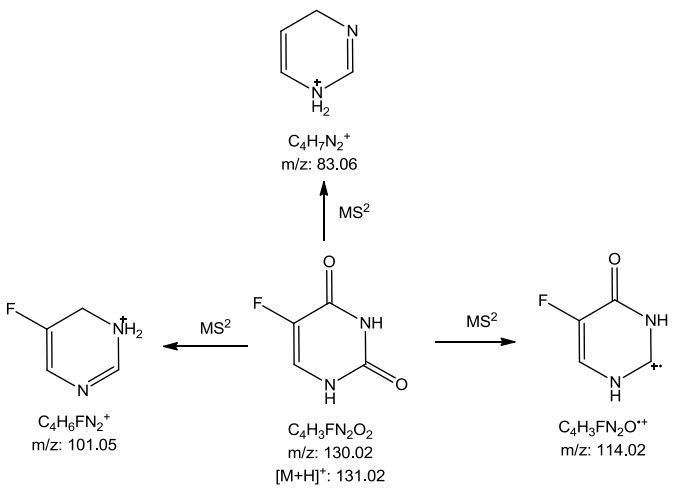


Text S5 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-IT-MS/MS.

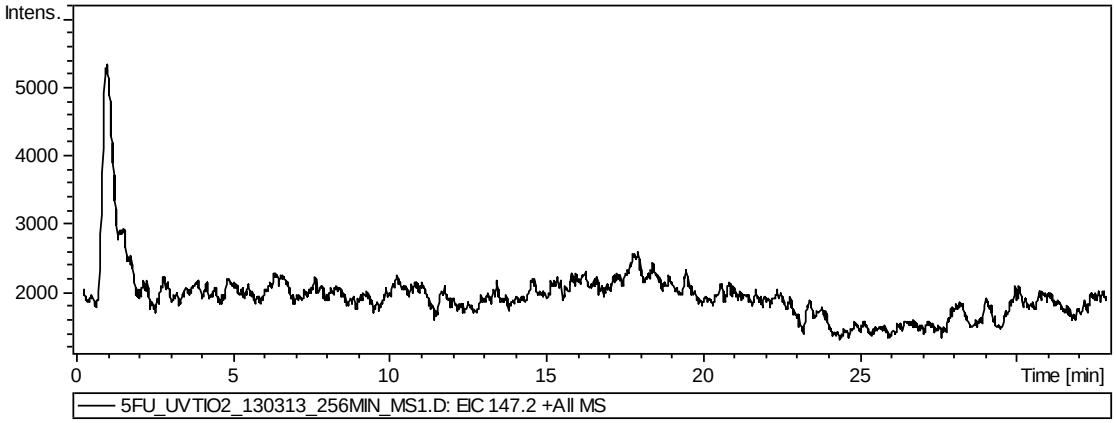
5-FU

