Whole mixture toxicity assessment accounting for genotoxicity of transformation products in mixtures derived from photolysis of pharmaceuticals

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3. **Anju Priya Toolaram**, Tarek Haddad, Christoph Leder, Klaus Kümmerer Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a combination of experimental and *in-silico* testing, *(submitted)*

4. **Anju Priya Toolaram**, Jakob Menz, Tushar Rastogi, Christoph Leder, Mandy Schneider, Klaus Kümmerer Genotoxicity and cytotoxicity characterization of mixtures generated from photolysis of the ß-blockers Atenolol and Metoprolol using a combination of experimental and (Q)SAR approaches, *(submitted)*

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Erklärung

Ich versichere, dass ich die eingereichte Dissertation <u>Whole mixture toxicity assessment accounting for genotoxicity of transformation products in mixtures derived from photolysis of pharmaceuticals</u> selbstständig und ohne unerlaubte Hilfsmittel verfasst habe. Anderer als der von mir angegebenen Hilfsmittel und Schriften habe ich mich nicht bedient. Alle wörtlich oder sinngemäß anderen Schriften entnommenen Stellen habe ich kenntlich gemacht.

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Anju Priya Toolaram

27th April, 2015

"Lil bai nah clim ladda fuh tun big man"

Guyanese proverb

"Thirty spokes share the wheel's hub;

It is the center hole that makes it useful.

Shape the clay into a vessel;

It is the space within that makes it useful.

Cut doors and windows for a room;

It is the hole that make it useful.

Therefore profit comes from what is there;

Usefulness from what is not there."

Lao Tsu, Tao To Ching

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List of Abbreviations

AOP	Advance oxidation process
ATL	Atenolol
CHO-K1	Chinese hamster ovary cells
CIP	Ciprofloxacin
CI	Combination Index analysis
CYC	Cyclophosphamide
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
FDA	US Food and Drug Administration
5-FU	5-Fluorouracil
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ISO	International organization for standardization
LC-MS	Liquid chromatography tandem mass spectrometry
MN	Micronucleus
MTL	Metoprolol
MTX	Methotrexate
NPOC	Non-purgeable organic carbon
OECD	Organization for Economic Co-operation and Development
ONPG	o-nitrophenyl-ß-D-galactopyranoside
PC	Parent compound
PPL	Propranolol
QSAR	Quantitative Structure-Activity Relationship
ROS	Reactive oxygen species
TD	Thalidomide
TP	Transformation product
UV	Ultraviolet
WWTP	Wastewater treatment plant

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Summary

Pharmaceuticals are a class of micro-pollutants occurring in the environment. Often times these pharmaceuticals are present in a cocktail mixture inclusive of their metabolites resulting from biotransformation and transformed products (TPs) formed within various treatment and environmental processes. The fate and effects of these cocktail mixtures are relatively unknown. From an environmental aspect, there are guidelines that sought to characterize the aquatic toxicity of the pharmaceuticals and metabolites but rarely does it focus on parameters such as genotoxicity since it is accepted that this is thoroughly investigated in the drug design phase. Further, environmental risk assessment of pharmaceuticals generally neglects the TPs. As such, toxicity risk assessment that includes TPs is now a growing field of research. However, not much of this research is focused on genotoxicity assessment to include TPs. Thus, this dissertation was designed to review the sporadic methodologies found in literature and to develop a genotoxicity characterization scheme for simple mixtures derived from treatment of single pharmaceuticals. The work described here is specifically focused on understanding whole mixture genotoxicity with an emphasis on understanding a change in mechanism of genotoxicity and proposing TPs that may pose a genotoxic risk.

The objectives of this dissertation were addressed in four research articles (Paper I-IV). The investigative work was based on an effect driven approach using a battery of genotoxicity assays namely, the Ames test, the umu test and the *in vitro* micronucleus (MN) test. This battery of genotoxicity assays were applied to the parent pharmaceutical and its UV photolysis mixtures after several treatment periods. Toxicological data were supported with HPLC analysis to determine parent compound (PC) elimination and LC-MS analysis to monitor TP formation and identification. Dissolved organic carbon (DOC) elimination was also determined to assess the degree of mineralization. Additionally, Quantitative Structure-Activity Relationships (QSARs) software were used to predict the genotoxicity and relevant physico-chemical properties of the individual photo-TPs. The developed scheme of genotoxicity risk characterization was applied to photolytic mixtures of pharmaceuticals from different classes with varying modes of action. The pharmaceutical classes investigated included antineoplastic agents (cyclophosphamide (CYC), 5- fluorouracil (5-FU), methotrexate (MTX)), immunosuppressive agents (thalidomide (TD)), antibacterial agents (ciprofloxacin (CIP)) and ß- blocking agents (atenolol (ATL), metoprolol (MTL), and propranolol (PPL)).

Paper I focused on genotoxicity characterization of antineoplastic agents within an environmental risk assessment framework. The investigated antineoplastic agents comprised a broad spectrum of mechanism of action in various genotoxicity test systems. Although the Ames test was the most common test used, very high concentrations in the mg/L range were necessary for most antineoplastic drugs to produce a mutagenic response. The umu test gave similar results. The *in vitro* mammalian cell lines were much more sensitive and demonstrated genotoxicity at lower concentrations. Based on a review of the environmental risk assessment strategies in paper I, a genotoxicity risk assessment of drugs and their TPs would have to include a combination of appropriate analytical methods, genotoxicity bioassays, (bio) degradability and computer based prediction methods such as QSAR studies. The findings in this paper led to the design of the genotoxicity risk characterization scheme described in the methodology of this dissertation.

Paper II described investigations on the mutagenicity of TD and its photo-TPs in the Ames test. Although the irradiated mixtures were not mutagenic, QSAR predictions revealed that a few TPs have the potential to be mutagenic. QSAR also predicted that some TPs were genotoxic for several endpoints including *in vitro* chromosome aberration and *in vivo* micronucleus test.

Paper III was based on genotoxicity monitoring of CIP and its photolytic mixtures using an extended test battery i.e. the entire battery of the selected genotoxicity assays as described above. The mixtures of TPs and CIP resulting from irradiation were neither mutagenic in the Ames test nor genotoxic in the *in vitro* MN test. The irradiated mixtures were *umuC* inducing. Combination index analysis revealed that the main contributor to the *umuC* induction in the irradiated mixture was CIP. QSAR predictions suggested that the TPs may be capable of inducing chromosome aberration and mammalian mutation.

Paper IV and supplementary study (appendix 5) concentrated on the genotoxicity of ATL, MTL and PPL and their individual photolytic mixtures. In this case, again all three genotoxicity bioassays were applied. While MTL and ATL and their photolytic mixtures were not genotoxic, the photolytic mixtures of PPL were *umuC* inducing and mutagenic in *Salmonella typhimurium* TA 100 without metabolic activation. Photolytic mixtures of PPL and MTL were also cytotoxic to CHO-K1 cells. Correlation between QSAR predictions and TP formation have proposed several TPs for further risk assessment.

In summary, the results demonstrate that

- A genotoxic risk characterization to include TPs is necessary for pharmaceutical risk assessment.

- A compound-by-compound investigation is necessary as compounds from the same pharmaceutical class can behave differently (Paper I, IV).

- The use of supporting analytical data and QSAR predictions coupled with mixture toxicity analysis for whole mixtures were able to identify the main contributor to the observed genotoxicity in photolytic mixtures (Paper III).

- The use of a battery of mechanistic genotoxicity assays was able to identify mixtures with similar (Paper III) and dissimilar (Supplementary study, Appendix 5) mechanism of genotoxicity.

- The major conclusion is that there is the need for a combination of selected bioassays, analytics and computer based prediction models to assess simple whole mixture genotoxicity so that changes in genotoxicity potentials and possible genotoxic TPs can be identified. (Paper III, IV, Supplementary study, Appendix 5).

- The suggested TPs from the scheme applied here should be further characterized in an exposure driven approach.

- The scheme applied here is not an environmental risk assessment but can be a precursor to such an extensive study once the TPs are characterized and can be identified in the environment.

Zusammenfassung

Arzneimittel sind eine Klasse von Mikro-Schadstoffen, die in der Umwelt vorkommen. Oft kommen diese Arzneimittel in einer cocktailartigen Mischung vor, zusammen mit Metaboliten resultierend aus Biotransformation und Transformationsprodukten (TPs), die aus verschiedenen Behandlungen und Umweltprozessen hervorgehen können. Der Verbleib und die Auswirkungen dieser Mischungen sind relativ unbekannt. Es gibt zwar Vorschriften für die Charakterisierung der aquatischen Toxizität von Arzneimittelwirkstoffen und deren Metaboliten, Parameter wie Genotoxizität werden in diesen jedoch kaum berücksichtigt, da diese in der Regel bereits im Rahmen der Arzneimittelentwicklung gründlich untersucht werden müssen. Darüber hinaus werden in der Umweltrisikobewertung von Arzneimittelwirkstoffen Transformationsprodukte grundsätzlich nicht berücksichtigt. Deshalb ist die Toxizitäts-Risikobewertung unter Berücksichtigung von Transformationsprodukten ein Forschungsgebiet dem ein zunehmendes Interesse zu Teil wird. Allerdings steht die Untersuchung der Genotoxizität von Transformationsprodukten hierbei nur selten im Mittelpunkt. In Anbetracht dieser Tatsache wurde eine Arbeit konzipiert, mit dem Ziel, die vereinzelt in der Literatur beschriebenen Methoden zusammenzufassen und ein Schema für die Charakterisierung der Genotoxizität von einfachen Mischungen, die aus der Behandlung von einzelnen Arzneimittelwirkstoffen herrühren, zu entwickeln. Die hier beschriebenen Arbeiten zielen insbesondere darauf ab, die Genotoxizität ganzer Mischungen zu verstehen. Hierbei liegen die Schwerpunkte vor allem darin Änderungen der Genotoxizität auf mechanistischer Ebene nachzuvollziehen und Transformationsprodukte, die möglicherweise ein Risiko darstellen zu identifizieren.

Die Ziele dieser Arbeit wurden in vier wissenschaftlichen Aufsätzen (Publikation I-IV) adressiert. Die Forschungsbemühungen basierten auf einem effektorientierten Ansatz, wobei eine Batterie von Genotoxizitätstests, bestehend aus dem Ames-Test, dem umu-Test und dem *in vitro* Mikrokerntest, angewandt wurde. Diese Batterie aus Genotoxizitätstests wurde angewandt, um Arzneimittelwirkstoffe und resultierende photolytische Gemische zu verschiedenen Behandlungszeitpunkten zu analysieren. Die Daten zu Toxizität wurden mittels HPLC-Analytik zur Bestimmung der Primärelimination der Muttersubstanz und LC-MS Analytik zur Überwachung der Bildung von Transformationsprodukten mit anschließender Identifizierung ergänzt. Die Elimination des gelösten organischen Kohlenstoffs (DOC) wurde ebenfalls bestimmt, um den Grad der Mineralisierung zu untersuchen. Zusätzlich wurden

Computerprogramme zur Analyse von quantitativen Struktur-Eigenschafts-Beziehungen (QSARs) verwendet, um die Genotoxizität und relevante physikochemische Eigenschaften der jeweiligen Photo-TPs vorherzusagen. Das zuvor entwickelte Schema zur Genotoxizitäts-Charakterisierung wurde auf photolytische Gemische von Arzneimittelwirkstoffen verschiedener Klassen mit unterschiedlichen Wirkmechanismen angewendet. Die untersuchten Wirkstoffklassen beinhalteten Zytostatika (Cyclophosphamide (CYC), 5-Fluorouracil (5-FU), Methotrexate (MTX)), Immunsuppressiva (Thalidomide (TD)), Antibiotika (Ciprofloxacin (CIP)) und ß-Blocker (Atenolol (ATL), Metoprolol (MTL), und Propranolol (PPL)).

Publikation I behandelte die Charakterisierung der Genotoxizität von Zytostatika vor dem Hintergrund einer Umweltrisikobewertung. Die dabei untersuchten Zytostatika umfassten ein breites Spektrum von Wirkmechanismen in verschiedenen Testsystemen für Genotoxizität. Obwohl der Ames-Test am häufigsten eingesetzt wurde, waren sehr hohe Konzentrationen im mg/L-Bereich notwendig um mutagene Effekte hervorzurufen. Der umu-Test brachte ähnliche Ergebnisse hervor. Die *in vitro* Tests mit Säugetierzelllinien waren deutlich sensitiver und führten zu Ergebnissen in geringeren Konzentrationen. Basierend auf der Literaturstudie über die Strategien der Umweltrisikobewertung, die in Publikation I beschrieben wird, sollte die Prüfung der Genotoxizität von Arzneimitteln und deren Transformationsprodukte eine Kombination aus geeigneten analytischen Methoden, Genotoxizitäts-Biotests, (Bio)abbaubarkeits-Versuchen und computerbasierte Vorhersagemethoden wie QSAR-Studien beinhalten. Die Erkenntnisse aus Publikation I führten zu der Entwicklung des Schemas zur Charakterisierung des Genotoxizitäts-Risikos, welches im Methodenteil dieser Arbeit beschrieben wird.

Publikation II beschreibt Untersuchungen zur Mutagenität von TD und den aus dem Abbau von TD resultierenden Photo-TPs im Ames-Test. Obwohl die bestrahlten Mischungen nicht mutagen waren, enthüllten QSAR-Vorhersagen, dass mehrere TPs mutagenes Potential besitzen. Zudem ergaben die QSAR-Vorhersagen zu verschiedenen Endpunkten, einschließlich *in vitro* Chromosomenaberration und *in vivo* Mikrokerntest, auch Hinweise auf eine Genotoxizität mancher Photo-TPs.

Publikation III behandelte die Genotoxizität von CIP und photolytischen Mischungen von CIP unter Verwendung einer erweiterten Testbatterie, d.h. die vollständige zuvor beschriebene Genotoxizitäts-Testbatterie. Die aus der Bestrahlung von CIP resultierenden photolytischen Gemische waren weder mutagen im Ames-Test noch genotoxisch im *in vitro* Mikrokerntest.

Die bestrahlten Mischungen waren *umuC*-induzierend. Die Berechnung des Kombinationsindex belegte, dass hauptsächlich Ciprofloxacin verantwortlich für die beobachtete *umuC*-Induktion der bestrahlten Mischung war. QSAR-Vorhersagen deuteten zudem darauf hin, dass die TPs möglicherweise Chromosomenaberrationen und Mutationen in Säugetieren hervorrufen könnten.

Publikation IV und Appendix 5 hatte die Genotoxizität von ATL, MTL und PPL einschließlich der jeweiligen photolytischen Mischung als inhaltlichen Schwerpunkt. In diesem Fall wurden erneut alle drei zuvor beschriebenen Genotoxizitäts-Biotests angewendet. Während MTL, ATL und die jeweiligen photolytischen Mischungen nicht genotoxisch waren, wurde für die photolytischen Mischungen von PPL sowohl eine *umuC*-Induktion, als auch eine Mutagenität ohne metabolische Aktivierung in *Salmonella typhimurium* TA 100 beobachtet. Photolytische Mischungen von PPL und MTL waren zudem zytotoxisch gegenüber CHO-K1 Zellen. Durch Korrelation mit QSAR-Vorhersagen und TP-Bildungskinetiken konnten mehrere TPs für eine tiefergehende Risikoanalyse vorgeschlagen werden.

Zusammenfassend konnte in dieser Arbeit folgendes demonstriert werden:

- Eine Charakterisierung des genotoxischen Risikos unter Berücksichtigung von TPs ist notwendig für die Risikobewertung von Arzneimitteln.
- Eine substanzbezogene, individuelle Untersuchung ist notwendig, da sich Verbindungen aus der gleichen pharmazeutischen Klasse unterschiedlich Verhalten können (Publikation I, IV).
- Mithilfe unterstützender Daten aus der chemischen Analytik und QSAR-Vorhersagen, in Kombination mit Mischungstoxizitäts-Analysen konnte der Bestandteil in den photolytischen Mischungen identifiziert werden, welcher hauptverantwortlich für die beobachtete Genotoxizität war (Publikation III)
- Eine Batterie aus mechanistischen Genotoxizitätstests konnte Mischungen mit ähnlichen (Publikation III) und unterschiedlichen (Appendix 5) Genotoxizitätsmechanismen identifizieren.
- Die wichtige Schlussfolgerung ist, dass eine Kombination von ausgewählten Biotests, chemischer Analytik und Vorhersagemodelle benötigt wird, um Gentoxizität auf der Ebene ganzer Mischungen zu bewerten, sodass Änderungen des genotoxischen

Potentials und mögliche genotoxische TPs identifiziert werden können. (Publikation III, IV,Appendix 5).

- Die unter Anwendung dieses Ansatzes als relevant eingestuften TPs sollten in einem effektorientiertem Ansatz weiter charakterisiert werden.

- Der hier beschriebene Ansatz stellt keine Umweltverträglichkeitsprüfung dar, kann jedoch als Vorläuferstudie zu einer umfangreichen Untersuchung dienen, nachdem zuvor identifizierte TPs ausreichend charakterisiert und in der Umwelt nachgewiesen wurden.

1.0 Introduction and Problem Statement

Pharmaceuticals are not only consumed by humans but are also readily used in veterinary medicine mainly as growth promoters and antibiotics. Generally, the quantity of pharmaceutical consumption varies from country to country. In 2001, about 38 000 tons of active compounds from pharmaceuticals were consumed in Germany, of which 6 000-7 000 tons per annum were of potential risk to the environment (Greiner and Rönnefahrt, 2003). In 2012, the amount of human pharmaceuticals consumed in Germany that were of potential risk to the environment rose to 8 120 tons (Ebert et al., 2014). This is only one example of the increasing trend in pharmaceutical consumption and these pharmaceuticals have numerous sources including washing or bathing oneself after topical application, excretion (in urine and feces), and disposal whether in municipal or hospital wastewaters to enter the environment (Daughton and Ruhoy, 2009).

Wastewater treatment plants (WWTPs) serve to eliminate or reduce the amount of pollutant released into the environment. However, an extensive review of Verlicchi et al. (2012) has emphasized that the degree of pharmaceutical removal in WWTPs varies mainly due to the different physico-chemical properties of the drugs and the operational conditions of the plants. In fact, some pharmaceuticals (e.g. Ibuprofen) are released untreated to environment (Verlicche et al., 2012). Additionally to treatment through conventional WWTPs, advanced oxidation processes (AOPs) such as ozonation, chlorination, photolysis and their various combinations and variants are actively considered as treatment processes for wastewater (Khetan and Collins, 2007). Further, biotic processes and abiotic processes such as sorption to sediments and photodegradation may change the concentration of pharmaceuticals in different environmental media (Halling-Sørensen et al., 1998; Heberer, 2002). Generally, the fate of pharmaceuticals from their treatment or presence in the environment can be either its complete mineralization to carbon dioxide and water resulting in no risk or its transformation to a more lipophilic compound that is not readily biodegradable or its conversion to a more hydrophilic persistent form (Halling-Sørensen et al., 1998). In the latter two cases, it becomes pertinent to evaluate their concentrations, fate and toxicity. All processes whether occurring in the environment or during treatment can transform pharmaceuticals and other micro-pollutants into many other possibly environmentally stable by-products commonly referred to as their transformation products (TPs). Occurrence of pharmaceuticals in surface waters and WWTPs effluent has been reported in the range of ng/l to μg/l (Halling-Sørensen et al., 1998; Kümmerer, 2001, Heberer,

2002, Verlicchi et al., 2012). Occurrence and characteristics of TPs are less known as this has been a relatively new field of research and therefore lack well established analytical methods for TP identification in the environment. Similarly, the extent of their effects are also relatively unknown.

The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) both have guidelines for environmental risk assessments of medicinal products. In both of these guidelines, standard toxicity testing is recommended with aquatic species of different trophic levels, i.e. representative of algae (OECD 201), invertebrates (OECD 211) and fish (OECD 210) (FDA, 1998, EMA, 2006). No genotoxicity testing is specifically stated but in both guidelines, tests deemed necessary based on the nature of the pharmaceuticals could also be used as long as they are appropriately justified and standardized (FDA, 1998, EMA, 2006). Wügler and Kramers (1992) defined genotoxins as "chemical and physical agents capable of inducing mutations and related genetic changes in living cells of living organisms." Change in the deoxyribonucleic acid (DNA) can result in negative consequences which may not only be genotoxic but also may be permanent and therefore mutagenic in nature. For compounds that are directly interacting with DNA there are no safe thresholds and as such, genotoxicity warrants a justified consideration in environmental risk assessment plans. In fact, many authors have shown that wastewaters especially hospital wastewater can be genotoxic (Gartiser et al., 1996; Hartmann et al., 1998, 1999; Giuliani et al., 1996; Wang et al., 2011).

The closest guidelines that explored genotoxicity of pharmaceuticals and their active metabolites are those used during the drug design phase. In fact, several genotoxicity tests have to be conducted to ensure the safety of the active pharmaceutical ingredients prior to their first in-human trials (Escobar et al., 2013). The International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidance on genotoxicity testing of pharmaceuticals intended for human use have specified the requirements of developing a battery of bioassays. The ICH recommended that the general features of a standard test battery included the assessment of mutagenicity in a bacterial reverse mutation test (Ames test) and genotoxicity in *in vitro* mammalian cells (recommended are the *in vitro* metaphase chromosome aberration (CA) assay, the *in vitro* micronucleus (MN) assay and/or the mouse lymphoma L5178Y) and/ or an *in vivo* assay (ICH, 2012). The combination of these tests can identify DNA damage and its fixation (ICH, 2012). The recommended battery of genotoxicity assays were also adopted in the regulatory guidelines of the EMA (in 2011) and the US FDA (in 2012).

In the environment, pharmaceuticals, their metabolites and their TPs are presented as mixtures with other micro-pollutants. These mixtures are very complex and understanding their toxicity including their genotoxicity can be difficult. The FDA guideline has stated that studies are required on the fate and effects of 'the active moiety and/or structurally related substances (SRSs), rather than on excipients, for example' (FDA, 1998).[1] Relevant SRS were deemed as those that are greater than 10% of the PC initial dose (FDA, 1998). This is as far as TPs are considered in the guidelines for environmental risk assessment. Researchers are now trying to understand the effects and risks of these TPs by studying much simpler mixtures of usually a single pharmaceutical treated from stimulated environmental and/or treatment processes. These simpler mixtures can investigate the change in toxicity between a parent pharmaceuticals and its TPs, mixture interactions, mixture toxicity thresholds and identify TPs of concerns for further characterization as well as the conditions under which they are formed. Escher and Fenner (2011) stressed the importance of assessing the toxicity of TPs since they often exhibit the same mode of toxic action and may even have an additive or synergistic effect in mixtures.

Although there is no established guideline on environmental risk assessment to include TPs, two approaches namely the exposure driven approach and the effect driven approach were proposed (Escher and Fenner, 2011). The first is the exposure driven approach that entailed the isolation and identification of the TPs formed during simulation studies, followed by toxicity or fate assessment. This method has been applied in a few studies on photolysis treated pharmaceuticals coupled with genotoxicity testing (Isidori et al., 2005, 2006, 2009). Identification and isolation of relevant TPs can be a difficult process and therefore may present a disadvantage to using this strategy. It is never clear whether all TPs were seen in the chromatographic analysis because of their unknown chromatographic behaviour as well as interference from the sample pretreatment. Each detector has also its own limitations, characteristics, specificity, and detection limits. Furthermore, different treatment conditions such as pH, concentration and others as well as different treatment procedures may result in the formation of different TPs at different concentrations. In any case, the identified TP may not be known and therefore no toxicity profile based on a known chemical structure would be available. The TP could be synthesized to characterize its biological effect (Schirmer, 2011), if the chemical structure could be established.

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[1] SRS was defined to encompass dissociated parent compound, metabolites, or degradants

The second approach is the effect driven approach that followed a tier system of analyzing toxicity of the TPs using a battery of bioassays as it moves from untreated to the treated phase. The most important aspect of this approach is the careful selection of the battery of bioassays. This approach has the advantage to explore mixture toxicity. However, it does not necessarily allow for the identification of the specific molecule(s) that may be responsible for the measured effect(s) and therefore lacks some information. It can be time consuming but it is mostly focused on detecting toxicity deviation from the PC (Schirmer, 2011). While this approach has been used by several authors such as Lunn et al. (1994), Hansel et al. (1996), Garcia-Käufer et al. (2012) and Vasquez et al. (2013) among others, rarely was the genotoxic fractions identified or further characterized. On the contrary, this approach has been better demonstrated in ecotoxicity testing in studies such as Neuwoehner et al. (2008), Escher et al. (2010) and Schirmer (2011).

Generally there has been a slower development in the genotoxicity assessment of micro-pollutants and their TPs when compared to ecotoxicity testing. The research literature all present varying methodology for genotoxicity assessment. For instance, Li et al. (2007) had performed liquid-liquid extraction of wastewater treated by photoelectrocatalytic degradation, dried and redissolved the 'concentrated' treated wastewater before testing it in the Ames test. Other authors such Burleson and Chambers (1982) and Chéltelat et al. (1996) have tested the treated wastewater without any form of concentration of the TPs. Hence, this dissertation was designed to review the sporadic methodologies found in literature and to develop a genotoxicity characterization scheme for simple mixtures derived from treatment of single pharmaceuticals. Moreover, the focus of the study is a characterization of the genotoxicity in photolytic mixtures with special emphasis on understanding the influence of TPs in the mixtures on the observed genotoxicity.

2.0 Research Goal

To develop and assess a whole mixture toxicity scheme that would take into consideration the genotoxicity of transformed products from photolysis treatment of pharmaceuticals.

2.1 Research Questions

(1)   What available test schemes are there that can consider the genotoxicity of pharmaceuticals, their metabolites and their TPs?

(2)   Which genotoxicity tests can be included in a battery of genotoxicity assays to investigate genotoxins formed from photolysis treatment of pharmaceuticals, and how effective are they?

(3)   Can pharmaceuticals and related TPs resulting from advanced oxidation treatment such as UV photolysis be assessed class-wise using a standard set of genotoxicity assays?

(4)   What other methods of investigations and toxicological analyses can be coupled to the genotoxicity whole mixture assessment of the pharmaceuticals and their treated mixtures to identify possible TPs of concern?

2.2 Research Objectives

(1)   To develop a scheme for assessing genotoxicity of mixtures of pharmaceuticals, their metabolites and their TPs.

(2)   To assess this scheme on whole mixture assessment of photolysis treated single pharmaceuticals of different classes with different mode of actions.

(3)   To incorporate analytical methods, structure identification, Quantitative Structure-Activity Relationship (QSAR) predictions and basic toxicological analyses with whole mixture genotoxicity assessment to identify possible TPs of concern.

3.0 Research Approach

Four research articles are presented in this dissertation that demonstrated the results in accordance to the objectives listed in section 2. These papers are herein referred to as Paper I to Paper IV. A list of the title of the research article corresponding to these paper can be found in Appendix 1-4.

The selection criteria for the investigated pharmaceuticals were as follows:

-        Antineoplastic agents (cyclophosphamide (CYC), 5- fluorouracil (5-FU), methotrexate (MTX) and immunosuppressive agents (thalidomide (TD)) - these are pharmaceuticals deemed as those with special importance as environmental pollutants (Kümmerer, 2001) and have an inherent genotoxic nature. (Paper I and II)

-        Antibacterial agents, Ciprofloxacin (CIP) - A known environmental genotoxin (Hartmann et al 1998, 1999) with an indirect genotoxic mode of action (Clerch et al., 1992; Albertini et al., 1995; Clerch et al., 1996). (Paper III)

-       Beta blocking agents (propranolol (PPL), atenolol (ATL), metoprolol (MTL)) - drugs with high sales volumes (Cleuver, 2005; Brambilla and Martelli, 2006; Küster et al., 2009) and high mass loading in wastewater treatment plants (Verlicchi et al., 2012). These drugs are also known non-genotoxins (Okine et al., 1983). (Paper IV, supplementary study, Appendix 5)

All of these substances were subjected to UV photolysis using TQ 150W medium pressure mercury lamp. All photolysis were done at high concentrations to accommodate the sensitivities of bioassays and identification of most TPs.

An extensive literature research was conducted on genotoxicity assessment of TPs. The results of which are chronicled in the review article (Paper I). The effect driven approach for risk assessment of TPs was selected to be expanded on to fulfill the objectives of this study. Paper I has outlined this research methodology. Figure 1 shows the general scheme of environmental risk assessment of pharmaceuticals to include TPs (a) and the general outlay of a genotoxic risk characterization scheme involving an effect driven approach (b).

a

Toxic ← Pharmaceutical → Biodegraded **b**

yes

No

Treatment under different conditions and varying treatment time

Degree of mineralization (< 20% NPOC) and PC elimination

Toxicity studies

Biodegradation studies

TP identification

Degree of degradation and PC elimination

QSAR studies

Toxicity studies

TP identification

Relevant TP isolation

Toxicity studies

Biodegradation studies

Treatment
- Initial concentration could be limited by; analytical method, bioassay sensitivity, MGC, non-genotoxic concentration
- Defined treatment time or multiple treatment time

Post-treatment
- To remove residual oxidants from test

Degree of mineralization
- Further analysis of mixtures only if NPOC > 20%

Genotoxicity assessment
- Treatment control (solvents treated and neutralized similarly to the samples)
- Positive control to validate test
- Negative control to validate test
- Solvent control for computation

Analytical Analysis
- Identification of TPs in mixture (LC-UVVis (and/or fluorescence) coupled with MS/MS)
- Monitoring of TP formation and development over time
- Estimation of the relative composition of the TPs and PC after a specific time of treatment

Analysis
- Mixture toxicity (additive, synergistic, antagonistic effects of mixture vs. PC only)
- Probable correlation of the TP development along treatment time to change in mixture toxicity over time
- Determination of the optimal treatment time and conditions for generation of non-toxic mixture

Further Analysis
- Possibilities for extraction and separation of TPs based on physico-chemical nature and toxicity assessment
- Combination of QSAR and toxicity testing to identify TPs of environmental concern and their magnitude of effect

Figure 1: (**a**) **General scheme of Environmental risk assessment of pharmaceuticals, metabolites and their TPs (b) An effect driven approach of genotoxic risk characterization for pharmaceuticals, metabolites and their TPs (Adopted from Paper I)**

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Since the focus of the research is on whole mixture genotoxicity characterization of the PC and its TPs, the decision was to use well established and standardized tests so that any changes between PC and TP mixtures can be better characterized. Hence, the two bacterial bioassays were selected and the *in vitro* micronucleus assay was performed using CHO-K1 cells, a cell line that has been used extensively in mammalian genotoxicity testing. The selected pharmaceuticals from different classes with different chemical structures, mode of action and genotoxic properties were applied to these bioassays.

In paper II-IV, the battery of genotoxic assays selected was based primarily on the ICH (2012) guideline and included:

(1)   Ames bacteria reversion assay or Ames test (Ames et al., 1973; OECD 471) using *Salmonella typhimurium* TA 100 and TA 98. This test was performed in a microplate format based on the fluctuation assay using the Ames MPF 98/100 Aqua test kit (Xenometrix AG). The Xenometrix test produced good correlation with the results of the standard Ames test (Flückiger-Isler et al., 2004). The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain. The strains that revert detect point mutations, either involving base substitution (TA 100) and/or frameshift mutation from addition or deletion of one or a few DNA base pairs (TA 98). Tests were performed with (+S9) and without (-S9) metabolic activation.

(2)   Umu test (Oda et al., 1985; ISO 13829) using *Salmonella typhimurium* TA1535 psk 1002. Genotoxins can produce a genetic lesion which would induce the *umuC* gene activating the SOS repair response system of the bacteria allowing for the repair of the DNA. The activation of the SOS repair system of the bacteria can be measured indirectly by photometrically measuring the o-nitrophenol (absorbance 420 nm) produced from the cleavage of the added o-nitrophenyl-ß-D-galactopyranoside (ONPG) substrate. Tests were performed with (+S9) and without (-S9) metabolic activation.

(3)   *In vitro* micronucleus (MN) test (OECD 487) using Chinese hamster ovary (CHO-K1). During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome or spindle damage leading to the formation of micronuclei in interphase cells. Harvested and stained interphase cells are analysed for the presence of micronuclei. This test detects chemicals that induce the micronuclei formation in the cytoplasm of interphase cells. It can detect clastogens (induces disruption or breakages of chromosomes) and aneugens (loss or gain of whole chromosomes). Micronuclei formation was measured by flow cytometry

using the Litron *Invitro* MicroFlow kit protocol (Litron Laboatories). Tests were performed without metabolic activation only.

Cytotoxicity is an important parameter for genotoxicity assessment. Therefore in addition to genotoxicity, cytotoxicity was assessed by growth inhibition in bacterial assays while relative survival and percentage apoptotic and necrotic cells were investigated in the *in vitro* micronucleus test.

The samples for toxicity processing in all papers (Paper II-IV, supplementary study, Appendix 5) were all handled in a similar way. All samples were left to stand for 24 h prior to preparation and storage and therefore the formation of short lived reactive oxygen species would be rather negligible. Further, all samples were sterile filtered and stored at -150°C to reduce sample degradation. PC stability was also tested. In some cases, photolytic mixtures were tested for peroxide using Merckoquant Peroxide test strips 0.5-25 ppm (VWR). All test methods were also the same between papers and were carried out at least twice with 2 replicates per sample per *in vitro* MN test and 3 replicates per sample per bacterial assay. These controls were necessary to ensure reproducible results were obtained.

As proposed for the effect driven approach of genotoxicity risk characterization, supporting data came from analytical analysis using HPLC-UV-VIS/FL, LC-ESI-MS/MS (ion trap) and LTQ-Orbitrap XL mass spectrometer for monitoring parent compound (PC) elimination and identifying TPs formed. Dissolved organic carbon (DOC) elimination assessed the degree of mineralization. Identification and proposal of TP structures enabled the prediction of physico-chemical and genotoxicity properties of the individual TPs using quantitative structure-activity relationships (QSAR). QSAR predictions were done using several software, namely, Case Ultra V.1.4.6.6 (MultiCASE Inc.), Leadscope software V.3.0.11-1 with training sets from 2012 SAR Genetox Database (Leadscope) and Oasis Catalogic software (module mutagenicity v.04) in *S. typhimurium* (Salmonella Catalogic model, SC) from Laboratory of Mathematical Chemistry, University Bourgas, Bulgaria. Physico-chemical parameters such as octanol-water partition coefficient (Log K_{ow}) and bioconcentration (BCF) were predicted using the EPI Suite software KOWWIN v1.68 model (Environmental Protection Agency, US). All of these supporting analysis to the genotoxicity testing were provided in Papers II-IV and were derived from the collaboration of the co-authors involved in each paper.

4.0 Results and Discussion

4.1 Summary of Papers

An extensive literature review of the risk especially genotoxic risk of anti-cancer drugs and their treated mixtures as environmental micropollutants showed that the effect driven approach was the most common of the two risk assessment methods to include TPs (Paper I). Since most pharmaceuticals, their metabolites and TPs are found as mixtures in the environment, the effect driven approach for risk assessment was expanded on. Paper I outlined several additions to this approach for a more comprehensive genotoxicity characterization for mixtures of pharmaceuticals and their TPs after treatment processes such as photolysis. These additional considerations included:

- Careful post-treatment methods and storage are necessary to remove (short lived) oxygen species resulting from treatment processes e.g. AOPs that are known to react with DNA and to ensure mostly stable TPs are tested.
- Non-purgeable organic content (NPOC) analysis is required to determine the degree of mineralization and therefore provide a first indication on the possibility of TPs formation.
- HPLC and LC-MS analysis is required to monitor primary elimination of the PC and identification of TPs and monitoring the kinetics of TPs formation.
- Careful identification and proposal of structural formula for the TPs formed.
- QSAR predictions should be included to assist in identifying individual TPs of possible concern.
- Standardized tests or well-documented procedures should be used since it is necessary to establish the conditions under which the TPs are formed and the effects they elicit.

For a more general environmental risk assessment, assessment of persistency in the form of biodegradation tests was also recommended since mixtures that are biodegradable would not pose a risk in the environment (Paper I). Paper I emphasized the need to carefully select the battery of bioassays for genotoxicity assessment and this would depend on the focus of the intended study. The ICH recommended a battery of assays focusing on identifying the mechanism of genotoxicity and mutagenicity. The OSPAR commission recommended additionally assays using native aquatic species or permanent cell lines geared towards eco-

genotoxicity testing.[2] Regardless, a battery of genotoxicity test should at least include a bacteria genotoxicity and an eukaryotic genotoxicity test (OSPAR, 2002). The bacteria genotoxicity tests, namely the Ames mutagenicity test and the umu test were recommended since they are well established and used successfully to identify a number of genotoxins (Paper I). The *in vitro* MN test has a lot of potential but selection of the cell line to use required careful considerations (Paper I).

Paper I showed that although the Ames test was the most common bioassay used, very high concentrations in the mg/L range of antineoplastic drugs were necessary to detect mutagenicity. The umu test was also similar. The mammalian cell lines were much more sensitive in detecting DNA damages at lower concentrations of antineoplastic drugs. However, it was also evident that selecting a test as part of a standard battery of genotoxicity tests for antineoplastic agents was very difficult since even within their respective classification groups there were variation in effective concentration ranges, mechanism of action and in the case of mammalian assays, sensitivity to cell lines. For example, the pyrimidine analogue antimetabolite cytarabine is mutagenic in *E.coli* WP2 strain but not another pyrimidine analogue 5-FU (Paper I). A tandem study to Paper I was conducted on photolysis mixtures to assess any changes in genotoxicity using the Ames and umu bioassays. Working with antineoplastic agents can be dangerous and required the appropriate personal protective equipment and as such, only up to 15 mg/L of 5-FU, CYC and MTX were tested these assays. No genotoxicity was observed in any of the bacterial tests but 5-FU was cytotoxic (relative growth < 50%) at concentrations > 0.4 mg/L. CYC did not achieve primary elimination but 5-FU and MTX were not detected after 256 min of photolysis. 5-FU and MTX were not completely mineralized after 256 min of UV photolysis indicating that there may be the formation of several TPs (Table 1). Like the PC, no genotoxicity was observed for the mixtures (Table 1). However, unlike 5-FU, its photolytic mixtures did not affect bacterial growth (Table 1). It is possible that the increase in relative growth could be due to removal of 5-FU in the photolysis sample at 256 min.

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[2] The OSPAR commission was set up to manage the Convention for the Protection of the Marine Environment of the North-East Atlantic or OSPAR convention

Table 1: Genotoxicity assessment of mixture from the UV photolysis of 20 mg/L of selected antineoplastic agents.

Sub-stance	Treat-ment time (min)	Dilut-ion Level	DOC %	Ames test				Umu Test			
				Number of Revertants				Growth		Induction Ratio	
				TA98		TA100					
				-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Millipore water	-	-	-	2±2	1±0	7±3	2±2	1.00±0.11	1.00±0.05	0.98±0.38	1.00±0.04
CYC	0	1.35		1±1	2±1	5±2	2±0				
		1.5						0.98±0.05	1.14±0.08	0.86±0.07	0.95±0.13
	256	1.35	99	1±1	1±1	7±1	2±2				
		1.5						1.01±0.07	1.04±0.04	0.82±0.11	0.99±0.08
MTX	0	1.35		1±1	2±1	7±4	3±2				
		1.5						0.97±0.05	1.01±0.10	0.75±0.07	0.88±0.06
	256	1.35	70	2±2	1±2	10±5	4±2				
		1.5						0.93±0.07	0.92±0.06	0.95±0.10	1.08±0.20
5-FU	0	*1.5[a]*						*0.30±0.01*	*0.26±0.02*		
		47		0±0	1±1	3±2	1±1	0.74±0.05	0.75±0.05	0.70±0.06	0.75±0.07
	256	1.5	82					1.02±0.09	0.98±0.08	0.76±0.06	0.99±0.14
		47		1±0	2±2	5±2	1±1				

[a] At this dilution level, 5FU is cytotoxic (growth < 0.5) and therefore no induction ratio was calculated. CYC: cyclophosphamide, MTX: methotrexate, 5-FU: 5- Fluorouracil

Paper II focused on TD and its photo-TPs. Less than 20% DOC was eliminated after UV-photolysis of 47 mg/L TD over 128 min. Several TPs were identified and structures proposed. TD or its UV photolysis mixtures were not mutagenic in *S. typhiumurium* TA 100 or TA98. TD is known not to be mutagenic in the Ames test (Ashby et al, 1997: Teo et al., 2000). However, QSAR based on the suggested structures had predicted that there were some mutagenic photo-TPs in the mixture. Experimentally, the photolysis mixtures were negative for mutagenicity but it cannot be excluded that perhaps the concentration of these photo-TPs in the mixtures may be too low to express a mutagenic effect or the possibility of antagonistic interactions within mixtures as possible reasons for the discrepancy with QSAR prediction. QSAR prediction did not specify in any effect concentrations and even if so without the standards for the TPs, the concentrations of the TPs within the mixture cannot be determined.

Further, QSAR is an estimation method and these estimations can be poor, even for well evaluated models (European Commission, 2003). Moreover, the QSAR model included a variety of Ames test strains that was not limited to TA 98 and TA 100 and therefore it was possible that these positive alerts were for strains other than TA 98 and TA 100. QSAR analysis also predicted genotoxicity for several other endpoints including *in vitro* chromosome aberration and *in vivo* MN but these endpoints were not experimentally investigated.