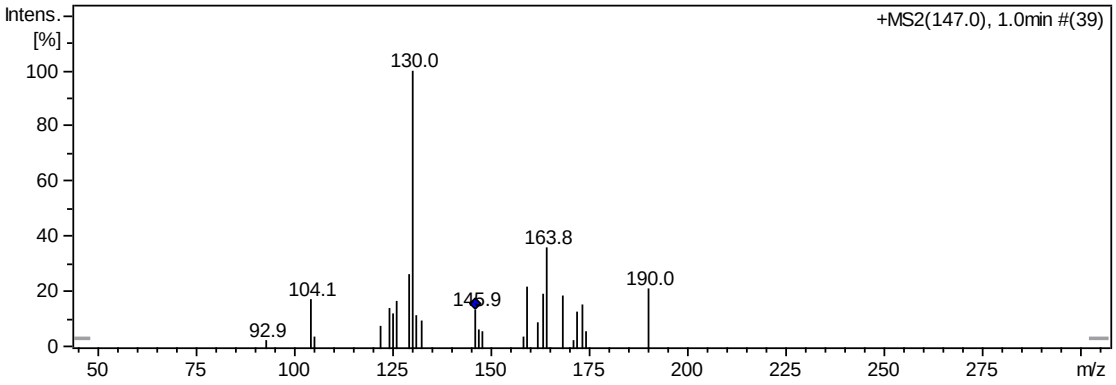


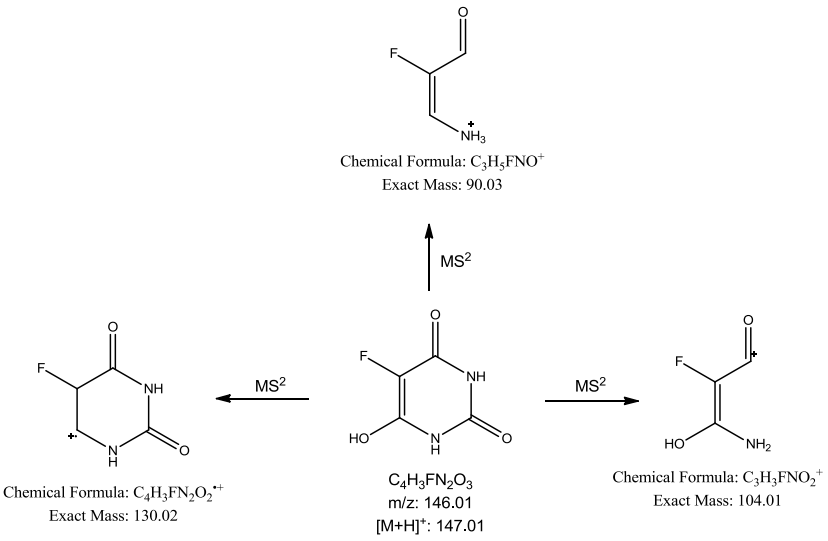




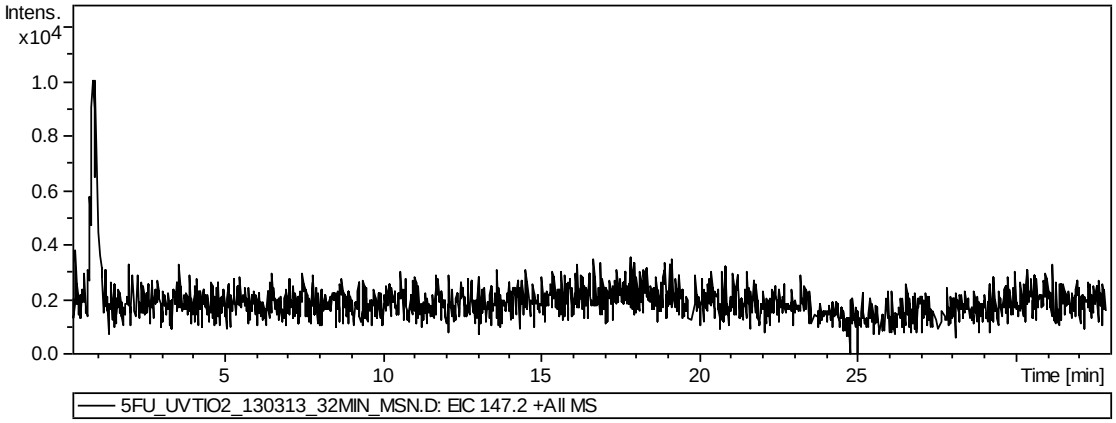
TP 146: Fragmentation pathway