Paper III demonstrated the usefulness/applicability of the proposed scheme (Paper I) based on the experience collected from preceding work (Paper I and II) by using the entire proposed battery of genotoxic assays. In the battery of genotoxic assays, CIP was not genotoxic in the *in vitro* MN test with CHO-K1 cells and was not mutagenic in the Ames test with strains TA 98 and TA 100 but is a known genotoxin in the umu test at environmentally relevant concentrations of 0.004 mg/L. CIP was also characterized in the umu test from the cytotoxic to non-cytotoxic concentration range to derive a dose-response curve. The photolysis of CIP after 128 min resulted in primary elimination of the PC but not complete mineralization. Testing the photolysis mixtures after different treatment times revealed that the photolysis mixtures were not mutagenic in the Ames test and did not induce MN formation *in vitro*. The *umuC* gene was induced in the presence of the irradiated mixtures. The trend in *umuC* induction for the irradiated mixtures followed the trend in primary elimination of CIP over the irradiation time. Therefore, under the assumption that the CIP was likely the main contributor for the observed *umuC* induction in the irradiated mixture, a further analysis using predictions from the dose-response curve of CIP and the combination index (CI) analysis was done. The CI revealed that this may most likely be the case, as the concentration of the residual CIP in the mixture (CIP mix) was not significantly different from concentration of CIP only that would produce the same measured effect. QSAR predictions for the umu test found in literature proposed that the TPs may induce the *umuC* gene at lower concentrations than CIP (Li et al., 2014). Other QSAR predictions revealed possible genotoxic and mutagenic risk inclusive of bacterial mutagenicity and unscheduled DNA synthesis for a few photo-TPs of CIP. These predictions should not be ignored especially in cases where the positive structural alert was not part of the CIP molecule. Structure identification also showed that the photo-TPs identified all retained the quinolone moiety but have alteration on the piperazine moiety and/or loss or substitution of the fluoride ion. The retention of the quinolone moiety may suggest that the TPs would bind to the DNA similarly as CIP but the alterations of the substituents may affect the affinity of TPs to DNA binding and subsequently their potency. Paper III was able show that although the battery of

genotoxicity assays employed here only covers a few endpoints with a few cell lines or bacterial strains, photolysis can provide a mean for the removal of CIP but the genotoxicity and cytotoxicity potential of the resultant mixtures could be dependent primarily on the concentration of residual CIP. No independent mechanism of genotoxicity was also experimentally observed in these mixtures although QSAR suggested otherwise.

Unlike the investigation with CIP, none of the beta blockers are genotoxic or mutagenic in the battery of genotoxicity assays selected (Paper IV and supplementary study: Appendix 5). In this case, even higher concentrations of 100 mg/L ATL and 400 mg/L MTL were subjected to UV photolysis and resulted in incomplete mineralization. ATL and MTL and their irradiated mixtures were not mutagenic in the Ames test, not *umuC* inducing and not micronucleus inducing *in vitro* (Paper IV). In the umu test, a statistical increase in the induction ratio was observed for mixtures after 256 min of photolysis for both beta blockers and therefore may indicate the possibility of formation of genotoxic TPs. QSAR predictions suggested that a few TPs were positive for several genotoxic endpoints such as *in vitro* chromosome aberration and mutagenicity in Salmonella. Perhaps due to mixture interaction, low occurrence of TPs or the use of a different strain in the Ames test, the irradiated mixtures were all negative for genotoxicity in the selected bioassays. A closer scrutiny of the structural alerts of the TPs in comparison to the structure of the PC allowed for the proposal of several TPs for further genotoxicity characterization.

The photolytic mixtures of MTL generated after 16 min and 256 min under the specified condition did result in lower relative cell survival and produced significantly more apoptotic and necrotic CHO-K1 cells (Paper IV). At 16 min, TPs kinetic showed that two TPs namely TP with m/z 238 (MTP238) and m/z 252 (MTP252) were peaking (Paper IV). MTP238 was selected as a most likely candidate which would contribute more to the cytotoxic nature at 16 min since it has the highest predicted log K_{ow} value of 2.5 (EpiSuite prediction) even when compared to the 1.88 experimental log K_{ow} of MTL. However, at 256 min, all other identified TPs were peaking and therefore would suggest that one or more of those TPs are responsible for cytotoxicity observed. The proposed structures for MTP 192, MTP 234_{1-2}, MTP 254 and MTP 284_1 were more hydrophilic (log K_{ow} >1). These TPs exhibited a high relative abundance peaking at 256 min and therefore could influence the observed cytotoxicity of the 256 min photolysis mixture to the CHO-K1 cells.

PPL on the contrary acted differently to its other structurally related beta blockers (supplementary study: Appendix 5). 100 mg/L PPL was subjected to UV photolysis resulting in formation of several TPs since it was not completely mineralized. Like ATL and MTL, PPL was not genotoxic in any of the selected bioassays. However, the mixtures generated after photolysis for 128 min or more were mutagenic and mixtures obtained after 64 min and 128 min treatment were *umuC* inducing. In fact, generally an increasing trend in revertants and *umuC* induction was noted for the photolysis mixtures in comparison to PPL. Analysis of proposed structures, TP formation kinetics and QSAR toxicity predictions revealed that it is possible that some of the structures proposed for TP 266, TP 292, TP 282 and TP 308 could have been responsible for the observed mutagenicity. The aldehyde found in these structures was hypothesized as the part of the molecules that could lead to formation of DNA adducts and therefore could cause the mutagenicity observed (Benigni et al., 2005). Interestingly, most of the TPs formed during photolysis of ATL and MTL predicted by QSAR to cause mutagenicity possessed the aldehydes as their structural alerts for mutagenicity. Photolysis can result in the formation of oxidative species such as peroxide that can affect the bioassays. The photolytic samples of all three ß-blockers contained ≤ 5-10 mg/L peroxide which is below the known threshold for peroxide induced genotoxicity in the umu test of 45 mg/L (Nakamura et al., 1987) and 17.8 mg/L in the *in vitro* MN test (Diaz et al., 2007). Nevertheless, further tests are been conducted to exclude the influence of the peroxide on the observed genotoxicity of the PPL photolysis mixtures in the Ames and umu tests. While none of the photolysis samples were MN inducing *in vitro*, samples from 8 min to 256 min were cytotoxic to CHO-K1 cells. Additionally, all the photolytic mixtures were more cytotoxic than the PPL affecting relative survival of CHO-K1 cells in particular. Cytotoxicity could be as a result of one or more of the TPs present in the mixtures. The irradiated mixture generated at 8 min was especially cytotoxic affecting both the relative survival and causing apoptosis and necrosis of CHO-K1 cells. Further work is ongoing in understanding the relation of hydrophobicity of the TPs with the observed cytotoxicity effect. Thus far, TP 276 (with a Log K_{ow} >1) that has a peak in formation around 8 min of irradiation was proposed to be a contributor to the observed cytotoxicity.

4.2 Discussion

Each of the research papers presented here demonstrated the advantages and disadvantages of using the proposed genotoxicity risk characterization scheme based on the effect driven methodology as applied to pharmaceuticals and their TPs.

Firstly, a requirement of good supportive analytical data and structure elucidation is generally crucial for interpretation of the toxicity data with respect to the influence of TPs. For instances, the lack in descriptive analytic data in the experimental study adjoined to Paper I would only allow for a mixture effect characterization for the photolysis mixture of 5-FU. In this case, the only conclusion that could be made was that the irradiated 5-FU mixture did not affect bacterial growth and this could be quite possibly because 5-FU was primarily eliminated or reduced beyond its minimum cytotoxic threshold or TPs with less cytotoxic potentials were formed. In cases, where the mixtures are analyzed to identify TPs, there is no certainty that all TPs can be detected using a standard analytical method developed for the PC. Moreover, the concentrations of the TPs cannot be determined unless a standard is available. The uncertainty in the concentration of the TPs resulted in conducting the photolysis studies at environmentally irrelevant concentrations controlled by water solubility, limit of detection of the analytical instruments and/or effective concentration range of PC in the selected bioassays (Paper II, III, IV, Appendix 5). With respect to the proposal of identified structures based on the MS^n spectra another limitation arises in that there may be more than one structure or structural isomers proposed for a mass and these structures may be predicted with QSAR to act differently from each other. This can limit the interpretation of the experimental work. Paper III demonstrated that with the use of the LTQ-Orbitrap XL mass spectrometer, a more accurate mass was determined and therefore more surety in proposed structures. This enabled the QSAR prediction in conjunction with experimental data to be used in a much more productive manner.

Secondly, photolysis as an AOP results in the formation of short lived reactive oxygen species (ROS) such as peroxides, hydroxyl radical and singlet oxygen (Chételat et al., 1996). ROS formed whether endogenous or exogenous are well known to cause DNA damage (Chételat et al., 1996; Cooke et al., 2003; Cadet and Wagner, 2013). In fact, ROS such as peroxides are known to affect the Ames, umu and *in vitro* MN tests (Nakamura et al., 1987; Abu-Shakra and Zeiger, 1990; Diaz et al., 2007). Several photolysis studies have reasoned that ROS may be contributors to the observed genotoxicity of their irradiated samples (Šojić et al., 2012; Garcia-Käufer et al., 2012; Vasquez et al., 2013). In the environment, the presence of ROS cannot be

discounted and as such would warrant their consideration in genotoxicity testing of environmental samples. However, if the focus of the study, as is presented here, is to propose stable TPs that may be genotoxic, steps such as post-treatment with a catalase have to be taken to minimize the effect from ROS. In other cases, quantifying ROS such as peroxides can help to ensure irradiated mixtures are tested at dilutions where there is no observable effect cause by the ROS on the test system (Paper IV, supplementary study: Appendix 5).

Thirdly, the use of a battery of bioassays for genotoxicity is generally preferable than to use only one genotoxic assay to characterize photolysis mixtures. Our experimental phase connected to Paper I tested the antineoplastic agents and their photolysis mixtures in the umu and Ames bioassays only. However, the literature review indicated that mammalian species were more sensitive for genotoxicity testing of antineoplastic agents. Therefore, the inclusion of such a test may have been more productive for assessment of the irradiated mixtures. Even so, 5-FU has a reported lowest observable adverse effect concentration of 400µM (~ 52 mg/L) in an automated *in vitro* MN test using CHO-K1 cells (Diaz et al., 2007). This reported concentration was beyond the concentration range of our tandem study to Paper I and therefore, emphasized the need to carefully select the initial concentration used in such an investigation. On the other hand, CYC has the capability of inducing MN formation at concentrations as low as 5 mg/L but requires metabolic activation to its active form (Bryce et al., 2010). In Paper II, the QSAR predictions of the photo-TPs of TD suggested that the inclusion of the *in vitro* MN test would have enhanced the genotoxicity characterization of the mixtures and possibly identified genotoxic TPs. The experimental work from both Paper I and II would suggest that the better case would be to use a battery of bioassays so that there is a greater possibility for detecting genotoxic activities for a broader range of chemicals (within and across classes) with varying physico-chemical and toxicological properties. This was also the rationale in the ICH (2012) guidelines for using a battery of genotoxicity assays for pharmaceuticals. Moreover, with the *in vitro* MN test using flow cytometry there would be the added benefit of distinguishing among clastogens and aneugens. The use of a battery of bioassays for genotoxicity was later illustrated in Paper III, IV and supplementary study (Appendix 5). Further, knowledge on the genotoxicity of the PCs and their mechanism of genotoxicity can assist in building a better battery of genotoxicity assays where at least one bioassay can monitor the changes in genotoxicity of the treated mixtures in comparison to the PC. Moreover, in cases where the PC does not have a genotoxic action, the used of the battery of bioassays can reveal the development of genotoxicity in the mixtures as a result of the treatment process. An

environmental genotoxicity risk assessment to include TPs would need to include bioassays such as comet assay or micronucleus test with fish cell lines that are better suited and sensitive for monitoring changes in the environment.

Fourthly, basic mixture toxicity analysis tools such as isobolograms and combination index analysis can be applied to better describe the relative genotoxicity of the mixtures to the PC or a reference compound. In paper III, this concept was used to identify the main *umuC* inducer as the PC. However, to conduct such an analysis, extensive testing for a dose-response curve of the PC and dilution-response curves for the irradiated mixtures across several treatment time needs to be done. Additionally, cytotoxicity testing is a crucial inclusion in the genotoxicity characterization scheme since genotoxicity is limited by cytotoxicity to the bacteria or cell line in these test systems. This is especially important for cytostatic and antibiotic drugs in interpreting genotoxicity for such an analysis. Extensive chemical analysis is also required to ensure data such as concentrations, are well correlated between mixtures and PC.

Finally, QSAR predictions are a valuable inclusion to this risk characterization scheme in understanding the toxicity of the mixtures and the roles of the TPs. QSAR are not without its limitations as discussed in Paper II but its addition to this scheme provided several advantages. For instance, it was possible that a mechanism of action could be hypothesized by using structural alerts for positive QSAR predictions as shown in study with PPL. Further, toxicity and physico-chemical predictions could help to understand the nature of toxicity in the mixture. As shown in Paper IV, the Log K_{ow} was an important descriptor for the cytotoxicity of the mixture and to propose the TPs likely to cause such an effect. In fact, QSAR prediction has been recently approved for genotoxicity characterization of degradation products and impurities formed or found in drug formulations in the ICH M7 (2014) guideline. This guideline has been adopted by the EMA and FDA. The guideline specifies how to interpret if a positive structural alerts of TPs warrants its further investigation. Although this is again used in the drug design phase, we have shown that it practically can be extended to the risk assessment of TPs as demonstrated in Paper III, IV. The addition of QSAR therefore could suggest the TPs for further characterization and goes beyond the consideration of TPs that account for 10% of the initial PC concentration as stated in the FDA guideline for pharmaceutical risk assessment (FDA, 1998). It is now possible to estimate the toxicity and physico-chemical characteristics for all the TPs found within the chromatographic analysis conducted.

Certainly, the aim of the approach presented here is two-fold in that it is used to characterize the genotoxicity of the mixture and to short list possible relevant TPs from an effect basis for further investigation. In the proposed scheme, the mixtures can be characterized with well-defined genotoxicity curves across several dilution range but the nature (number, type and concentration of TPs) of the photolysis mixtures generated from such high initial concentration and in much simpler matrix would differ drastically from their presence in the environment. Hence, the results of the mixture toxicity cannot be easily read across to an environmental risk but the implications of studies of this nature can productively contribute to the debate on environmental risk assessment and management of chemicals to include TPs. This work signals that treatment processes for micropollutants such as pharmaceuticals can produce mixtures with fundamental changes in the physico-chemical and toxicological natures. It is quite possible that the compounds created in these processes can be more toxic with the capacity to cause DNA damage while it is also likely that some can be less toxic to organisms. The determination of the magnitude of effect would require more targeted analysis but at least the potential hazards can be identified in studies like this one. There are no safe threshold for DNA damaging toxins and this emphasizes the importance of such a genotoxicity characterization schemes as this. In fact, the scheme proposed here can serve as a preliminary risk characterization to determine if it is necessary and what aspects is necessary to investigate further as part of an environmental risk assessment.

5.0 Conclusions and Outlook

This work has demonstrated a scheme for a genotoxicity assessment of photolysis treated mixtures based on an effect driven approach. Key findings included the necessity of using a battery of genotoxicity assays that may or may not include one bioassay that can detect the mechanism of genotoxicity exhibited by the PC. Mechanism based *in vitro* tests are valuable towards providing an initial characterization of the mixtures containing a number of TPs that may differ in physico-chemical and toxicological properties. For the *in vitro* micronucleus test, the selection of cell line is important. Sticking to well established cell lines and test procedure is sufficient for an initial genotoxicity characterization.

The battery of genotoxicity test applied here was capable of monitoring the removal of a specific genotoxic effect that is known to the PC as well as to detecting genotoxicity effects in the treated mixtures that are unknown to the PC. The entire scheme can be used to determine

similar and dissimilar mechanism of genotoxicity between PC and treated mixtures. Additionally, mixture toxicity analysis tools can be adopted and used to identify one or more genotoxin of interest but this will require extensive work on characterizing mixtures obtained after each treatment time and a general knowledge of the test system and the investigated endpoints.

Supporting analysis derived from using several analytical methods, structure elucidation and identification of TPs and DOC elimination studies are crucial towards understanding what is present and the changes occurring in the treated samples. If there is access to better analytical methods such as LTQ-Orbitrap XL high resolution mass spectrometer, it should be fully utilized since the better the determination of the accurate mass and subsequently better structure proposal, the more information can be correlated to the toxicity data. QSAR prediction while having its drawbacks since it is a prediction method can still provide an excellent supportive tool in whole mixture genotoxicity assessment with the capacity to propose TPs for further risk characterization and assessment.

The scheme proposed here provides only an initial characterization and can short list TPs of concerns. It is a preliminary risk characterization applied to simpler mixtures than environmental samples and can be used to monitor effectiveness of treatment methods for removal of toxicity of pharmaceuticals and other pollutants. In fact, it was clearly shown that the degradation of the PC by treatment processes such as UV photolysis does not necessarily translate to the removal of toxicity but can sometimes lead to the development of more toxic mixtures. It is therefore necessary to conduct exposure based assessment inclusive of isolating or synthesizing the TPs, establishing analytical protocols for their detection and investigating their biodegradation potentials if a more comprehensive environmental risk assessment is desired.

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Publication list and Scientific conference contribution

Article	Title	Authors	Author Status	Weighing factor	Publication status*	Conference contributions
I	Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings	Anju Priya Toolaram, Klaus Kümmerer, Mandy Schneider	Co-author with predominant contribution (Überwiegender Anteil)	1.0	Mutation Research/Reviews in Mutation Research, 2014, 760, 18-35 (IF= 7.326 (2013))	Environmental fate of Methotrexate: photodegradation, biodegradability and mutagenicity assessment. Pharmaceutical products in the environment: Is there a problem? 3rd – 4th June, 2013, Nimes, France
II	Identification of phototransformation products of thalidomide and mixture toxicity assessment: An experimental and quantitative structural activity relationships (QSAR) approach	Waleed M.M. Mahmoud, Anju P. Toolaram, Jakob Menz, Christoph Leder, Mandy Schneider, Klaus Kümmerer	Co-author with equal contribution (Gleicher Anteil)	1.0	Water Research, 2014, 49, 11-22 (IF= 5.323(2013))	1. Photodegradation of thalidomide: Identification of transformation products by LC-UV-FL-MS/MS, assessment of biodegradability, cytotoxicity and mutagenicity in 4th EuCheMS chemistry congress, 26th – 30th August, 2012, Prague, Czech Republic 2. Identification and initial toxicity assessment of Thalidomide and its phototransformation products in EuCheMS International Conference on Chemistry and the Environment, 25th – 28th June, 2013, Barcelona, Spain
III	Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a	Anju Priya Toolaram, Tarek Haddad, Christoph Leder,	Co-author with equal contribution (Gleicher Anteil)	1.0	Submitted	1. Identification of photo-transformation products of ciprofloxacin and evaluation of their genotoxicity using *in silico* methods and in vitro assays in Pharmaceutical products in the environment: Is there a

	combination of experimental and *in-silico* testing	Klaus Kümmerer				problem? 3rd – 4th June, 2013, Nimes, France 2. Evaluating the genotoxic potential of ciprofloxacin and its transformation products after photolysis treatment in SETAC North America 34th Annual Meeting, 17th - 21st November, 2013, Nashville, Tennessee, U.S.A
IV	Genotoxicity and cytotoxicity characterization of mixtures generated from photolysis of the ß-blockers Atenolol and Metoprolol using a combination of experimental and (Q)SAR approaches	Anju Priya Toolaram, Jakob Menz, Tushar Rastogi, Christoph Leder, Mandy Schneider, Klaus Kümmerer	Co-author with equal contribution (Gleicher Anteil)	1.0	Submitted	Genotoxicity and ecotoxicity screening of photolytic mixtures from the selective β1-receptor blockers Atenolol and Metoprolol in SETAC Europe, 24th Annual Meeting, 11th - 15th May, 2014, Basel, Switzerland
Vᵃ	Photolysis of propranolol leads to the formation of cytotoxic transformation products with increased genotoxic potential *(tentative title)*	Jakob Menz, Anju Priya Toolaram, Tushar Rastogi, Christoph Leder, Mandy Schneider, Klaus Kümmerer	-	-	Planned	Photodegradation of Propranolol: Identification of transformation product, assessment of their biodegradability, bacterial toxicity and mutagenicity. Gemeinsame Jahrestagung der SETAC GLB und der Fachgruppe Umweltchemie und Ökotoxikologie der GDCh, 10th – 12th September, 2012, Leipzig, Germany

*IF=Impact factor 2013 from ISI Web of Science; [a] The data to be present in this paper that is relevant to this dissertation is presented in the Supplementary study (Appendix 5)

Curriculum Vitae

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Born 30th January, 1987, in Georgetown, Guyana

Education:	Leuphana University Lüneburg, Lüneburg, Germany **Dr.rer.nat. Candidate**, June, 2015 (expected) Hamburg University of Technology (TUHH), Hamburg, Germany **Masters of Science**, October 2010 Joint European Masters in Environmental Studies University of Guyana (UG), School of Earth and Environmental Sciences, Turkeyen, Guyana **Bachelors of Science (Distinction)**, December, 2007 Environmental Studies
Work and Internship Experiences:	**Research Scientist** Leuphana University Lüneburg, Institute for Sustainable and Environmental Chemistry, Lüneburg, Germany, April 2011-July, 2014 **Intern** Hamburg University of Technology, Institute of Environmental Technology and Energy Economics (Ecotoxicology Group), Hamburg, Germany, June-November, 2010 **Intern** Hamburg University of Technology, Institute of Environmental Technology and Energy Economics (Bioconversion and emission reduction Group), Hamburg, Germany, February-May, 2010 **Assistant Researcher** National Agricultural Research and Extension Institute (NAREI), Guyana, October, 2007- August, 2008 **Intern** University of Guyana, School of Earth and Environmental Studies, Turkeyen, Guyana, January, 2007- June, 2007 **Intern** Guyana Forestry Commission, Guyana, August 2003-September 2003
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Peer reviewed Publications:

Anju Priya Toolaram, Tarek Haddad, Christoph Leder, Klaus Kümmerer Monitoring the course of genotoxicity of Ciprofloxacin and its transformation products formed during photolysis using the whole mixture approach incorporating combination index analysis and quantitative structure activity relationship (QSAR) predictions, (*submitted)*

Anju Priya Toolaram, Klaus Kümmerer, Mandy Schneider (2014) Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings, Mutation Research/Reviews in Mutation Research, 760, 18-35

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Anju Priya Toolaram, Iris Raquel Gutiérrez, Wolfgang Ahlf (2012) Modification of the umu-assay (ISO 13829) accounting for cytotoxicity in genotoxicity assessment: A preliminary study, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 747 (2), 190-196

Conference contributions:

Mandy Schneider, **Anju Priya Toolaram**, Jakob Menz, Tushar Rastogi, Klaus Kümmerer Genotoxicity and ecotoxicity screening of photolytic mixtures from the selective β1-receptor blockers Atenolol and Metoprolol. SETAC Europe, 24th Annual Meeting, 11th - 15th May, 2014, Basel, Switzerland

Anju Priya Toolaram, Tarek Haddad, Mandy Schneider, Klaus Kümmerer, Evaluating the genotoxic potential of ciprofloxacin and its transformation products after photolysis treatment. SETAC North America 34th Annual Meeting, 17th - 21st November, 2013, Nashville, Tennessee, U.S.A

Waleed M. M. Mahmoud, **Anju Priya Toolaram**, Jakob Menz, Christoph Leder, Mandy Schneider, Klaus Kümmerer, Identification and initial toxicity assessment of Thalidomide and its phototransformation products. in: *EuCheMS International Conference on Chemistry and the Environment: Book of Abstracts* (S. 366). European Association for Chemical and Molecular Sciences / Division of Organic Chemistry. 25th – 28th June, 2013. Barcelona, Spain

Anju Priya Toolaram, Ewelina Baginska, Carlos Lutterbeck, Kham Dieu Huynh, Mandy Schneider, Klaus Kümmerer, Environmental fate of Methotrexate: photodegradation, biodegradability and mutagenicity assessment. *Pharmaceutical products in the environment: is there a problem ?* 3rd – 4th June, 2013, Nimes, France

Tarek Haddad**, Anju Priya Toolaram**, Christoph Leder, Klaus Kümmerer, Identification of photo-transformation products of ciprofloxacin and evaluation of their genotoxicity using *in silico* methods and *in vitro* assays. *Pharmaceutical products in the environment:: is there a problem ?* 3rd – 4th June, 2013, Nimes, France

Tushar Rastogi, Jakob Menz, **Anju Priya Toolaram**, Christoph Leder, Mandy Schneider, Richard Bolek, Klaus Kümmerer, Photodegradation of Propranolol:

Identification of transformation product, assessment of their biodegradability, bacterial toxicity and mutagenicity. *Gemeinsame Jahrestagung der SETAC GLB und der Fachgruppe Umweltchemie und Ökotoxikologie der GDCh,* 10th – 12th September, 2012, Leipzig, Germany

Waleed M. M. Mahmoud, **Anju Priya Toolaram**, Mandy Schneider, Klaus Kümmerer (2012). Photodegradation of thalidomide: Identification of transformation products by LC-UV-FL-MS/MS, assessment of biodegradability, cytotoxicity and mutagenicity. Presented in the session Environmental Chemistry-emerging contaminants, POPs, phototransformation in the 4th EuCheMS chemistry congress, 26th – 30th August, 2012, Prague, Czech Republic

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Additional Skills:	ArcGIS, Flow cytometry, Photolysis Simulation, Ready Biodegradability tests (OECD 301), *C. elegans* growth, fertility and reproduction test (ISO 10872), Solid contact assay (ISO 18187)
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Appendices

Appendix 1: Paper I

Anju Priya Toolaram, Klaus Kümmerer, Mandy Schneider (2014) <u>Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings,</u> Mutation Research/Reviews in Mutation Research, 760, 18-35

Appendix 2: Paper II

Waleed M.M. Mahmoud, Anju P. Toolaram, Jakob Menz, Christoph Leder, Mandy Schneider, Klaus Kümmerer (2014) <u>Identification of phototransformation products of thalidomide and mixture toxicity assessment: An experimental and quantitative structural activity relationships (QSAR) approach,</u> Water Research, 49, 11-22

Appendix 3: Paper III

Anju Priya Toolaram, Tarek Haddad, Christoph Leder, Klaus Kümmerer <u>Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a combination of experimental and *in-silico* testing,</u> *(submitted)*

Appendix 4: Paper IV

Anju Priya Toolaram, Jakob Menz, Tushar Rastogi, Christoph Leder, Mandy Schneider, Klaus Kümmerer <u>Genotoxicity and cytotoxicity characterization of mixtures generated from photolysis of the ß-blockers Atenolol and Metoprolol using a combination of experimental and (Q)SAR approaches,</u> *(submitted)*

Appendix 5: Supplementary study

<u>Genotoxicity and cytotoxicity characterization of Propranolol.</u>

Appendix 1

Paper I

Review

Environmental risk assessment of anti-cancer drugs and their transformation products: A focus on their genotoxicity characterization-state of knowledge and short comings

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ABSTRACT

Anti-cancer drugs are chemotherapeutic agents that are designed to kill or reduce proliferating cells. Often times, they interfere directly or indirectly with the cell's deoxyribonucleic acid (DNA). Some of these drugs can be detected in the ng/L concentration range in the aquatic environment and have the potential to be very persistent. Environmental risk assessment is available for only a few anti-cancer drugs, derived mainly from predicted data and excluding information on their metabolites and transformation products (TPs). Notably, there is no defined strategy for genotoxicity risk assessment of anti-cancer drugs, their metabolites and TPs in the environment. In fact, the presence of anti-cancer drugs in hospital and municipal wastewaters has not been clearly related to the genotoxic nature of these wastewaters. The few available studies that have sought to investigate the genotoxicity of mixtures derived from treating anti-cancer drugs prior to disposal seem to share the commonality of coupling analytical methods to measure concentration and genotoxic bioassays, namely the Ames test to monitor inactivation. Such limited studies on the environmental fate and effects of these drugs presents an area for further research work. Most importantly, there is a need to characterize the genotoxic effects of anti-cancer drugs towards aquatic organisms. Given current environmental risk assessment strategies, genotoxicity risk assessment of these drugs and their TPs would have to include a combination of appropriate analytical methods, genotoxicity bioassays, (bio) degradability and computer based prediction methods such as QSAR studies.

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1. Introduction

Cancer is credited with been the leading cause of human deaths worldwide, accounting for 7.6 million deaths in 2008 and is expected to rise to 13.1 million deaths in 2030 [1]. Antineoplastic or anti-cancer drugs are one of the main chemotherapeutic agents used in the fight against cancer. Most of these drugs kill or control the proliferating cells by mainly interfering with deoxyribonucleic acid (DNA) through various mechanisms [2]. These drugs can also exhibit unwanted effects to normal cells and are potentially immunosuppressive to humans and animals. Some anti-cancer drugs have shown potential to act as carcinogen, teratogen and/or mutagen [3,4]. Evidence of their genotoxic effects has so far been established in situations where there is likelihood of higher exposure such as in a health care setting [5–9].

Anti-cancer drugs are administered under controlled situations at hospitals and now at increasing levels at home by out-patients' consumption [10]. The main environmental source of anti-cancer drugs comes from excretion in the form of urine and faeces from chemotherapeutic patients. An ongoing move towards out-patient treatment and the fact that hospital effluent often time leads into the municipal sewer system would now make municipal wastewater an important source for the introduction of these drugs into the environment. There is some knowledge on the presence of these compounds in the aquatic environment but rather limited knowledge on their effects on humans and wild life once they enter the environment.

Thus far, there have been some efforts in characterizing the potential risk of anti-cancer drugs in the environment. Publications on detection of these compounds in the aquatic environment started since the late 1980s with the works of Richardson and Bowron [11] and Ahrene et al. [14], among others. Since then it has been found that different anti-cancer drugs usually occur in ng/L or below in the environment [11–13]. Recently, a number of reviews have chronicled the efforts of researchers in characterizing the presence and fate of these drugs in the environment [13,15–17]. The review of Kosjek and Heath discussed the state of analytical procedures for detecting anti-cancer drugs in the aquatic environment [13]. Zhang et al. focused on methods of removal of anti-cancer drugs from wastewaters [17]. Many authors including Kümmerer et al. [18–20], Kümmerer and Al-Ahmad [21], Al-Ahmad et al. [22], Steger-Hartmann et al. [23,24] and Al-Ahmad and Kümmerer [25] have investigated the environmental fate of some anti-cancer drugs. Besse et al. provided extensive data on exposure of several anti-cancer drugs for surface waters in France [15]. The review of Xie additionally contained data on ecotoxicity and approaches for effluent treatment [16]. Presently, there are two ongoing projects, funded by the European Union (EU), namely the Pharmas (http://www.pharmas-eu.org) and Cytothreat (http://www.cytothreat.eu/) projects that are focused on determining the risks from the presence of anti-cancer drugs, their metabolites and their transformation products in the aquatic environment.

So far we know some of these drugs are present and stable in the aquatic environment [11–24]. Data on acute toxicity testing usually suggest that anti-cancer drugs are toxic at 3 fold or higher concentration than their known environmental concentrations [16,17]. Most of the reviews mentioned above conclude that there is a need for more chronic ecotoxicity testing of these drugs since they are present in low concentrations and are rather persistent in the aquatic environment. Only a few rough risk assessments are available and only for a few compounds such as Cyclophosphamide (CPA) and Ifosfamide (IF). Moreover, though it is known that many of these compounds are transformed through human metabolism, limited studies have sought to identify and characterize their human metabolites. Furthermore, additional transformation products (TPs) can result from various treatment processes or from abiotic and biotic environmental processes such as biotransformation, hydrolysis or photolysis. For them even less is known.

In this paper, emphasis is not placed on the occurrence and fate of these compounds. In this respect, we aim to simply highlight the presence of these compounds as contaminants in the aquatic environment. The main focus of this work is on determining the status of current research on genotoxic and mutagenic potentials of these drugs, their human metabolites and their TPs as part of their environmental risk assessment. Emphasize is placed specifically on the current methods used for genotoxicity risk assessment and their suitability to assess the effects of anti-cancer compounds and their TPs in the aquatic environment.

2. Understanding the potential risk of anti-cancer drugs as environmental micro-pollutants

Anti-cancer drugs are classified by the Anatomical Therapeutic Classification (ATC) system according to their chemical structures and therapeutic properties as class L, Antineoplastic and immunomodulating agents [26]. Table 1 shows the classes of antineoplastic drugs as defined by the ATC and a general description of their mode of action. Understanding the different modes of action can support the idea that by design, these drugs can interact directly or indirectly with DNA causing DNA damage and/or inhibit DNA synthesis as well as affecting mitosis and inhibiting cell proliferation. These actions can be unspecific inhibiting normal cells thereby presenting a danger to environmental organisms.

2.1. Usage and physico-chemical properties as an indicator of environmental fate

To understand the potential risk of these drugs to the environment, a closer look at the consumption patterns and the physico-chemical nature of the drugs are the least of requirements. According to Bergmann et al., Germany has experienced an increase of 58% in the consumption (mass) of active ingredients of various anti-cancer drugs from 2002 to 2009 [30]. Even though not all drugs are consumed equally, the gross effect is likely to be an increased input into the environment. Kosjek and Heath in their review mentioned that 5-Fluorouracil (5-FU) followed by Gemcitabine (GEMc), IF, CPA, and Methotrexate (MTX) were the most widely administered cytostatic drugs globally [13]. Interest should also be given to the newly formulated anti-cancer drugs such as Imatinib (IB) since little to no information exists on their environmental fate. In Germany, there was a 478% increase in consumption of IB from 2002 to 2009 [30] while in France, there was a 50% increase between 2004 and 2008 [15]. Furthermore with increasing life expectancy and increasing standard of living on a global scale it has to be expected that the input of anti-cancer drugs into the environment will increase further. Some drugs are used for anti-cancer treatment but also for other treatments. MTX, for example, is used in anti-cancer treatment and the treatment of rheumatism. There seems to be also a trend of increasing usage of anti-cancer drug treatment for pets such as dogs and cats in several countries. This has to be accounted for when data of usage are assessed.

Physico-chemical parameters such as the dissociation constant (pK_a), bioconcentration factor (BCF), octanol–water partition coefficient (K_{ow}), organic carbon partition coefficient (K_{oc}), atmospheric OH reaction rate, solubility, Henry's coefficient and the vapour pressure are all instrumental in risk assessment analysis. Since many reviewers [13,15–17] have provided extensive data on the physico-chemical nature, the occurrence and fate of these compounds, only data pertaining to the five main

Table 1
Classification of anti-cancer drugs (Class L) according to the Anatomical Therapeutic Classification (ATC) system.

L 01 Antineoplastic agents	Subcategory	Examples	Mode of action
L01A Alkylating agents	L01AA Nitrogen mustard analogues L01AB Alkyl sulfonates L01AC Ethylene imines L01AD Nitrosoureas L01AG Epoxides L01AX Other alkylating agents	Cyclophosphamide, Ifosfamide Busulfan, Treosulfan Thiotepa, Triaziquone Carmustine, Lomustine Etoglucid Mitobronitol, Temozolomide	Replaces a hydrogen atom with an alkyl group that can slow or block DNA replication [27].
L01B Antimetabolites	L01BA Folic acid analogues L01BB Purine analogues L01BC Pyrimidine analogues	Methothrexate, Pemetrexed Mercaptopurine, Fludarabine Cytarabine, Fluorouracil	Structurally similar to endogenous nucleic acids and can be incorporated into the metabolic pathways instead of the endogenous purine and pyrimidines, thereby affecting the enzyme dependent synthesis of DNA and cell reproduction [27].
L01C Plant alkaloids and other natural products	L01CA Vinca alkaloids and analogues L01CB Podophyllotoxin derivatives L01CC Colchincine derivatives L01CD Taxanes L01CX Other plant alkaloids	Vinblastine, Vincristine Etoposide, Teniposide Demecocline Paclitaxel, Docetaxel Trabectedin	Interacts with the microtubules or the tubulins leading to inhibition of synthesis of proteins and nucleic acids, disruption of the mitotic spindle and eventually cell death [28].
L01D Cytotoxic antibiotics and related substances	L01DA Actinomycines L01DB Anthracyclines and related substances L01DC Other cytotoxic antibiotics	Dactinomycin Doxorubicin, Daunorubicin Bleomycin, Mitomycin	Mechanism of action involves direct toxic action on cellular DNA, interfering with DNA replication and protein synthesis [16].
L01X Other antineoplastic agents	L01XA Platinum compounds L01XB Methylhydrazines L01XC Monoclonal antibodies L01XD Agents used in photodynamic therapy L01XE Proteinkinase Inhibitors L01XX Other antineoplastic agents L01XY Combinations of antineoplastic agents	Cisplatin, Carboplatin Procarbazine Edrecolomab, Rituximab Porfimer sodium, Verteporfin Imatinib, Gefitinib Asparaginase, Irinotecan	E.g. Imatinib as a proteinkinase inhibitor can block the breakpoint cluster region-Abl tyrosine kinase and therefore inhibit proliferation and induces apoptosis of chronic myelogenous leukemia cells [29].

Source: ATC (http://www.whocc.no/atc_ddd_index/), other references are included in [].

anti-cancer drugs and IB are presented here (Table 2) for an illustrative purpose.

The pK_a value reveals the extent to which the compound would dissociate at a particular pH. MTX has a relatively low dissociation constant and thus would most likely be dissociated in the aquatic environment and therefore increase its mobility there because of its higher polarity. A log $K_{ow} < 1$ suggests that the compounds are highly mobile in the aquatic environment. Therefore, the likely behaviour of the selected anti-cancer drugs with the exception of IB (log K_{ow} = 3) is to remain in the water phase and less likely to sorb onto particles, sediments or sludge in the environment. Thus, far the fate of IB in the environment is widely unknown. The guideline on the environmental risk assessment of medicinal products for human use by the European Medicines Agency (EMA) states that if a drug has a log $K_{ow} > 4.5$ only then should it be screen for persistence, bioaccumulation and toxicity [34]. The US Food and Drug Administration (FDA) guideline has set an even lower threshold of log $K_{ow} > 3.5$ [35]. The K_{oc} value of these selected anti-cancer drugs also suggested limited sorption to sediments and suspended materials in the environment. Additionally, since the BCF factor is also low, none of these cytostatic drugs are expected to bioaccumulate in aquatic organisms. In cases where only the log K_{ow} is known, if the log $K_{ow} > 3$ a high BCF has to be assumed and therefore bioconcentration by bioaccumulation and biomagnification is assumed.

The combination of information gained from the physico-chemical properties can thus provide an insight into how difficult it would be remove these compounds from the aquatic environment once they have entered it. Moreover, given that drugs such as MTX and IB have a large proportion of the administered drug excreted unchanged and others such as CPA are excreted as still active
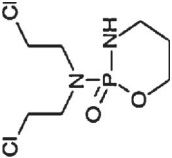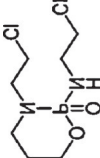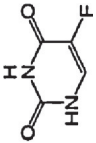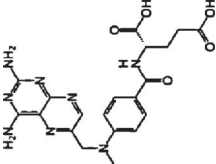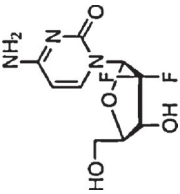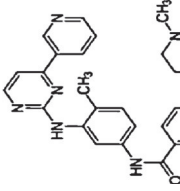
metabolites places further emphasizes on the urgent need to gain a better understanding of their fate and effects in the environment (Table 2).

2.2. Predicted environmental concentration (PEC) and measured environmental concentration (MEC)

The first stage of an environmental risk assessment requires the predicted environmental concentration (PEC) or the expected introduction concentration (EIC) in surface water for the active drug substance only [34,36]. The PEC or EIC is dependent on several factors including the consumption pattern and direct disposal into wastewater. No emphasize is placed on metabolism or biodegradation in the sewage treatment plant at this stage. Refined PEC calculation in phase II according to the EMA guideline and the FDA's expected environmental concentration (EEC) includes removal rates and excretion rates to compensate for some of these deficiencies [34,36]. However, the activity of metabolites is not included in these refinements. The PEC for anti-cancer drugs CPA, 5FU, MTX and IB calculated for France in 2008 was >1.74, 7.91, 1.54 and 4.99 ng/L, respectively [15]. If the PEC value is ≤0.01 μg/L according to EMA [34] or ≤1 μg/L as required by FDA [35], and providing no other environmental concerns are expected then persistence, bioaccumulation and toxicity (PBT) tests are not required. For compounds that are directly interacting with DNA there are no safe thresholds. That is not covered by the above mentioned guidelines. Therefore, there seems to be a gap in the environmental risk assessment of some of the anti-cancer drugs in respect to genotoxicity assessment.

Table 3 gives some examples of measured concentrations of some anti-cancer drugs in various environmental compartments.

Table 2
Physico-chemical and Biological properties of selected anti-cancer drugs.

Parameters	Properties	Cyclophosphamide	Ifosfamide	5-Fluorouracil	Methotrexate	Gemcitabine	Imatinib
Physical/chemical	Structure	(structure)	(structure)	(structure)	(structure)	(structure)	(structure)
	pK_a	2.84	1.45	$pK_{a1} = 8.0$; $pK_{a2} = 13$	4.7	3.6	$pK_{a1} = 8.07$; $pK_{a2} = 3.73$; $pK_{a3} = 2.5$; $pK_{a4} = 1.52$
	log K_{ow}	0.6	0.86	−1	−1.85	−1.22	3
	Solubility (mg/L)	4.00×10^4	4×10^3	1.11×10^4	0.26×10^4	5.14×10^4	1.0×10^3
	Henry's law constant (atm-cum/mole)	1.4×10^{-11}	1.4×10^{-11}	1.66×10^{-10}	1.54×10^{-31}	1.70×10^{-17}	N.D.
	Vapour pressure (mmHg)	4.45×10^{-5}	3.0×10^{-5}	2.7×10^{-6}	2.1×10^{-9}	1.7×10^{-9}	N.D.
	K_{oc}	52	70	8	1	1.4	7.9
	BCF	3	3	3	3.2	1	1
Biological (human)	Biological half life	7 h	6–8 h	5–20 min	low doses: 3–10h; high doses: 8–15 h	1.49 h	18–40 h
	% excretion unchanged	20%	6.6%	10%	80–90%	<10%	81%

N.D. represents no data. *Sources:* HSDB (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB), Kosjek and Heath [13], Xie [15], Zhang et al. [17], Cohen et al. [29], Bagley et al. [31], Heggie et al. [32], Kümmerer et al. [33]

Even though the predicted values given above for France are case specific, these anti-cancer drugs may actually occur at higher levels in the environment (Table 3). This is especially the case of CPA and IF because of their highly persistent nature (see Section 2.3). It is important to note that the measured concentrations of CPA and IF can be 3–20 fold higher than the 0.01 μg/L PEC stipulated in the EMA guidelines. Landfill leachate is particularly a concern since it can leach into groundwater supplies and seep into surface waters. For developing countries where landfills are often times in the form of dumpsites with no bottom capping, no pre-treatment of waste and/or collection of leachates, there the risk to the environment is higher.