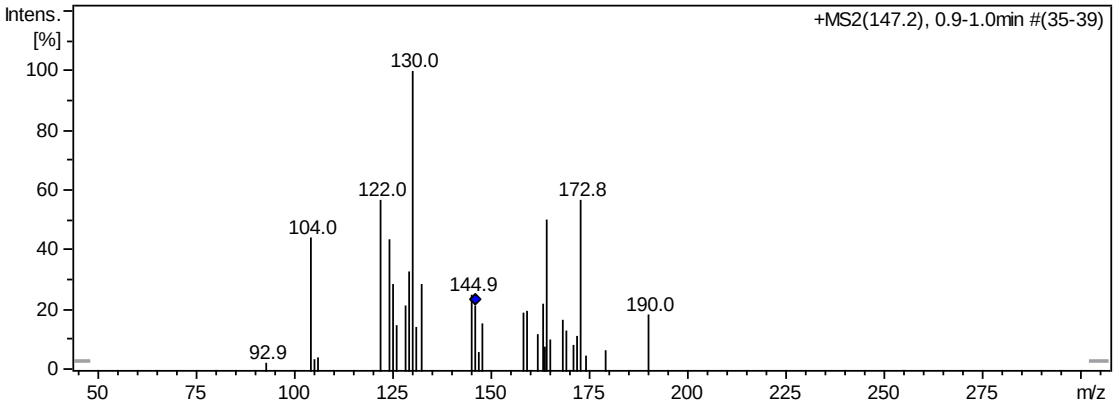


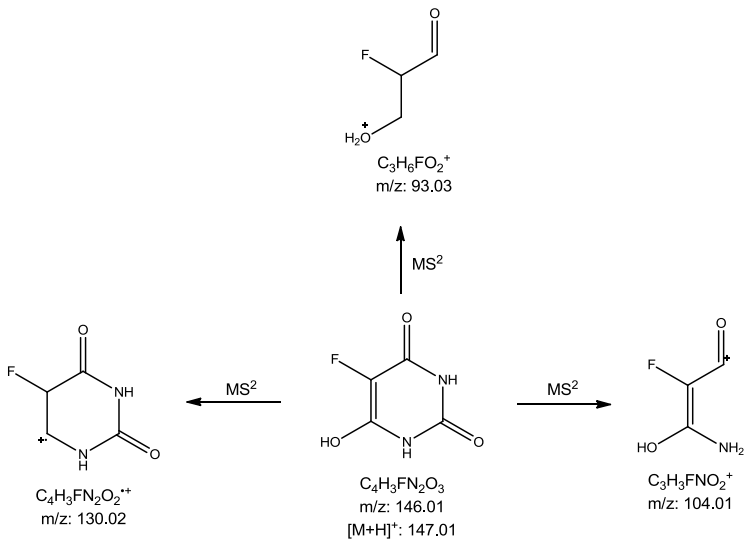




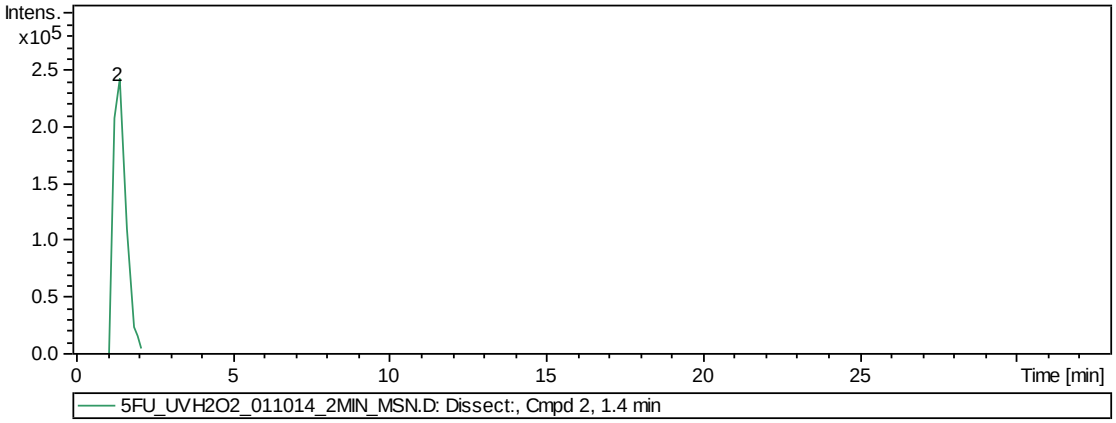
TP 148: Fragmentation pathway

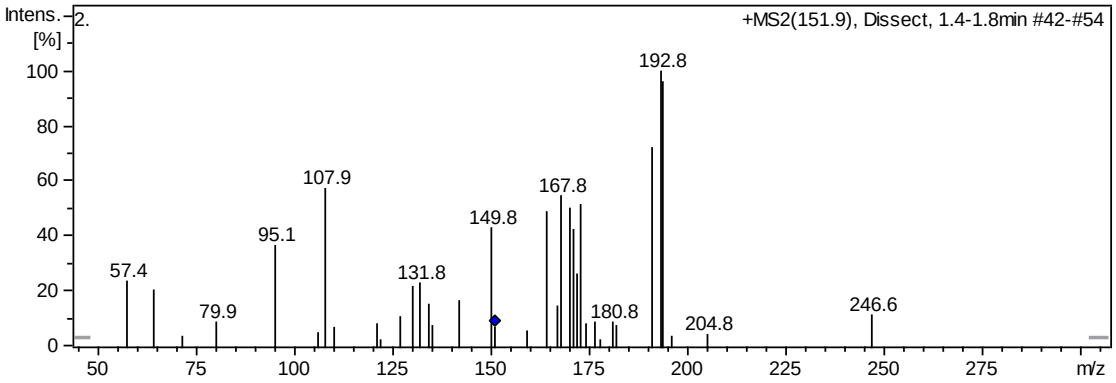


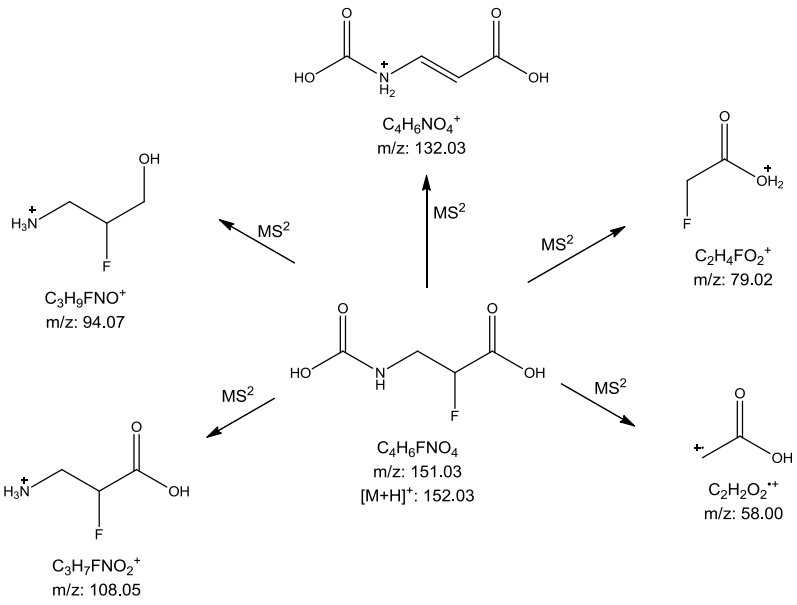




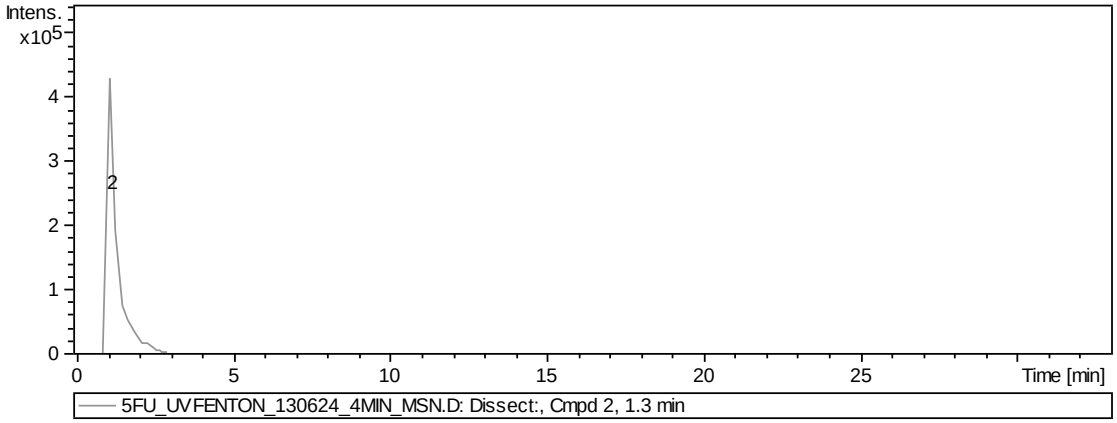
TP 151: Fragmentation pathway