Johnson et al. have compared the PEC for CPA across Europe to its MEC in sewage effluents for different countries to find a 60% agreement [36]. Of course, local and regional factors such as variation in consumption levels, excretion rates, removal rates, size of the receiving water body, and limits of detection in analytical procedures can account for differences in the values. Nonetheless, this shows that evaluating the risk of anti-cancer drugs would be better done if the PEC and MEC values are done on a country or area specific basis. Johnson et al. in their evaluations have revealed that the use of anti-cancer drugs can vary within a country and from country to country [36].

2.3. Persistence, bioaccumulation and toxicity (PBT) in the environment

CPA is one of the most frequently found anti-cancer drugs in the environment. Persistency evaluation of CPA as a component of wastewater based on several OECD guidelines revealed it is rather difficult to biodegrade and is released to the environment unchanged [19,23,24,38,43]. IF is another rather persistent anti-cancer drug. It has limited biodegradability [19,23,38,43]. For example, Buerge et al. were able to show in laboratory simulation tests that degradation in lake water under dark conditions results in a half-life of 80 days for CPA and limited degradation for IF [38]. However, in irradiated lake water, degradation progressed at a faster rate with a half-life of ∼44 days for CPA and ∼144 days for IF [38]. Given their estimated half-life and their continuous introduction into the aquatic environment, it is therefore no surprise that these drugs are frequently detected in the aquatic environment. 5-FU, MTX, Cisplatin (CP) and Cytarabine (CA) were also investigated for persistency using the OECD screening test and the OECD confirmatory test to reveal that 5-FU was eliminated but is inversely dependant on the initial concentration while MTX was eliminated regardless of the initial concentration [43]. CP was not eliminated while CA underwent elimination depending on its initial concentration [43]. Kümmerer and Al-Ahmad suggested that their difference in biodegradability is related to the chemical structures of 5-FU, CA and GEMc as investigated in the Closed-Bottle test (CBT: OECD 301D) and Zahn-Wellens test (ZWT: OECD 301B) [21]. CA was more biodegradable than 5-FU and GEMc mainly because it contains pyrimidine and arabinose while 5-FU has no such easily biodegradable sugars and GEMc has fluorinated arabinose [21]. Biodegradation studies as a measure of persistency remains a fundamental aspect in risk assessment since the rapid degradation of a drug leads to less environmental exposure to humans and other organisms. For anti-cancer drugs there seems to be a wide range in degree of persistency across different classes of anti-cancer drugs.

The toxicity of these drugs as components of the hospital wastewater released into the environment is less clearly determined. Gartiser et al. found genotoxicity in several of their hospital wastewater samples using the Ames test with *Salmonella typhimurium* TA 98 and TA100, and chromosome aberration in Hamster V79 cells [44]. Even though the authors found some

Table 3
Environmental occurrence of selected anti-cancer drugs.

Environmental source	Cyclophosphamide	Ifosfamide	5-Fluorouracil	Methotrexate	Gemcitabine
Landfill effluent	97–192 ng/L [37]	32–42 ng/L [37]	N.D.	N.D.	N.D.
Surface waters	<50 pg/L–11 ng/L [37–39]	<0.3–29 ng/L [19,38]	N.D.	<6.25 µg/L [41]	N.D.
Wastewater/Hospital wastewater	19 ng/L–4.5 µg/L [24]	<6–1.9 µg/L [19,24]	<8.6–124 µg/L [40]	1 µg/L [41]	<0.9–38 ng/L [42]
Air	45–13 mg/m^3 [12]	N.D.	N.D.	N.D.	N.D.

References are provided in []. N.D. represents no data.

anti-cancer drugs (CPA, IF and 5-FU) in their samples, they could not attribute the genotoxic response of the samples specifically to these drugs [44]. Similarly, Guiliani et al. found their hospital wastewater samples positive for the Umu C induction [45]. The authors concluded after determining the dose-response relationship of some anti-cancer drugs (Mitomycin C and CP) that the possibility of these drugs causing the genotoxic response of their samples should not be excluded [45]. In 1997, Steger-Hartmann et al. combined the Umu C induction test and analytical methods for hospital wastewater with CPA spiked influent and effluent of a laboratory scale sewage treatment plant to determine if CPA was the causative agent for the genotoxicity response of the hospital wastewater [24]. The authors were able to conclude that CPA was not responsible for the genotoxic response in the hospital wastewater at the concentration it was detected [24]. In light of the body of knowledge presented here, presently there can be no concrete conclusion as to the significance the genotoxic effect of anti-cancer drugs has in wastewater.

In most cases, the PBT data are scarcely available for anti-cancer drugs and therefore most risk classification of anti-cancer drugs or pharmaceuticals on the whole are based on predicted data. Schulmann et al. performed a human health risk assessment of the presence of CPA in drinking water [46]. The authors revealed that the measured CPA was much below the derived threshold value for the ambient water quality criteria and therefore may not be of a risk to human health [46]. Webb et al. stressed that there are no threshold doses for not causing cancer [47]. They have also highlighted that the threshold values are derived from pharmacological data which are based on therapeutic dose and not toxicological threshold data [47]. The Swedish Association of the Pharmaceutical Industry in 2005 preformed an environmental assessment of several pharmaceuticals including anti-cancer drugs [48]. They used PBT from both estimated and measured data to characterized the risk with a resulting 36 antineoplastic and immune system modulating agents (including CPA, IF and MTX) identified as 'cannot be excluded' as a risk to the environment [48]. Several researchers have sought other ways to quantify a risk for these compounds. Johnson et al. used the PEC and predicted no effect concentration (PNEC) to determine the risk of 5-FU in the environment to humans and the environment for UK [10]. They have concluded that even though the estimation of 5-FU concentration in the environment was below the threshold of toxicological concern, there was no idea if this would have an effect on the foetus of pregnant women [10]. Kümmerer and Al-Ahmad had estimated the risk of cancer to humans from the presence of CPA and IF in surface water [25]. They had derived two methods of estimation using the PEC$_{regional}$ (inclusive of consumption data, removal rate, population, volume of wastewater per capita per day and dilution factor) and the PEC$_{local}$ which is modified based on annual consumption data in selected hospitals and the measured and calculated data on influent and effluent concentrations [25]. Based on data of secondary bladder cancer caused by CPA and IF, their results led to the conclusion that the additional risk of cancer from the presence of CPA and IF in surface water cannot be dismissed and therefore there should be proper wastewater treatment procedures to reduce this risk as much as possible [25].

Lately, Besse et al. also used PEC and a refined PEC (includes excretion rates) values to identify anti-cancer drugs of interest in France [15]. The preferential molecules identified that require further investigations include Hydroxycarbamide, Capecitabine, CPA, IF, Mitotane, IB, Tamoxifen, Lapatinib, Flutamide [15].

While there have been some efforts to classify these compounds based on environmental risk, much of it is based on estimated data with limited knowledge on the behaviour and effects on the environment. The deficiency in current information on toxicological effects of these compounds is reiterated in the works of Johnson et al. [10], Besse et al. [15] and Kümmerer and Al-Ahmad [21]. What is more is that we know that several hospital wastewaters containing anti-cancer drugs were found to have genotoxic potential. However, in most studies the amount of anti-cancer drugs expected or measured in hospital effluents could not explain the measured toxic effects. Furthermore, there are also other mutagenic and genotoxic compounds such as disinfectants (e.g. aldehydes or peroxides) and some antibiotics (e.g. ciprofloxacin) present in the 'cocktail' mixture of wastewaters that may exert some of the measured effects individually or in combination. Additionally, one has to be aware that anti-cancer drugs in hospital effluent most often comprise less than 1% of the total municipal sewage flow and that the loads of pharmaceuticals emitted by hospitals is in nearly all cases much lower than the one emitted by the general public [49]. Because of increasing out-patient treatments this does and will hold for anti-cancer drugs too. Therefore, what we are uncertain of at the moment is if anti-cancer drugs have a major influence on the genotoxic potentials of wastewaters. Further, these drugs are not only present as the parent compounds (PC) in the environment but in mixtures with their metabolites and environmental transformation products. CPA and 5-FU as pro-drugs needs to be metabolized to its more active form and therefore, it is necessary to investigate the effects of their metabolites. In fact, Besse et al. argued that the metabolites of some anti-cancer drugs such as MTX can be more active, toxic and less biodegradable than their PC [15]. Similarly, the transformation products can be even more polar and therefore of higher mobility in the aquatic cycle, may be structurally similar, more persistent and maybe more toxic than the PC [50,51]. As such, it is necessary to investigate the transformation products (TPs) and metabolites in addition to the PC if a complete environmental risk characterization is to be achieved. Of course genotoxicity would be an important toxicological criterion given the inherent properties of these drugs.

3. Genotoxicity assessment of anti-cancer drugs and their TPs

It is recommended to use a battery of genotoxicity bioassays when conducting genotoxicity assessment. In 2008, the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use produced the guidance on genotoxicity testing of pharmaceuticals intended for human use in which the requirements of developing a battery of bioassays were specified. The ICH recommended that the general features of a standard test battery includes the assessment of mutagenicity in a bacterial reverse mutation test (Ames test)

and genotoxicity in *in vitro* mammalian cells (recommended are the *in vitro* metaphase chromosome aberration assay, the *in vitro* micronucleus assay and/or the mouse lymphoma L5178Y) and/ or *in vivo* [52]. Toxicity databases such as the Hazardous Substances Data Bank (HSDB; http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB) contains data from a number of *in vitro* genotoxicity bioassays namely the Ames test, *in vitro* micronucleus assay (CHO K1, V79, human lymphoblastoid TK6 cell lines) and a few with *Saccharomyces cerevisiae* and *Escherichia coli* WP2 assays for anti-cancer drugs. So far these have been the most widely used test systems for characterizing anti-cancer drugs from a human regulatory perspective.

3.1. Genotoxicity assessment of PCs and human metabolites

A brief summary of the genotoxic nature of 16 anti-cancer drugs commonly found in the environment is presented in Table 4. The data presented were extracted from various databases and included studies with only positive results from the late 1970s to the 2000s. The tested concentration ranges are provided when available and indicates that often times the positive results are achieved at concentrations considerably higher than those found in the environment (Table 4). Most of the earlier studies are *in vitro* studies aiming to investigate the inherent genotoxicity to reveal the mechanistic properties or modes of action of these drugs. For example, the alkylating agents are detectable in the Ames test with bases substitution strains such as *S. typhimurium* TA 100, TA 1535 and *E. coli* WP2. The use of S9 extracts in *in vitro* test systems to metabolize the drug to its active form provided preliminary information on its metabolites as is the case of CPA and IF. The most common *in vitro* standardized test for mutagenicity is the Ames test using several *S. typhimurium* strains. However, majority of the cytostatic drugs, such as, Procarbazine, Imatinib, MTX, Cytarabine, 5-FU, Epotoside, Vincristine, Bleomycin and GemC proved negative in this test. The use of human or mammalian cell lines are more sensitive to these drugs giving rise to positive results for several endpoints including micronucleus (MN), chromosome aberration (CA) and sister-chromatid exchange (SCE) (Table 4). The few available *in vivo* studies presented the expressed genotoxicity often time leading to micronucleus formation and/or chromosome aberration. The *in vivo* studies are especially important for the pro-drugs since it provides multiple enzymes and target sites for their metabolism.

The genotoxicity data presented here are mostly for the parent compound. Some data are also available for the genotoxicity of the human metabolites of these anti-cancer drugs. For example, CPA exhibits carcinogenic, teratogenic, and mutagenic properties mediated by its metabolites [3,53]. Studies of the metabolites of CPA (phosphoramide mustard, 4-OH-peroxy-CPA, nor-nitrogen-mustard, carboxyphosphamide, 4-keto-CPA) revealed that they are more genotoxic than CPA in Ames test (*E. coli* WP2, *S. typhimurium* TA 1535) and for SCE frequency in human peripheral lymphocytes and CHO cells [54,55]. Often times, the metabolites are more toxic at lower concentrations than CPA itself, i.e., the dose response curves of the metabolites are steeper and start to be genotoxic at lower concentrations than CPA itself. For example, phosphoramide mustard is mutagenic at 25 μg/plate (±S9) while CPA needs to be more than 50 μg/plate and metabolically activated (+S9) in the Ames test (*S. typhimurium* TA1535) before it gives a mutagenic response [54]. SCE in human peripheral lymphocytes exposed to CPA showed that SCE induction occurs at higher than 1×10^{-4} M/L but the metabolites phosphoramide mustard, 4-OH-peroxy-CPA and nor-nitrogen-mustard can produce SCE already at 1×10^{-6} M/L and higher [55]. Nau et al. in their review noted that *E. coli, S. cerevisiae* and to a certain extent peripheral lymphocytes may be able to metabolize CPA by themselves [53]. CPA and its metabolite 4-keto-CPA does not

exhibit significant *in vivo* or *in vitro* cytotoxicity unlike its metabolites, 4-OH-peroxy-CPA, phosphoramide mustard and nor-nitrogen mustard, of which one or a combination of these compounds are responsible for the cytotoxic nature of CPA [56]. In this particular case, the evidence suggests that emphasises should also be placed on characterizing relevant metabolites—in the best case as single compounds as well as mixtures according to their excretion pattern for genotoxicity if we are to fully understand and assess the risk presented by the presence of these compounds in the environment.

On an environmental basis, eco-genotoxicity testing involves two distinct areas of assessment, the initial testing for hazard characterization using *in vitro* bioassays and the use of *in vivo* testing as a part of biomonitoring [57]. In our literature search, eco-genotoxicity of anti-cancer drugs was assessed mostly using the Umu C test or SOS chromotest and *in vivo/in situ* micronucleus (MN) test using several fish species. Grisolia and Cordeiro tested the MN formation after intra-abdominal injection of CPA (20 mg/kg), 5-FU (2.5 mg/kg), Bleomycin (12.5 mg/kg) and Mitomycin (1 mg/kg) in 3 species of fish to find that CPA was the most potent of the 4 anti-cancer drugs even though all produced micronuclei [58]. The review of Al-Sabti and Metcalfe showed that it was possible to detect micronucleus formation for Mitomycin C in a number of other fish species using liver cells, erythrocytes, and embryo cells after intraperitoneal exposure [59]. Generally, the frequency of MN formation varies among fish species. Further, these anti-cancer drugs were administered intra-abdominally and therefore represented the worst-case scenario rather than an environmental exposure [58,60]. Thus far, there are no *in situ* MN tests with fish for environmental biomonitoring of anti-cancer drugs but this method has shown potential for biomonitoring studies of river and paper mill effluent [60]. There are even less studies on *in vitro* MN test using fish cell lines to assess genotoxicity of anti-cancer drugs. In 2000, Sánchez et al. performed the *in vitro* MN test using rainbow trout (*Oncorrhynchus mykiss*) gonadal tissue (RTG-2) cell line to find that Mitomycin C and Vincristine sulphate both showed significant MN formation from concentration as low as 0.25 and 0.0025 μg/mL, respectively [61]. Mitomycin C MN formation shared an inverse relationship to concentration and exposure time test while Vincristine sulphate has a normal dose response relationship. In this test, Mitomycin C showed MN formation at concentrations similar to those that cause an effect in human and mammalian cells [61].

In 2006, Yasunaga et al. tested 18 anti-cancer drugs from the antimetabolites, alkylating agents and cytotoxic groups with the Umu C test [62]. The authors concluded that 15 of them were positive for Umu C induction. The concentration ranges of the drugs tested were all above the PEC thresholds of 0.01 and 1 μg/L established by the EMA and the FDA, respectively. Nevertheless, the cytotoxic anti-cancer drugs Mitomycin C, Bleomycin, Dauno-mycin and Peplomycin were active in the Umu C test at concentrations as environmentally relevant as 20 μg/L to as high as 5000 μg/L [62]. All other anti-cancer drugs to test positive in the Umu C test were at the environmentally irrelevant concentrations of $\geq 2 \times 10^{6}$ μg/L [62]. The authors concluded that the Umu C test can successfully identify anti-cancer drugs that can interfere with DNA through inhibition of DNA synthesis (antimetabolites), DNA base alkylating, DNA strand breaks and DNA adduct to induce the SOS response system but is difficult to assess genotoxicity of anthracycline antibiotics such as aclarubicin and chromomycin A3 [62]. Zounkova et al. tested 5FU, CP, CPA, Doxorubin and Etoposide for genotoxicity with the SOS chromotest and the eukaryotic yeast GreenScreen Assay (GSA) [63]. The results showed minimum genotoxic concentrations (MGC) ranging from 0.3 mg/L for Doxorubin (−S9) to 250 mg/L for Etoposide (+S9) for the Umu C test and 0.02 mg/L for 5-FU to 470 mg/L for CPA in the GSA [63]. No genotoxicity was reported for CPA when tested up to 1000 mg/L in

Table 4
Genotoxicity, carcinogenicity and teratogenicity of selected anti-cancer drugs.

Anti-cancerous drugs	Mutagenicity/genotoxicity						IARC carcino-genicity classification[*]	Evidence for terato-genicity[**]
	In vitro					In vivo		
	Bacteria	Eurkaryotes						
	Ames[a]	Human[b]	Mammalian[b]	Yeast[c]	Others			
Alkylating agents								
Nitrogen mustard analogues								
Cyclophosphamide	20–2500 µg/plate: S. typhimurium TA 1535 (+S9), TA 100 (+S9)	Up to 25 µg/mL: Human peripheral lymphocytes (+S9)	0.1–4 µg/mL: Chinese Hamster Ovary (CHO) (±S9) up to 8000 µg/mL: Mouse lymphoma L5178Y (TK+/TK−) (±S9)	Saccharomyces cerevisiae: 10 mg/mL	Drosophila melanogaster[d]	[CA]Bone marrow cells (Rat): up to 50mg/kg (i.p.)	1 (2012)	Y
	2628–10,000 µg/plate: S. typhimurium TA 1535 (−S9), TA 100 (−S9)	From 1×10^{-5} M: human leukocytes (+S9)	Up to 1000 µg/mL: UDS rat hepatocytes			[MN]Bone marrow cells (Mouse): up to 135 mg/kg (i.p.)		
	200–800 µg/plate or 1 mg/mL: E. coli WP2		Up to 100 µM: bovine lymphocytes (+S9) Other cell lines: Chinese hamster lung (V79) cell, Mammalian polychromatic erythrocytes			[MN]Bone marrow cells and peripheral blood (Dog): up to 25 mg/sq m (i.v.)		
Ifosfamide	20–600 µg/plate: S. typhimurium TA 100 (±S9), TA1535 (±S9)				Drosophila melanogaster[d]	[MN]Bone marrow polychromatic erythrocyte (PCE) assay (Mouse): up to 35 mg/kg (i.p.)	3 (1987)	Y
Other alkylating agents								
Dacarbazine	Up to 2500 µg/plate: S. typhimurium TA 1535 (+S9), TA 100 (+S9), TA 97 (±S9), TA 98 (±S9); up to 0.2 µM: TA 100 (±S9), TA 92 (±S9),G46 (±S9) Up to 0.02 µM: E. coli WP2, WP2 UVRA, TM6, TM9	2×10^{-3} M: CHO cells			Drosophila melanogaster[d]		2B (1987)	Y
Antimetabolites								
Folic acid analogues								
Methotrexate	Up to 0.25 mg/mL: E. coli WP 2	Up to 300 µg/mL: human lymphoblastoid TK 6	Up to 1.25 µg/mL: Mouse lymphoma L5178Y (TK+/TK−) (±S9)			[CA]Bone marrow cells (Mouse): up to 10mg/kg (i.p.)	3 (1987)	Y
Pyrimidine analogues								
Cytarabine	Up to 2000 µg/plate: E. coli WP2	Up to 200 µM: human lymphocytes Up to 300 µg/mL: human lymphoblastoid TK6	Up to 10 µg/mL: mouse lymphoma L5178Y (TK+/TK−) Up to 2 µM: Chinese hamster V79					

Fluorouracil		Up to 100 µg/mL: human lymphoblastoid TK6	Up to 8 µg/mL: Mouse lymphoma L5178Y (TK+/TK−) (±S9); Up to 5000 µg/mL: Chinese hamster V79; Up to 12.5 µg/mL: CHL/IU	MN Bone marrow cells (Mouse): up to 15 mg/kg (i.p.); MN Blood erythrocytes (Mouse): up to 50 mg/kg (i.p.); MN Micronucleated hepatocytes (Rat): up to 40 mg/kg (i.p.)	3 (1987)	Y
Gemcitabine		Up to 0.004 µg/mL: human peripheral lymphocytes		MN, CA Bone marrow cells (Mouse): up to 8 mg/kg (i.p.)		
Plant alkaloids and other natural products						
Vinca alkaloids and analogues						
Vincristine		Up to 1 µg/mL: human lymphoblastoid TK6	Up to 2.5 mg/mL: mouse lymphoma L5178Y (TK+/TK−)		3 (1987)	Y
Podophyllotoxin derivatives						
Etoposide		Up to 1 µg/mL: human lymphoblastoid TK 6	up to 2 µM: Chinese hamster V79; Up to 5 µg/mL: Chinese hamster ovary K1 (±S9); Up to 0.05 µg/mL: CHO XRS-5; Up to 1.5 µg/mL: mouse lymphoma L5178Y (TK+/TK−)	CA Bone marrow cells (Mouse): up to 20 mg/kg (i.p.); MN Bone marrow polychromatic erythrocytes (Mouse): up to 20 mg/kg (i.p.)	1 (2012)	
Cytotoxic antibiotics and related substances						
Anthracyclines and related substances						
Doxorubicin	Up to 10 µg/mL or 10 µg/plate: S. typhimurium TA 98 (±S9), TA1538, TA 1978/PMK101, TA 2637, TA 102 (±S9)	Up to 0.15 µg/mL: human lymphocytes	Up to 1 µg/mL: Chinese hamster V79	CA Bone marrow cells (Rat): up to 90 mg/kg (i.p.)		
Daunorubicin	Up to 100 µg/plate: S. typhimurium TA 98, TA 100, TA1538; Up to 10 µg/plate: S. typhimurium TA 1978/PMK101, TA 2637, TA 102 (±S9); TA 1537, GW257	5×10^{-9} M: Human lymphocytes; Up to 0.12 µM: Chinese hamster V79	Up to 0.1 µg/mL: CHO; 0.16–0.32 µg/mL: CHO cells	MN Bone marrow erythrocytes (Mouse): up to 15 mg/kg (i.p.)	2B (1987)	
Other cytotoxic antibiotics						
Bleomycin	Up to 4 µg/plate: S. typhimurium TA 102	Up to 210 µg/mL: human peripheral lymphocytes; Up to 100 µg/mL: Chinese hamster V79	Up to 7.5 µg/mL: CHE; *Saccharomyces cerevisiae*	MN Peripheral blood reticulocytes (Mouse): up to 50 mg/kg (i.p.)	2B (1987)	

Table 4 (*Continued*)

Anti-cancerous drugs	Mutagenicity/genotoxicity						IARC carcino-genicity classification[*]	Evidence for terato-genicity[**]
	In vitro					In vivo		
	Bacteria	Eukaryotes						
	Ames[a]	Human[b]	Mammalian[b]	Yeast[c]	Others			
Other antineoplastic agents								
Platinum compounds								
Cisplatin	Up to 50 µg/plate: *S. typhimurium* TA 98 (±S9),TA 100 (±S9), TA 1535 (±S9), TA 1538 (±S9), TA 98 (±S9), TA 102 (±S9), TA 2410 (±S9), TA 92 (±S9) Up to 50 µg/plate: *E. coli* WP2	Up to 12 µM: MT1, TK6, HCT116, lymphocytes	Up to 40 µM: CHO/ HGPRT, Chinese hamster V79, CHO k1, UDS rat hepatocytes		*Arabidopsis* species[e]	[MN]Peripheral blood cells (Mouse): up to 6 mg/kg (i.p.) [CA]Bone marrow cells (Mouse): up to 8 mg/kg (i.p.)	2A (1987)	
Carboplatin	Up to 30 µg/plate: *S. typhimurium* TA102 (±S9)		Up to 100 µM/plate: Chinese hamster V79					
Methylhydrazines								
Procarbazine			Up to 12,000 µg/mL: Chinese hamster V79 (±S9)	*Saccharomyces cerevisiae*			2A (1987)	
Other antineoplatic agents								
Imatinib		Up to 20 µM: Normal human dermal fibroblasts (NHDF)	Up to 20 µM: CHE, Indian muntjak fibroblasts (IMF)					

Sources: CCRIS (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS), HSDB (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB), GENTOX (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?GENETOX), IARC (http://monographs.iarc.fr/ENG/Classification/) Accessed on: 02–03, 2013). MN=micronucleus, CA=chromosome aberrations, Y=indicates there is evidence for teratogenicity.

[a] Ames by standard plate method or pre-incubation or fluctuation method.
[b] One or more of these endpoints investigated: Micronucleus test in vitro, chromosome aberrations, Sister–chromatid exchange (SCE) in vitro.
[c] One or more of these endpoints investigated: forward gene mutation, mitotic recombination or gene conversion, reverse gene mutation.
[d] Sex-linked recessive lethal gene mutation.
[e] Gene mutation.
[*] IARC classifications are Group 1: Carcinogenic to humans, Group 2A: Probably carcinogenic to humans, Group 2B: Possibly carcinogenic to humans, Group 3: Not classifiable as to its carcinogenicity to humans, Group 4: Probably not carcinogenic to humans; ()=year of classification.
[**] Ref.: Some Antineoplastic & Immunosuppressive Agents, IARC Monographs, Volume 26, 1981.

the Umu C test but it was positive for genotoxicity at high concentration to the yeast cells [63]. Lately, Zounkova et al. used the Umu C test to determine genotoxicity of 5-FU, CA, GemC and their respective metabolite [64]. CA (167 mg/L (−S9); 333 mg/L (+S9)) and GemC (167 mg/L (−S9); 42 mg/L (+S9)) were genotoxic while their respective metabolites were not genotoxic. Due to high cell cytotoxicity, 5-FU was negative in the Umu C test but its metabolite revealed a genotoxic response at 667 mg/L [64].

Generally, genotoxicity of anti-cancer drugs in short term *in vitro* toxicity testing is reported at very high concentrations. While this may be relevant from a human health perspective and in determining the mechanism of toxicity, environmental organisms may be at risk to chronic dosage of these compounds and their TPs. Most of the time, the metabolites are simply included as part of the PC mixture after metabolism *in vivo* or via S9 mix in *in vitro* test systems. However, this does not provide information on which of the metabolites are toxic. This represents an important area for further research especially since some of these drugs are not excreted predominantly as the PC (Table 2) while others act as pro-drugs having metabolites that are more toxic than the PC.

It is noteworthy to mention that the limited *in vivo/in situ* studies using various fish species has shown potential for use in biomonitoring studies of anti-cancer drugs after intra-abdominal or intra-peritoneal exposure. However, the exposure route does not provide a realistic approach of environmental exposure and therefore, there is a general need to establish the sensitivity of various fish species to these drugs using more realistic approaches. Aquatic organisms are smaller in size, have different metabolism and spend their entire life cycle exposed in waters, therefore, the maximum tolerant dose to these organisms would be different from the better established doses for humans and other mammals. Further, the lack of *in vitro* genotoxicity testing using fish cell lines leads to the inability to determine its correlation to *in vivo* fish genotoxicity testing. However, it is believed that fish cells lines have genotoxic response to the same chemical mutagens and clastogens as mammalian cells lines [65]. Now only the sensitivity of aquatic organisms to these drugs needs to be established. Thus far, only few cases of genotoxicity testing of known metabolites were reported [54,55,64].

3.2. Genotoxic assessment of mixtures derived from abiotic treatment of anti-cancer drugs formulation

Pharmaceuticals in the environment can undergo chemical and biological transformations during wastewater treatment, raw water treatment used as drinking water and in the environment. Their concentration can be lowered by sorption on sediments and suspended materials. However, if PCs and TPs are very polar, elimination by sorption will be of lower importance. Several treatment processes including chemical treatment and advanced treatments such as UV photolysis, ozonation and/or chlorination among others are also investigated as possible options for removing anti-cancer drugs prior to release in the environment [16,17]. The extent to which the primary and secondary treatment of WWTPs or the various advanced treatment methods eliminates these anti-cancer drugs in the environment is not discussed here nor is there much emphasis on identification of TPs. Instead, the focus is given to the methods of genotoxicity assessment of the TPs formed from these processes. However, it is believed that most of these treatments result in the formation of hitherto unknown TPs of most often higher polarity and therefore higher mobility in the aquatic cycle.

In 1985, anti-cancer drugs were considered in a program initiated by the International Agency for Research on Cancer (IARC) to investigate the chemical treatment of waste containing carcinogens. In this program strong oxidants such as potassium permanganate were used in the treatment process [66–68]. Later on, chemical treatment using less harmful oxidants such as sodium hypochlorite (bleach) were found as an effective means of treating waste containing anti-cancer drugs [69–71]. These studies are the first of studies to consider the mutagenicity of mixtures or residues derived from treating anti-cancer drugs. In fact, the program emphasized the need to closely use analytical methods to determine the degree of elimination of the PC and mutagenicity testing to determine its inactivity. A summary of the results of this program is given in Table S1. A closer look at the strategy used in this program revealed several trends including:

- High initial concentration of the PC. In this case, most of the treatment are directed towards hospital waste and therefore may be in the ranges of the pharmaceutical preparation of the drugs. In fact, Lunn et al. showed that the tested concentrations of some anti-cancer drugs were mutagenic before treatment [67]. Further, the initial concentration and amount of residue generated could also affect the chances of detecting a positive response in the mutagenicity test of the TPs mixtures [69,70,72].
- Mutagenicity testing was done with Ames test using several strains. In some cases, only strains known to positively respond to the drug were used [68] while in other cases a series of strains were used [67,69].
- Drug preparation can have an effect on the efficiency of the chemical treatment. The effect of using different solvents was clearly shown in the experiments conducted by Lunn et al. [67] and Hansel et al. [69].
- The quenching of oxidants and neutralization of pH after reaction is necessary for the mutagenicity test.
- Treatment controls consisting of the solvent treated and deactivated similarly to the spiked samples are necessary for mutagenicity computation. Hansel et al. [69] were able to show that their treatment controls were negative for mutagenicity but Benvenuto et al. [68] showed that their treatment controls led to an increase in revertants over the background levels. Benvenuto et al. then expressed their positive results as percentage mutagenicity increase over the treatment control while their negative results as that similar to the background levels [68].
- High performance liquid chromatography (HPLC) was used to monitor PC primary elimination in the sample. Of course the degree of degradation of the drug is based on the limit of quantification of the method and the limitations in detection such as altered UV-spectrum of TPs. Similarly the identification of TPs using gas chromatography coupled with mass spectrometry (GC–MS) is also dependent on the limit of detection of the method and the extractability of the unknown TPs. In most cases, TPs were not considered.

In addition to the use of chemical oxidation methods, Burleson and Chambers [73] and Lunn et al. [72] utilized ozonation and photolysis for treatment of anti-cancer waste (Table S1). Although Burleson and Chambers were unsuccessful in producing non-mutagenic residues, they showed another strategy for monitoring mutagenicity of the residues [73]. They used a single strain that can detect the mutagenicity of CPA to test the residue derived from ozonation of CPA at the maximum non-mutagenic concentration. They were able to deduce that the products of the ozonation were more mutagenic than the PC and further suggested that it could be the metabolite of CPA. In fact, Fernández et al. showed from their ozonation investigations that the main resultant product after treatment was the metabolite 4-keto-CPA [74]. Given that the metabolites of CPA that is a pro drug are more toxic and may be the most likely to be excreted, their presence in the environment may

be of a greater concern. Further, it is even possible that these metabolites may be TPs of CPA resulting from biotic or abiotic processes, hence the need for genotoxic assessment of CPA after various treatment processes. Benvenuto et al. also used a single strain that is able to detect the mutagenicity of the PC to assess the mutagenicity of their mixtures and successfully show that 8 out of the 10 treated mixtures were mutagenic by the same strain as the PC [68] (Table S1).

Lunn et al. took another route to understand the mutagenicity of the residues or mixtures after treatment by evaluating both the treated mixture and isolated TPs separately using a number of strains [67]. For example, although Carmustine (positive in the Ames test using TA 98, TA100, TA 1535 and TA 1530) was completely eliminated, the resulting mixture after treatment by hydrogen bromide in glacial acetic acid was mutagenic to strain TA 100, TA 1535 and TA100 (Table S1). Testing the isolated TPs showed that these compounds were responsible for the mutagenic response of the mixture after treatment.

In the studies presented here, genotoxicity assessment was done using *in vitro* test for the inherent genotoxicity of the drugs and their TPs' mixtures. In each case, the studies were directed towards inactivating the toxicity of the anti-cancer drug prior to its release to a WWTP and subsequently the environment. The few examples given here suggest that in most cases, the toxicity of the treated mixtures often time mimics the PC and therefore would suggest TPs with similar mode of actions. Commonly, the treated mixture was positive for genotoxicity but the TPs were not identified or further investigated. Therefore, it was often time not possible to determine if there was a single TP that was responsible or a combination of TPs. It is noteworthy to understand that all of the anti-cancer drugs used in these tests are responsive to the Ames test for mutagenicity using one or more strains with the only exception being MTX. As such, the high initial concentration used made it possible to couple mutagenicity testing with analytical methods. However, the relevance of this choice of bioassay for determining genotoxicity inactivity may not be the same for other anti-cancer drugs such as 5-FU, GEMc, among others that are negative in this test (Table 4). Nevertheless, the little evidence here suggests the need to investigate genotoxicity of the treated waste containing anti-cancer drugs. The question now is when and how to incorporate this in the environmental risk assessment of these drugs.

4. Incorporating genotoxicity assessment into environmental risk assessment of anti-cancer drugs and their TPs

On a regulatory basis, there are some regulations that stipulate the necessity of an environmental risk assessment of certain drugs before they are marketed. The FDA produced the guidance for industry for the environmental assessment of human drug and biologics applications [35]. In the European Union, Directive 2001/83/EC [75] resulted in the creation and approval of a guideline on the environmental risk assessment of medicinal products for human use by the EMA in 2006 [34]. In both of these guidelines, toxicity testing is recommended with aquatic species of different trophic levels, i.e. representative of algae, invertebrates and fish. In most cases the tests performed are the algae growth inhibition test (OECD 201), *Daphnia sp.* reproduction test (OECD 211) and the fish early life stage toxicity test (OECD 210) [34]. Of course in both guidelines, tests deemed necessary based on the nature of the pharmaceuticals could also be used as long as it is appropriately justified and is a standardized test.

The FDA guideline requires studies to be focused on the fate and effects of 'the active moiety and/or structurally related substances (SRSs), rather than on excipients, for example'. SRS was defined to encompass dissociated parent compound, metabolites, or degradants and therefore included transformation products mainly as degradants formed from environmental processes such as hydrolysis. Relevant SRS were deemed as those that are greater than 10% of the PC initial dose [35]. TPs formed by the metabolism of organisms (bio TPs) in the environment were also considered in this guideline where it specifically stated that 'Chronic toxicity testing should be considered if the compound has the potential to bioaccumulate or bioconcentrate, if indicated based on Tier 1 and/or Tier 2 testing, or if there are other indications that the compound undergoes biotransformation to more toxic compounds.' The European counterpart, the EMA guideline, provided less guidance on risk assessment involving TPs as it clearly states that 'Refinement of the risk assessment using data on transformation of the substance within the environment (i.e. the water/sediment systems) is not further considered here and is subject to expert judgement.' Therefore, limited regulations exist on the inclusion of TPs in risk assessment. Hence, the concern now is how to incorporate the TPs into risk assessment strategies.

4.1. General approaches for environmental risk assessment to include TPs

In 2011, a review on the environmental risk assessment of TPs was compiled by Escher and Fenner [76]. The authors stressed the importance of assessing the toxicity of TPs since they often exhibit the same mode of toxic action and may even have an additive or synergistic effect in mixtures. The authors outlined two approaches for assessing the risks of TPs. The first is the exposure driven approach that entails the isolation and identification of the TPs formed during simulation studies followed by toxicity or fate assessment. Identification and isolation of relevant TPs can be a difficult process and therefore may present a disadvantage to using this strategy. Moreover, it is never clear whether all TPs were seen in the chromatographic analysis because of their unknown chromatographic behaviour as well as in the sample pre-treatment. Moreover, each detector has its own limitations, characteristics, specificity, and detection limits. Furthermore, different treatment conditions such as pH, concentration and others as well as different treatment procedure may result in the formation of different TPs at different concentrations. In any case, the identified TP may not be known and therefore no toxicity profile based on a known chemical structure available. The TP could be synthesized to characterize its biological effect [50], if its chemicals structure could be established. Isidori et al. demonstrated the usefulness of the exposure driven approach in their studies of light (Xe lamp) treated pharmaceuticals (Naproxen, Furosemide and Ranitidine) followed by genotoxicity assessment [77–79]. In their studies, the PC was subjected to photolysis after which the TPs were identified and isolated. Both the PC and the isolated TPs were then profiled for genotoxicity using the SOS chromotest (*E. coli* PQ37) and the Ames test (*S. typhimurium* TA 98 and TA100). With this approach they were able to:

- Separately determine the genotoxic concentration of the PC and the isolated TPs and their magnitude of effect.
- Estimate the relevance of the genotoxic effect based on environmental concentration of PC.
- Compare the mechanism of toxicity of the PC to the isolated TPs. For example, Furosemide was not mutagenic in the Ames test but its photoproduct was mutagenic in strain TA 98.

The second approach proposed by Escher and Fenner is the effect driven approach that follows a tiered system of analysing toxicity of the TPs using a battery of bioassays as it moves from untreated to the treated phase [76]. The most important aspect of this approach is the careful selection of the battery of bioassays.

This approach has the advantage to explore mixture toxicity. However, it does not necessarily allow for the identification of the specific molecule(s) that may be responsible for the measured effect(s) and therefore lacks some information. It can be time-consuming but it is mostly geared towards detecting toxicity deviation from the PC [50]. Schirmer demonstrated the effect driven assessment approach for the herbicide diuron treated by four different oxidative methods [50]. Sample mixtures were taken at regular time intervals during treatment to be bioassayed for photosynthesis and algal growth. Key information extracted from effect driven approach included:

- Effective concentration of PC.
- Effect of unknown TP mixtures at varying time points of treatment.
- The computation of the toxic equivalent concentrations (TEC)—effects of total mixtures to be compared with that of the parent compound.
- The comparison of TEC and concentration of parent compound.

Escher and Fenner also explained that in cases where the treated mixture behaved differently from the PC, it is possible to fractionate the mixture based on physico-chemical properties such as hydrophobicity and then investigate the toxicity of the extract to explain the deviation [76]. However, again the disadvantage here is the uncertainty that no constituent of the sample is lost, especially as most TPs will probably be very similar, e.g. with respect to the position of an OH-group, e.g. at an aromatic ring and therefore of similar hydrophobicity.

4.2. Characteristics of genotoxicity assessment in an environmental risk assessment framework for TPs

With regards to genotoxicity risk assessment, the majority of the studies on anti-cancer drugs and their TPs presented in Table S1 can be grouped into the effect driven approach for risk assessment. They all carry the characteristic treatment for a specified time followed by the genotoxic assessment of the treated mixture. Other authors used photolysis to treat antibiotics and then evaluated the genotoxicity in the *in vitro* micronucleus test (Hep G2 cells) of the mixture sampled at different time points during treatment rather than a single endpoint after treatment [84,85]. In this case, the genotoxic assessment of multiple treatment time allowed for a better determination of the treatment residence time to reduce the activity of the mixture. Analytical methods such as LC–MS/MS were used to identify TPs and to monitor their development across the treatment time [84,85]. The use of LC–MS as opposed to the GC–MS method used in the IARC program provided an added benefit since GC is only suitable for volatile, low polar compounds. For the identification of TPs, LC combined with UV–vis/mass spectrometry is necessary. Further, in order to establish the chemical structure of TPs high-resolution mass spectrometry is highly recommended.

In addition to having analytical methods to monitor the elimination of the PC, it is also important to monitor other parameters such as the dissolved organic carbon (DOC) content to determine the degree of mineralization of the PC. DOC is combination with monitoring PC concentration can serve as an initial benchmark for determining the presence of TPs and the requirement for further genotoxicity studies. A drawback of such an approach is the high concentration necessary for the analytical methods to measure DOC removal and to safely identify TPs. With the advent of high resolution MS at least for the identification of TPs at lower concentrations would be possible. However, to assess the degree of mineralisation which is an important piece of information within the whole assessment process still needs

concentrations that are high compared to the concentrations of anti-cancer drugs found in the environment. Therefore it might be necessary to have a treatment at high and low concentrations of the PC and to monitor whether the kinetics and type of TP formation is the same at the different concentrations. Nevertheless, TP risk assessment studies are done up to now with the initial concentration ranges of PC determined by the limit of detection of the analytical method and the sensitivity of the bioassay used.

For genotoxicity testing, post-treatment treatment after AOP is usually done to remove reactive oxygen species (ROS) and other free radicals stemming from treatment. Some researchers showed that post treatment methods such as use of the de-chlorination agents sodium sulfite or sodium thiosulfate mask the mutagenic effect after the sample is concentrated [80,81]. However, at the same time Schneck et al. [82] and Gartiser et al. [83] noted that also the generation of mutagenic artifacts is possible during concentration from the interaction of disinfectants with resins if no post treatment is done. Vasquez et al. noted that both post treatment and storage of the mixtures prior to toxicity testing can remove ROS and other free radicals while ensuring only stable TPs are tested [84]. The choice of post treatment method is also dependent on the bioassay used and the aims of the study, i.e. whether it is to establish the effects of certain compounds or to investigate the mixture toxicity. Regardless of the method chosen, it is advisable that appropriate test and treatment controls are prepared to test the effect of the treatment and post treatment on the bioassay.