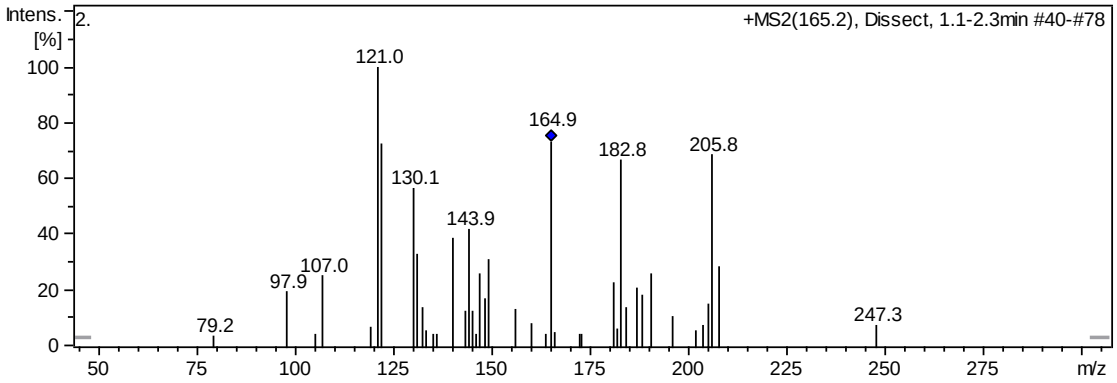


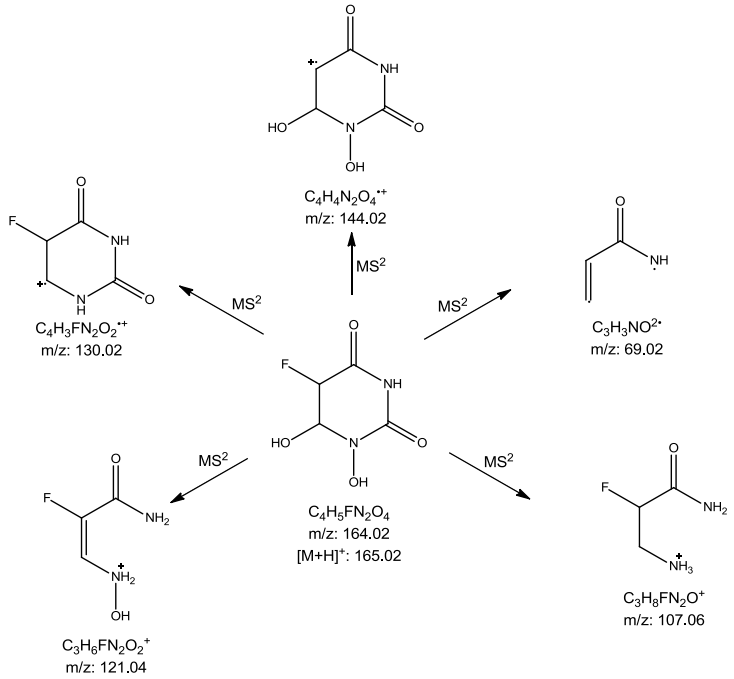




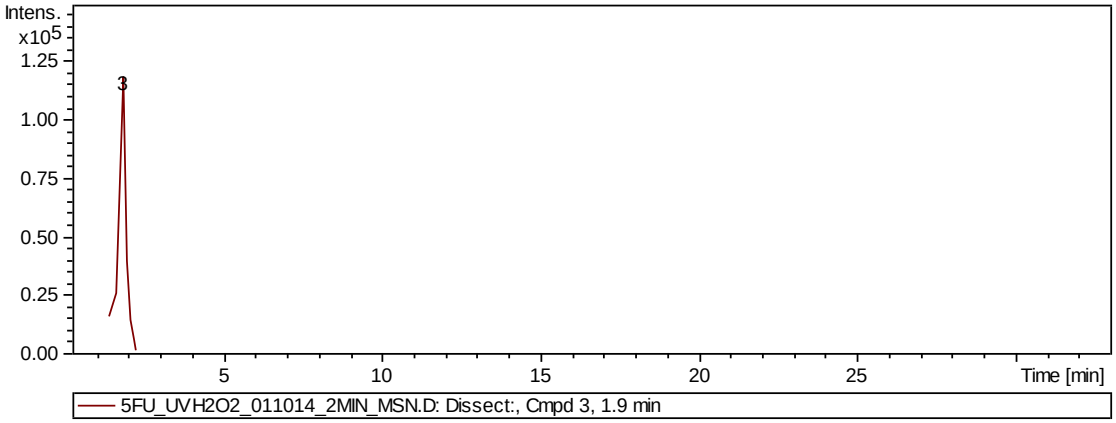
TP 164: Fragmentation pathway