Although the effect driven approach is preferable for mixture toxicity, it can sometimes be very difficult to identify the TPs responsible for genotoxicity in the mixture from simply comparing the development of single TPs to the mixture genotoxicity across time points. In fact, both Garcia-Käufer et al. and Vasquez et al. were unable to attribute the genotoxicity of mixtures at different time points to any of the identified TPs [84,85]. A possible addition to this approach is the use of multispecies modelling to predict the toxicity of the identified TPs based on quantitative structure-activity relationships (QSARs). A prerequisite despite the reliability of the predictions is the safe establishment of the chemicals structure of the TPs. Escher and Fenner have proposed the use of the toxicity related structural alerts of the PC as the basis for the predicting relative effect of the TPs based on their structure similarities or differences [76]. Neuwoehner et al. and Mahmoud et al. have demonstrated the usefulness of this method to determine the relevant TPs for higher tier toxicity testing [86,87]. Serafimova et al. reviewed QSAR models for predicting mutagenicity and carcinogenicity stressing that as with all computational models, the predictions should be carefully interpreted by experts knowledgeable in the pros and cons of the model [88]. As with much of the toxicity databases available for genotoxicity of chemicals, these models are based on genotoxicity data from Ames test and/or *in vitro/in vivo* tests for MN, CA and other endpoints using mammalian cells. Hence, this is another reason why these common genotoxicity bioassays would be selected for monitoring changes in mixture toxicity. The higher tier toxicity testing of TPs with structural alerts varying from the PC consisted of a bioassay that can identify the mode of action of the PC and an unspecific bioassay [76]. This is specifically useful in identifying TPs which exhibit independent action from PC. This was demonstrated by Lunn et al. when they were able to show that the mixture was mutagenic in the Ames test with strains similar to the isolated TPs and not to all the strains responsive to the PC [67].

Each of the approaches proposed by Escher and Fenner [76] have been used in genotoxic risk characterization studies of pharmaceuticals and their TPs with defining set of characteristics (Fig. 1). As of now the general lack in knowledge of the occurrence, fate and effects of TPs in the environment necessitates studies of these two approaches that are directed towards understanding the

TP formation and effects in lab-scale simulation tests. Until sufficient information of TPs is gathered, then can we consider 'environmental' risk assessment of known and relevant TPs.

In addition to treatment followed by toxicity testing, it is important to understand that TPs that will readily biodegrade in the environment may be not of relevance. Therefore, before testing the toxicity of TPs it may be advisable to perform a biodegradation test with the treated samples in order to see which TPs are to be expected to be stable in the environment. The Organisation for Economic Co-operation and Development (OECD) guidelines for testing chemicals (Section 3) has standards for testing biodegradability (e.g. OECD 301, 302B) and direct photolysis (OECD 316) [89]. In recent years, analytical methods to identify and analyse

transformation products of anti-cancer drugs are also now better developed [13,21,43]. If the chemical structure of TPs is known this can also be done using computational methods. Again, the use of QSARs and other chemoinformatical approaches for predicting biodegradability and their results should be carefully handled by experts [90]. Besides biodegradability, other treatment processes are not standardized and publications often lack full information on the conditions of the treatment. Therefore each researcher used their own method to conduct the treatment. For example, Fatta-Kassinos et al. provided evidence of the complexity in comparing photolytic degradation across studies because of the lack of a standard reporting method [91]. The authors concluded that at least parameters such as description of photo reactor, the light
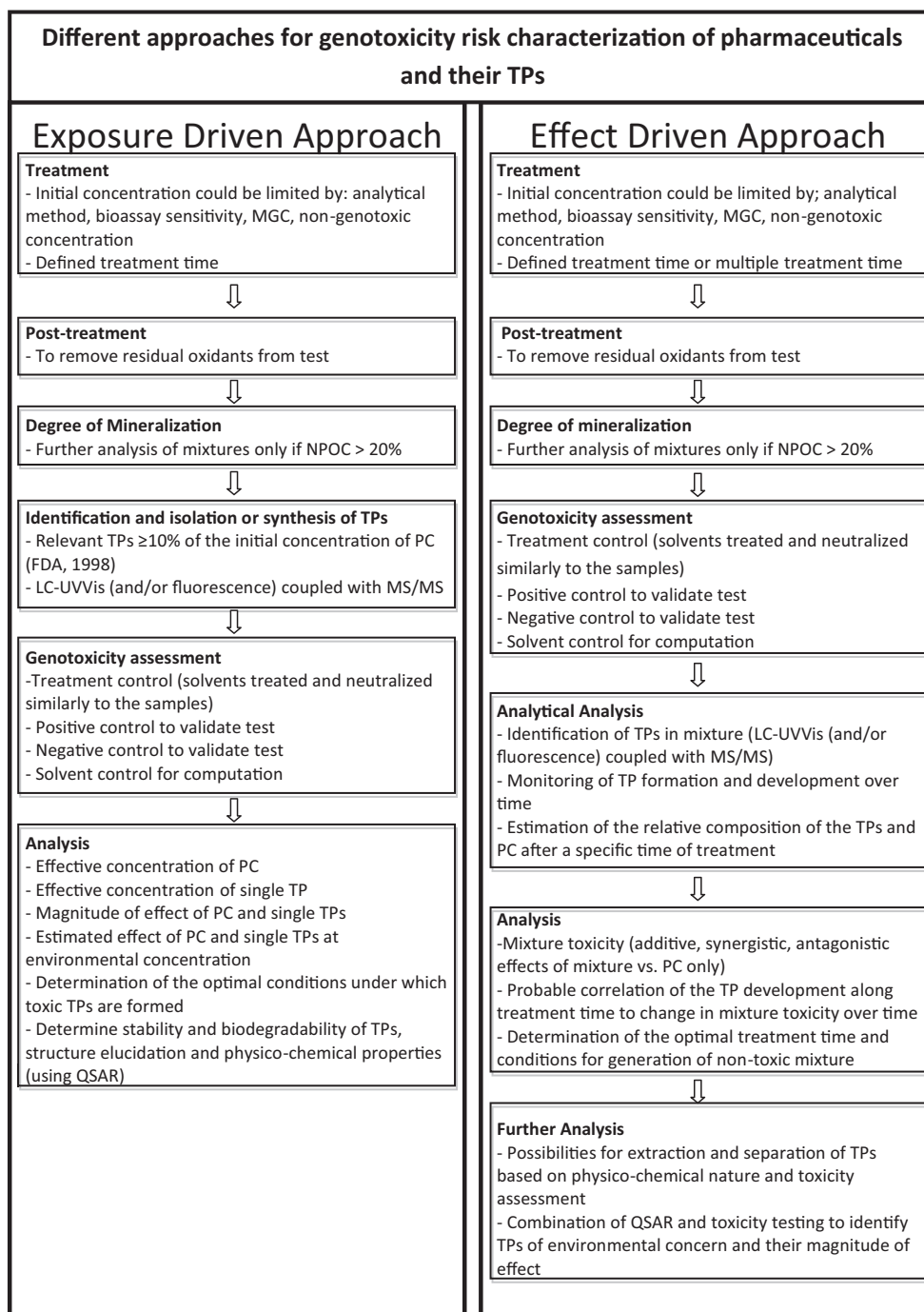
Fig. 1. Characteristics of the two main approaches of genotoxic risk assessment of pharmaceuticals and their transformation products using the two approaches of Escher and Fenner [76].
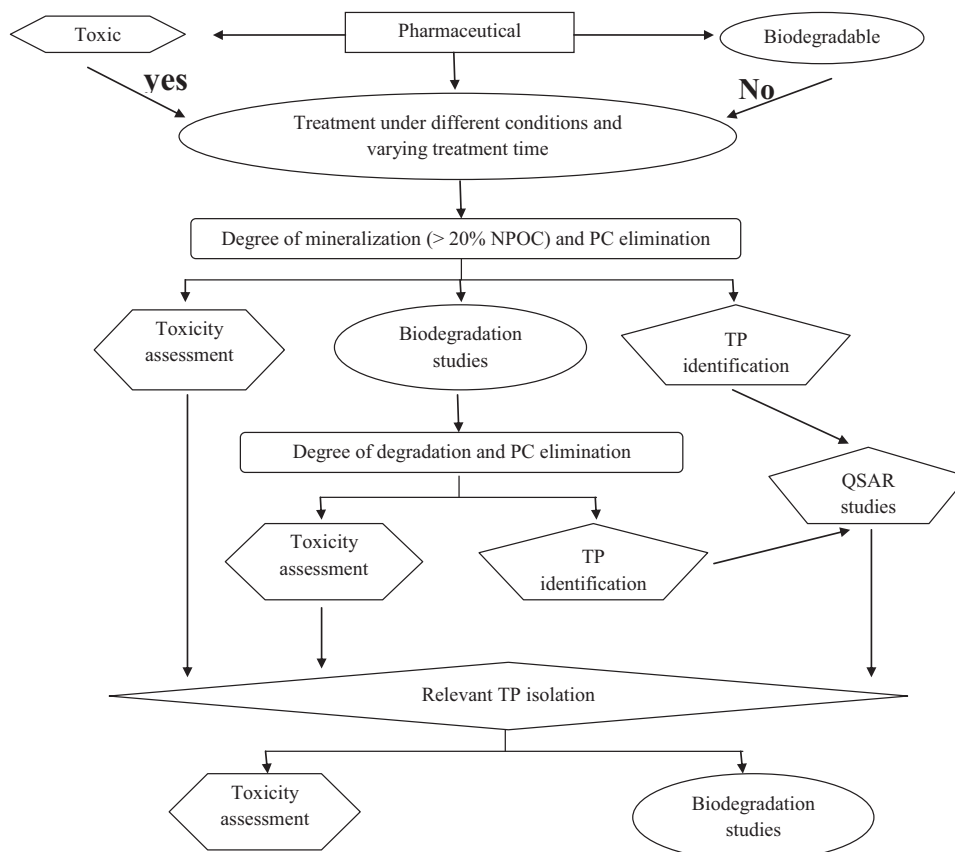
Fig. 2. Flow chart a possible strategy of environmental risk assessment of pharmaceutical drugs.

source, water matrix and initial concentration should be reported [91]. To assess the intensity of the source of radiation is a time consuming process and is therefore often not performed. However, it has a high impact on the type of TPs formed as well as the kinetics. Therefore, it is important that the treatment procedure is explicitly and extensively performed and reported to understand the conditions under which certain TPs are formed and would likely to be detected in the environment.

Thus far there is no standardized protocol for risk assessment of TPs. Even more critical is that the few studies that have sought to characterize these drugs and their TPs are scattered over the years and have varying methodologies for investigation (Table S1). Therefore summarizing all that is currently known, a working strategy for the risk assessment of pharmaceuticals in general is given in Fig. 2. For genotoxicity risk assessment, not only the type of genotoxicity bioassay chosen is an important factor but also in our opinion proper documentation of the circumstances and procedures (how the material and samples thereof were generated, e.g. the specific conditions of the treatment process in the case of advanced effluent treatment as well as the treatment of the samples, e.g. enrichment or not and if yes—how) and quality control of the treatment processes and sample treatment are important towards understanding the circumstances under which certain TPs are formed and can cause certain effects.

4.3. Selection of bioassays for genotoxicity risk characterization

The choice of risk assessment approach would largely be based on the focus of the study whether it is to profile the toxicity of PCs and/or isolated TPs or to elucidate the formation and contribution of individual or all TPs to mixture toxicity. Regardless of the approach taken to study the effects of PCs and their TPs, there are

several areas such as the type of bioassay that need careful considerations when designing the research method for genotoxic risk characterization.

So far much of the genotoxicity monitoring of treated pharmaceuticals in general involve bacterial assays. In addition to the bacterial *in vitro* test systems, the OSPAR commission survey of genotoxicity tests applicable for testing wastewater listed tests for eukaryotic cells for genotoxicity testing not only from a human prospective but also from an environmental perspective (eco-genotoxicity) [92]. The survey has listed in addition to the *in vitro* micronucleus assay and *S. cerevisiae*, the comet assay and sister chromatid exchange using several cell lines including fish, algae and/or protozoa that were used for both surface and waste water samples tested as whole effluent.

Generally, the selection of a bioassay for TP risk assessment is usually dependant on the mode of action of the PC tested. As such, the inherent genotoxicity of the TP mixture is the primary target for these analyses and hence the reliance on the Ames test in most of these studies in order to find out the mode of action of the mixture (i.e. its constituents) and by this the inherent toxicity of the mixture. The major drawback of using the Ames test is that there are several strains available, each with a different mode of action. At the same time this is its greatest strength as it can appropriately define the mode of action of the TPs based on the strain detecting its activity. The mutagens detected in the Ames test have had good correlation with carcinogenicity in experimental animals [93,94]. According to a review by Walmsley and Billinton, the sensitivity (the ability of a test to identify positives, i.e. genotoxic carcinogens) and the specificity (the ability of a test to identify negatives, i.e. non-genotoxic carcinogens) are 60% and 77% respectively for the Ames test [95]. Recently, the Ames fluctuation test as a micro-plate format (MPF) was standardised as

ISO 11350 [96]. This version of the Ames test would be able to detect genotoxicity in wastewater and water applying a low dilution factor (1.25) in the test system. An international round-robin study using the ISO 11350 protocol for testing river water and genotoxin spiked river water samples showed an overall sensitivity of 100% and specificity of 90% [96]. Escobar et al. provides a review of the other versions of the Ames test highlighting their weaknesses and strengths [97].

The Ames test has clear limitations for genotoxic risk characterization of anti-cancer drugs since some anti-cancer drugs cannot be detected by the standard *S. typhimurium* strains of TA 100 and TA 98. Ferguson and Denny have provided an overview of cases where applying the Ames test to evaluate mutagenicity of anti-cancer drugs may be misleading [27]. This includes inappropriate metabolic activation, the lack of the *uvr*B gene that can affect detection of some alkylating agents, the dependence on bacterial gyrases to detect topo II inhibitors and the presence of metabolites in the media that can affect detection of antimetabolites' mutagenic properties. Further, Jolibois and Guerbet have repeatedly shown that in testing wastewater using the Ames test, the TA102 strain is the most sensitive [98–100]. In fact, the authors have alluded that sensitivity of TA102 to wastewater mutagenicity is based on its ability to detect a variety of mutagens and most anti-cancer drugs since the histidine mutation is introduced into a multicopy plasmid rather than the bacterial chromosome [98–100]. Table 4 has highlighted that *S. typhimurium* TA102 and/or *E. coli* strains are two of the strains to commonly detect the mutagenicity of the selected anti-cancer drugs (Table 4). An alternative genotoxicity screening tool with similar specificity and sensitivity as the Ames test is the Umu C test [101] or the SOS chromotest. This test assesses genotoxicity based on the ability of the toxin to induce the SOS repair response system upon DNA damage. It has been used in the genotoxic risk assessment of pharmaceuticals and their TPs [20,77–79,83]. Yasunaga and co-workers have proved that the umu test can detect genotoxicity for a number of anti-cancer drugs with different mode of actions [62]. Further the umu is an inexpensive test with the possibility of high throughput. However, the major limitation to using any of the bacterial assays is that the concentration required to cause an effect is much higher than the environmental concentration of anti-cancer drugs. This can therefore limit their applicability for environmental monitoring. Generally, the *in vitro* genotoxicity test with mammalian cell lines can detect genotoxicity activity at lower concentrations of anti-cancer drugs (Table 4). In fact, *in vitro* mammalian test for CA and mutation have better sensitivity than the Ames test but are less specific [95]. Ideally, the use of *in vitro* tests using aquatic cell lines would be more beneficial to aquatic risk assessment of anti-cancer drugs and their TPs than mammalian cell lines.

The lack in genotoxic profiling of anti-cancer drugs using eco-genotoxicity testing systems represents a research gap that needs to be urgently filled. Already there is some evidence that fish cell line can be used to monitor surface water genotoxicity. In fact, Reifferscheid and Grummt had used several genotoxic assays including Ames and Umu C bacterial tests as well as the DNA unwinding assay, alkaline elution, comet assay and the unscheduled DNA synthesis assays with fish, clams and algae to analyse surface waters in Germany [102]. The authors concluded that in addition to the bacterial test systems the use of the comet assay with fish cell lines (rainbow trout RTG-2 or RTL-W1) would be a good inclusion for a genotoxicity test battery system for surface water screening [102]. Since surface water contains low concentrations of environmental pollutants this is therefore a promising approach that indicates it may be possible to use fish cell lines to monitor pharmaceuticals (and other chemicals) and their TPs at lower concentrations. The study by Sánchez et al. (see Section 3.1)

showed the potential to detect the genotoxicity of some anti-cancer drugs with fish cells (*in vitro*) at relatively low concentration [61]. Generally, care must be taken in selecting the fish cell lines for genotoxicity screening since the amount of P-450 enzymatic activity varies among the cell type and can affect the detection of metabolic activated genotoxins [65]. Johnson et al. alluded that genetic damage by cytotoxic drugs to fish are probably of the same type as those described for mammalian species [10].

For higher tier studying of TP mixtures, *in vivo and/or in situ* studies using aquatic species may also be possible to assess the expressed genotoxicity of the mixture. Recently, Kushwaha et al. have successfully used the comet and MN assay *in situ* with two fish species to test for genotoxicity of polluted waters [103]. In this review, evidence of *in situ* MN activity of anti-cancer drugs was presented (Section 3.1) and therefore this assay provides an opportunity for biomonitoring of anti-cancer drugs and their TPs. Fish cells have low mitotic activity, large number of small chromosomes and a low amount of DNA per cell [59,60,104]. Therefore, the micronucleus assay with various fish species is a good alternative for biomonitoring to detect the clastogenic activity of various toxins. There are limited data available on using aquatic organisms for eco-genotoxicity testing and this is a fairly new area of research. On the one hand, there is a need for a standardized test protocol and difficulties in reproducibility due of factors such as age, sex, diet and others have to be overcome. On the other hand, *in situ* and *in vivo* studies provide the opportunity for testing low dosage of mixtures of pharmaceuticals and their TPs that would not be possible otherwise. Johnson et al. postulated that since many cytotoxic drugs are hydrophilic, their bioconcentration in non-target organisms is not expected [10]. However, the authors believe that this needs to be confirmed before it can be concluded concretely that there is no genotoxic risk from low levels of cytotoxic drugs in the environment. The use of toxicokinetic modelling to predict bioavailability of PCs and relevant TPs to certain tissue sites in aquatic organisms may be necessary, especially in instances where there is positive indication in *in vitro test* but the endpoint fails to be expressed in the *in vivo* test. Furthermore, independently from possible bioaccumulation, persistent PCs and TPs cause possibly a livelong exposure of environmental organisms and include the possibility of the transfer of these chemicals and their TPs into the drinking water cycle.

5. Conclusions

Pharmaceuticals in general and anti-cancer drugs as a subgroup of these are present as so called micro-pollutants in low concentrations in the aquatic environment. It is also possible that not only the PC but also the metabolites and TPs are present in the environment. Little is known on their presence and fate in water and still less in soil. They may persist there because of a lack of elimination and/or continuous introduction. There is a lack of studies on the occurrence of the metabolites and TPs. Given the inherent nature of anti-cancer drugs, that is, to kill or inhibit the growth of cells, it is important to establish the risk from the presence of these compounds, their metabolites and their TPs. The risk of anti-cancer drugs to the environment and their risk to humans because of their presence in the environment is not very clear mainly because of the lack of toxicity testing in terms of approach and in terms of tests to be used.

Over the years, several strategies have been developed for risk assessment of pharmaceuticals and their related TPs. Although these strategies are mostly directed towards lab-scale simulation tests, they represent the only available methods at this time to characterize the risk of these compounds. Only when the relevant TPs are identified and methods developed to characterize them can

there be an assessment of their actual environmental occurrence, fate and effects. Hence, the direction of genotoxic risk assessment of anti-cancer drugs and their TPs could be either to identify and profile the relevant TPs or to understand the toxicity of their mixtures. As of now the coupling of appropriate analytical methods, genotoxicity bioassays, (bio) degradability and QSAR studies provides the best, however, also time consuming approach for understanding PCs and their mixture of TPs. It is important to note that such an approach needs expertise from different fields in order to get reliable and meaningful results.

Regardless of the approach taken, it is always necessary to careful select bioassays that can monitor the development of genotoxicity in these simulation tests and that are appropriate for the compounds selected. Each anti-cancer drug is structurally different and can behave differently and would therefore require the selection of bioassay to be based on its MOA. This might be impossible when testing TPs. As of now, the Ames test is the most frequently bioassay for these studies as the interest seems to be in monitoring the inherent genotoxic nature of these compounds. However, anti-cancer drugs often require relatively higher concentrations to be detected in this test and therefore other bioassays should be considered. Unfortunately, from an environmental perspective, very few studies has been done in assessing genotoxicity with aquatic organisms both *in vitro* and *in vivo*. This represents an area that needs to be focused on so that appropriate bioassays can be developed to monitor the effects of these drugs in the environment.

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.mrrev.2014.02.001.