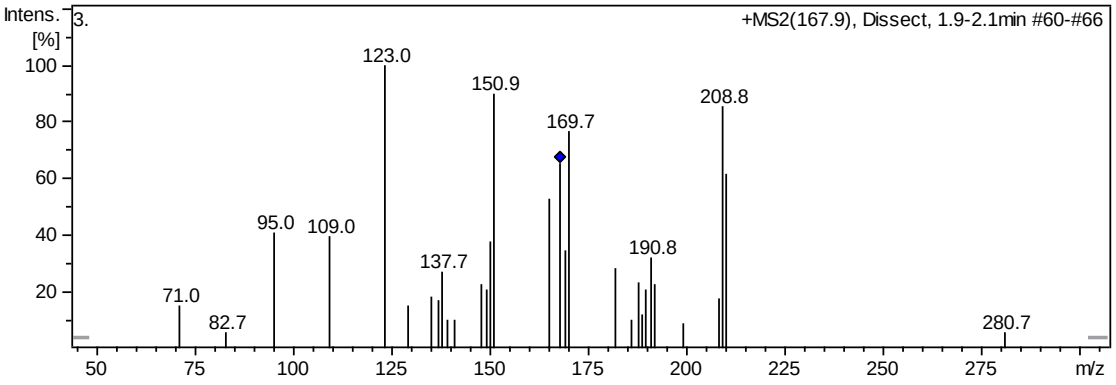


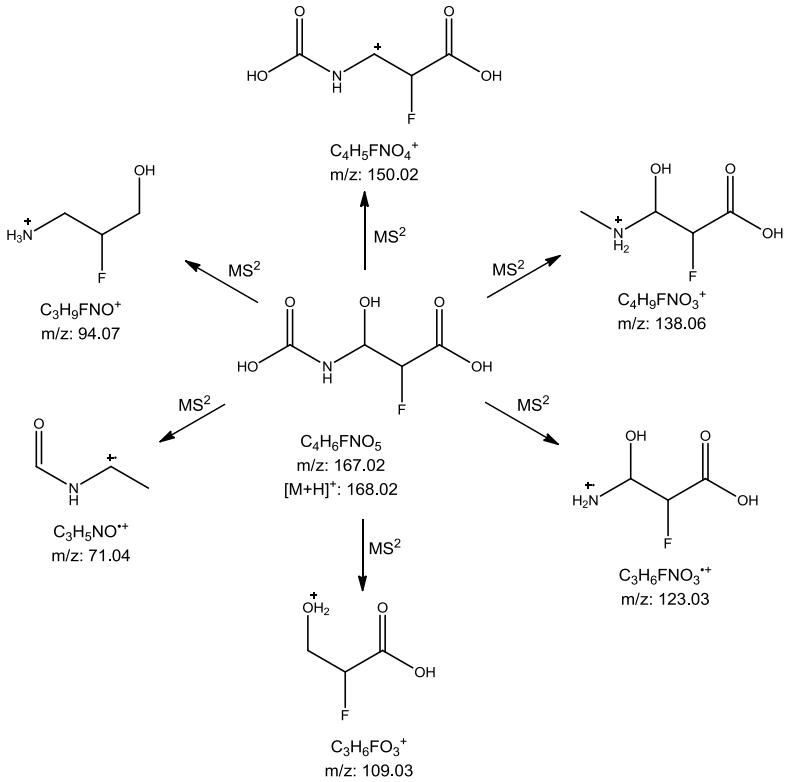




TP 167: Fragmentation pathway







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