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Supplementary Data

For

Environmental risk assessment of anti-cancer drugs and their transformation products: a focus on their genotoxicity characterization –

state of knowledge and short comings

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Table S1: Genotoxicity risk assessment of anti-cancer drugs and their TPs after lab-scale treatment

Anticancer drug	Treat-ment Concen-tration	Solvents	Treatment procedure					Mutagenicity Testing			Comment	Reference
			Method	Time	PC degrada-tion[a]	TPs Identifica-tion	Post treat-ment	Ames Muta-genicity test[c]	Results			
					Alkylating Agents							
Cyclophos-phamide	4 mg/ml	1. Dextrose 5% 2. 0.9% sodium chloride (NaCl)	Hydrogen peroxide (H$_2$O$_2$) oxidation	1 h	Complete	No	Solid sodium bisulfite to remove oxidant and neutralization with 12 mol/l hydrochloric acid (HCl)	TA 97a, TA 98, TA 100 and TA 102 (+/-S9)	Non-mutagenic residues in 4 strains (+/-S9)	1. Treatment controls for Ames test were done using solvent with negative results for mutagenicity in all strains (-/+S9)	Hansel et al., 1997
	4 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Sodium hypochlorite oxidation	1 h	Complete	No			Non-mutagenic residues in 4 strains (+/-S9)		
	4 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Fenton's Reagent oxidation	1 h	Complete	No			Mutagenic residues	2. H$_2$O$_2$ treated samples were expected to show mutagenicity but results were negative due to the low levels degraded	
	—	—	Potassium permanganate oxidation (1.70 mg KMnO$_4$/ mg drug)	24 h	75.9 %	No	Sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA 100 (+S9)	16% mutagenic increase over controls	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
	—	—	Alkaline potassium permanganate oxidation and Nucleophilic substitution (1.70 mg KMnO$_4$/ mg drug)	30 min	Complete	No	Neutralized with HCl		Non-mutagenic residues		

	Concentration	Solvent	Treatment	Time	Degradation	Mutagenic (by chemical test)	Neutralization	Ames strains	Residues	Notes	Reference
	5 mg/ml	water	Alkaline hydrolysis (0.5 M KOH/CH₃OH)	24 h	No degrada-tion	No	Neutralized with HCl		—		
	10 mg/ml	water	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol; with GC	—		Non-mutagenic residues	1. Tested at mutagenic concentrations of PC, detected by TA 100 (+S9), TA1535 (+/-S9), TA 1530 (+/-S9)	Lunn et al., 1989
	10 mg/ml	saline	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	No	—	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Non-mutagenic residues		
	5 mg/ml	1 M HCl	Acid reflux with HCl for 1 h then Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	No	—		Non-mutagenic residues	2. Degradation with KOH only led to mutagenic residues	
	10 mg/ml	water	Ozonation 73.8 mg O₃/min	3 min	—	No. Suggested to be metabolites of PC due to mutagenic activity	—	TA 1535 (+/-S9)	Mutagenic residues	1. Tested at non-mutagenic concentrations of PC, detected by TA1535 (+/-S9) 2. Control samples of mutagen were treated in a similar way with the only exception been the use of oxygen instead of ozone.	Burleson and Chambers, 1982
Ifosfamide	27 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Hydrogen peroxide oxidation	1-3 h	Complete	No	Solid sodium bisulfite to remove oxidant and neutralization with 12 mol/l HCl	TA 97a, TA 98, TA 100 and TA 102 (+/-S9)	Non-mutagenic residues	1. Treatment controls for Ames test were done using solvent with negative results for mutagenicity in all strains (-/+S9) 2. H₂O₂ treated samples were expected to show mutagenicity but results were negative due to the low levels degraded	Hansel et al., 1997
	27 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Sodium hypochlorite oxidation	1-3 h	Complete	No			Mutagenic residues formed with dextrose 5% solvent (-S9)		

27 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Fenton's Reagent oxidation	1-3 h	Complete	No			Non-mutagenic residues	3. Dextrose 5% consumes some of the oxidizing potential of the H_2O_2
—	—	Potassium permanganate oxidation (1.82 mg $KMnO_4$/ mg drug)	24 h	Complete	No	sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide		33% mutagenicity increase over controls	
—	—	Alkaline potassium permanganate oxidation and Nucleophilic substitution(1.82 mg $KMnO_4$/ mg drug)	30 min	Complete	No	Neutralized with HCl	TA 100 (+S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
5 mg/ml	water	Alkaline hydrolysis (0.5 M KOH/CH_3OH)	24 h	No degrada-tion	No	Neutralized with HCl		2-3 fold increase mutagenic residues over controls	
10 mg/ml	water	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol; with GC	—	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Non-mutagenic residues	1. Tested at mutagenic concentrations of PC, detected by TA1535 (+S9), TA 1530 (+S9) 2. IF dissolved in methanol were not completely degraded and produced mutagenic residues after treatment	Lunn et al., 1989
Melphalan 2 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Hydrogen peroxide oxidation	1 h	Complete	No	Solid sodium bisulfite to remove oxidant and neutralization with 12 mol/l HCl	TA 97a, TA 98, TA 100 and TA 102 (+/-S9) with samples dissolved	Non-mutagenic residues	1. Treatment controls for Ames test were done using solvent and each treatment method with negative results for mutagenicity in all strains (-/+S9)	Hansel et al., 1997
2 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Sodium hypochlorite oxidation	1 h	Complete	No			Non-mutagenic residues	

Drug	Concentration	Solvent	Method	Time	Degradation	Recovery	Further treatment		Test strains	Mutagenicity	Notes	Reference
	2 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Fenton's Reagent oxidation	1 h	Complete	No		in Dextrose 5%		Non-mutagenic residues		
	1 mg/ml	0.1 mol/liter sulphuric acid/water	Oxidation by potassium permanganate:	1 h	Complete[b]	No	Decolourization and neutralization with sodium bisulfite		TA 98, TA 100 and TA 1535 (+/-S9)	Non-mutagenic residues	—	Barek et al., 1987
Chlorambucil	10 mg/ml	water	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol; with GC				Non-mutagenic residues	1. Tested at mutagenic concentrations of PC, detected by TA 100, TA1535, TA 1530 (+/-S9)	Lunn et al., 1989
	10 mg/ml	none	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	—	—		TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Non-mutagenic residues	2.Incomplete degradation when dissolved in methanol and produced mutagenic residues after treatment	
	10 mg/ml	water	Sodium bicarbonate solution	overnight	Complete	—				Non-mutagenic residues		
Carmustine	3.3 mg/ml, 1 mg/ml, 0.1 mg/ml	Ethanol/water	Method: solution were tested in presence of a constant airflow with and without the addition of 30% hydrogen peroxide Lamp description: 200 W medium pressure mercury lamp (λ 200-1400 nm) attached to a cooling water system	1 h	Complete at all concentration	GC-MS could not identify products in solutions with Complete destruction . Suggested products are carbon dioxide, water and other inorganic compounds.	Quenching with sodium metabisulfite		TA 97a, TA 100 and TA 102 (+/-S9)	At 3.3 mg/ml and 1 mg/ml: mutagenic residues At 0.1 mg/ml: Non-mutagenic residues	—	Lunn et al., 1994

	Concentration	Solvent	Method	Time	Completion	Product identification	Neutralization/Quenching	Ames strains	Residue result	Notes	Reference
	33.3 mg/ml	ethanol	Acidic Potassium permanganate oxidation	24 h	Complete	No	Neutralized with potassium hydroxide and excess potassium permanganate then quenched with 1 % sodium bisulfite	TA 100 (-S9)	Mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
	33.3 mg/ml	ethanol	Acid hydrolysis (30.4 ml 1 N HCl/g drug)	24 h	68%	No	Neutralized with sodium hydroxide		73% mutagenic increase over controls		
	—	—	Hydrogen bromide in Glacial acetic acid	3 h	Complete	^{13}C NMR spectroscopy of dichloromethane extract identified as bis(2-chloroethyl)urea, 1-(2-chloroethyl)imidazolidinone and N-(2-chloroethyl)-4,5-dihydro-2-oxazolamine	Dissolving extracted mixture in distilled water, then neutralizing with sodium bicarbonate	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	extracted mixture were mutagenic with strain TA 100, TA 1530, TA 1535	1. Mutagenicity test with isolated TPs revealed mutagenicity with strain TA 100, TA 1530 and TA 1535	Lunn et al., 1989
	10 mg/ml	methanol	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol and ethylamine; with GC	—		Mutagenic residues with strain TA 1535	2. Tested at mutagenic concentrations of PC, detected by TA 98 , TA 100, TA1535, TA 1530 (+/-S9)	
	3.33 mg/ml	ethanol/ water	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	No	—		Non-mutagenic residues		
Lomustine	10 mg/ml	methanol	Method: solution were tested in presence of a constant airflow with and without the addition of 30% hydrogen peroxide	1 h	Complete	GC-MS could not identify products . Suggested products are carbon dioxide, water and other	Quenching with sodium metabisulfite	TA 97a, TA 100 and TA 102 (+/-S9)	Non-mutagenic reaction mixture after treatment with and	—	Lunn et al., 1994

		Lamp description: 200 W medium pressure mercury lamp (λ 200-1400 nm) attached to a cooling water system			inorganic compounds.			without H2O2		
28.7 mg/ml	dimethylfo rmamide	Acidic Potassium permanganate oxidation	24 h	Complete	No	Neutralized with potassium hydroxide and excess potassium permanganate then quenched with 1 % sodium bisulfite	TA 100 (-S9)	Mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
28.7 mg/ml	dimethylfo rmamide	Acid hydrolysis (25.7 ml 1 N HCl/g drug)	24 h	43%	No	Neutralized with sodium hydroxide		62% mutagenic increase over controls		
—	—	Hydrogen bromide in Glacial acetic acid	3 h	Complete	^{13}C NMR spectroscopy of dichloromethane extract identified as 1-cyclohexyl-3-(2-chloroethyl)urea	Dissolving extracted mixture in distilled water, then neutralizing with sodium bicarbonate	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Extracted mixture was mutagenic with strain TA 1530 (+S9)	1. Mutagenicity test with isolated TPs from extract revealed mutagenicity with strain TA 1530 and TA 1535 (-/+S9)	Lunn et al., 1989
10 mg/ml	methanol	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol, cyclohexylamine, 2-chlorodiazoethan e, diazoethane and ethylamine; with GC	—		Non-mutagenic residues	2. Tested at mutagenic concentrations of PC, detected by TA 98 , TA 100, TA1535, TA 1530 (+/-S9)	
10 mg/ml	methanol	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	No	—		Non-mutagenic residues		

Compound	Concentration	Solvent	Degradation method	Time	Degradation	Products identification	Quenching/neutralizing	Strains	Mutagenicity	Additional test	Reference
Semustin	—	—	Hydrogen bromide in Glacial acetic acid	3 h	Complete	^{13}C NMR spectroscopy of dichloromethane extract identified as 1-(4-methylcyclohexyl)-3-(2-chloroethyl)urea	dissolving extracted mixture in distilled water, then neutralizing with sodium bicarbonate	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Non-mutagenic extract	1. Mutagenicity test with isolated TPs from extract revealed mutagenicity with strain TA 1530 and TA 1535	Lunn et al., 1989
	10 mg/ml	methanol	Nickel-aluminium alloy in Potassium hydroxide (KOH) oxidation	overnight	Complete	Ethanol, 4-methylcyclohexy lamine and ethylamine; with GC	—		Non-mutagenic residues	2. Tested at mutagenic concentration of PC, detected by TA1535, TA 1530 (+/-S9)	
Streptozocin	14 mg/ml	1. Dextrose 5% 2. 0.9% NaCl	Method: solution were tested in presence of a constant airflow with and without the addition of 30% hydrogen peroxide Lamp description: 200 W medium pressure mercury lamp (λ 200-1400 nm) attached to a cooling water system	2h	Complete	GS-MS could not identify products . Suggested products are carbon dioxide, water and other inorganic compounds.	Quenching with sodium metabisulfite	TA 97a, TA 100 and TA 102 (+/-S9)	Non-mutagenic reaction mixture with booth solvents after treatment with and without H2O2	—	Lunn et al., 1994
	100 mg/ml	water	Sodium bicarbonate solution	overnight	Complete	Methanol, 4-methylamine and diazomethane; with GC	—	TA 1535, TA 1530, TA 100 and TA98 (+/-S9)	Non-mutagenic residues	Tested at mutagenic concentration of PC, detected by TA 100, TA1535, TA 1530 (+/-S9)	Lunn et al., 1989
	—	—	Hydrogen bromide in Glacial acetic acid	3 h	Complete	No			Non-mutagenic residues		
Thiotepa	0.016 mg/ml	5% D-glucose	Hydrogen peroxide (H$_2$O$_2$ 30%) oxidation	1h, 4h, 16h	Complete	No	Quenching with solid sodium bisulphite and neutralise with concentrated hydrochloric acid	TA 97a, TA98, TA 100 and TA 102 (+/-S9)	Non-mutagenic residues	—	Barek et al., 1998
			Sodium hypochlorite (NaOCl 5%) oxidation	1h, 2h, 16 h	Complete	No			Non-mutagenic residues		
			Fenton's reagent (30%) oxidation	30 min	not detected	No			Non-mutagenic residues		

Drug	Concentration	Buffer	Degradation Method	Time	Degradation	Products Identified	Quenching	Strains	Mutagenicity	Other Results	Reference
Dacarbazine	10 mg/ml, 0.1 mg/ml	Citric acid/manni tol buffer	Method: solution were tested in presence of a constant airflow with and without the addition of 30% hydrogen peroxide	1 h	At 10 mg/ml: incomplete after 2 h. Solution had to be filtered after 1 h of photolysis to remove coloured substance and then undergo photolysis for another 1 to be completely degraded	GS-MS could not identify products in solutions with Complete destruction. Suggested products are carbon dioxide, water and other inorganic compounds.	Quenching with sodium metabisulfite	TA 97a, TA 100 and TA 102 (+/-S9)	At 10 mg/ml: mutagenic reaction mixtures without the addition of H_2O_2. Non-mutagenic in presence of H_2O_2	—	Lunn et al., 1994
			Lamp description: 200 W medium pressure mercury lamp (λ 200-1400 nm) attached to a cooling water system		At 0.1 mg/ml: complete degradation of PC after 1 h without filtration				At 0.1 mg/ml: non-mutagenic reaction mixtures		

Antimetabolites

Drug			Degradation Method	Time	Degradation	Products Identified	Quenching	Strains	Mutagenicity	Other Results	Reference
Methotrexate	—	—	Potassium permanganate oxidation (1.04 mg $KMnO_4$/ mg drug)	24 h	Complete	No	Sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA 100, TA 1535 (+/-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9) 2. Methotrexate is not mutagenic in the Ames test for the tested strains	Benvenuto et al., 1993
			Bleach (3.9 ml sodium hypochlorite/ mg drug) oxidation		Complete	No	Quenching with 1% sodium bisulfite		Non-mutagenic residues		

			Treatment	Time	Degradation		Quenching/Neutralization	Strain	Mutagenicity	Comments	Reference
Etoposide	—	—	Potassium permanganate oxidation (2.42 mg KMnO$_4$/ mg drug)	24 h	Complete	No	sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA UTH8413 (-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
			Bleach (20.1 ml sodium hypochlorite/ mg drug) oxidation		Complete	No	Quenching with 1% sodium bisulfite		Non-mutagenic residues		
			Acid hydrolysis (25.5 ml 1 N HCl/g drug)	24 h	30%	No	Neutralized with sodium hydroxide		38% mutagenic increase over controls	2. Mutagenicity after acid hydrolysis maybe due to structural vulnerability	
			Nucleophilic substitution with Sodium thiosulfate (13.4 ml Na$_3$O$_3$S$_2$ /g drug)	24 h	18%	No	—		109% mutagenicity increase over controls		
Teniposide	—	—	Potassium permanganate oxidation (2.65 mg KMnO4/ mg IF)	24 h	Complete	No	sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA UTH8413 (-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
			Bleach (28.0 ml sodium hypochlorite/ mg drug) oxidation		Complete	No	Quenching with 1% sodium bisulfite		Non-mutagenic residues		
			Acid hydrolysis (22.8 ml 1 N HCl/g drug)	24 h	Complete	No	Neutralized with sodium hydroxide		3 % mutagenic increase over controls	—	
			Nucleophilic substitution with Sodium thiosulfate (48.2 ml Na3O3S2 /g drug)	24 h	50%	No	—		155 % mutagenic increase over controls		

Cytotoxic antibiotics and related substances											
Doxorubicin	0.4 mg/ml	Dextrose 5%	Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997
	—	—	Hydrogen peroxide oxidation (30% H$_2$O$_2$)	24 h, 48 h	60% of PC degraded after 24 h; 68% of PC degraded after 48 h	No	Quenching with sodium bisulfite; adjust pH to 6-7		—	2. Previous experiments showed mutagenicity for TA98 after treatment with bleach but in this case the concentration tested may have been too low for detection.	
	—	—	Fenton's Reagent oxidation	1 h	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues		
Daunorubicin	5 mg/ml		Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997
	0.02 mg/ml	0.9% NaCl	Hydrogen peroxide oxidation (30% H$_2$O$_2$)	24 h	60%	No	Quenching with sodium bisulfite; adjust pH to 6-7		—	2. Previous experiments showed mutagenicity for TA98 after treatment with bleach but in this case the concentration tested may have been too low for detection.	
	5 mg/ml		Fenton's Reagent oxidation	30 min	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues		
Idarubicin	0.5mg/ml	Dextrose 5%	Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997

—	—	Hydrogen peroxide oxidation (30% H₂O₂)	24 h	60% of PC degraded after 24 h	5 TPs, HPLC	Quenching with sodium bisulfite; adjust pH to 6-7		—	
—	—	Fenton's Reagent oxidation	1 h	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues	
Epirubicin	0.2-2 mg/ml	0.9% NaCl	Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues	
—	—	Hydrogen peroxide oxidation (30% H₂O₂)	1-24 h	Complete degradation of PC after 1 min with formation of a red solution after 1 h. After 24 h more products were detected	2 TPs after 1 h, HPLC	Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residual solution but red solids formed in media were not tested.	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997
—	—	Fenton's Reagent oxidation	1 h	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues	
Pirarubicin	1 mg/ml	Dextrose 5%	Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997
—	—	Hydrogen peroxide oxidation (30% H2O2)	24 h	<97 %	No	Quenching with sodium bisulfite; adjust pH to 6-7		—	

Drug	Conc.	Vehicle	Method	Time	Degradation	Products identified	Neutralization/Treatment	Ames strains	Result	Notes	Reference
—	—	—	Fenton's Reagent oxidation	1 h	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues		
Aclarubicin	0.5 mg/ml	Dextrose 5%	Bleach (5.25% sodium hypochlorite) oxidation	1 h	Complete	No	Bubbling with nitrogen then Quenching with sodium bisulfite; adjust pH to 6-7	TA 97a, TA 98, TA 100 and TA 102 (+/- S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using solvents with negative results for mutagenicity	Castegnaro et al., 1997
	—	—	Hydrogen peroxide oxidation (30% H_2O_2)	24 h	<97%	No	Quenching with sodium bisulfite; adjust pH to 6-7		—		
	—	—	Fenton's Reagent oxidation	1 h	Complete	No	Quenching with sodium bisulfite; adjust pH to 6-7		Non-mutagenic residues		
Bleomycin	1 mg/ml	water	1. UV Photolysis, in presence of a constant airflow with and without the addition of 30% hydrogen peroxide 2. Lamp description: 200 W medium pressure mercury lamp (λ 200-1400 nm) attached to a cooling water system	1, 2 or 4 h	Complete degradation after 1 h in presence of H2O2	GC-MS could not identify products. Suggested products are carbon dioxide, water and other inorganic compounds.	Solutions were treated with sodium metabisulfite	TA 97a, TA 100 and TA 102 (+/- S9)	Non-mutagenic reaction mixture after treatment presence of H_2O_2	—	Lunn et al., 1994
	—	—	Potassium permanganate oxidation (0.34 mg $KMnO_4$/ mg drug)	24 h	Complete	No	Sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA 102 (-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background	Benvenuto et al., 1993

Drug	Conc.	Matrix	Degradation method	Time	Degradation		Quenching/Neutralisation	Ames strain	Mutagenicity	Comments	Reference
—	—		Bleach (7 ml sodium hypochlorite/ mg drug) oxidation		Complete	No	Quenching with 1% sodium bisulfite		Non-mutagenic residues	mutagenicity in all strains (-/+S9)	
—	—		Acid hydrolysis (7.1 ml 1 N HCl/g drug)	24 h	30%	No	Neutralized with sodium hydroxide		113% mutagenicity increase over controls	2. Bleomycin may have been activated by acid	
Mitomycin C	—	—	Potassium permanganate oxidation (0.91 mg KMnO$_4$/ mg drug)	24 h	Complete	No	Sodium bisulfite to remove oxidant and centrifuge to remove manganese oxide	TA 102 (-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
			Bleach (3.0 ml sodium hypochlorite/ mg drug) oxidation		Complete	No	Quenching with 1% sodium bisulfite		Non-mutagenic residues		
			Nucleophilic substitution with Sodium thiosulfate (145.3 ml Na3O3S2 /g drug)	24 h	63%	No	—		26 % mutagenicity increase over controls		

<div align="center">**Other antineoplastic agents**</div>

Drug	Conc.	Matrix	Degradation method	Time	Degradation		Quenching/Neutralisation	Ames strain	Mutagenicity	Comments	Reference
Cisplatin	1 mg/ml	saline	Sodium Diethyldithiocarbonate (DDTC) complexation	24 h	HPLC confirms complexation but can not determine degree of PC degradation	No	—	TA UTH8414 (-S9)	Non-mutagenic residues	1. Treatment control for Ames test were done using distilled water with > 2-fold increase of mutagenicity over the background mutagenicity in all strains (-/+S9)	Benvenuto et al., 1993
Amsacrine	0.15 mg/ml	5% D-glucose	Hydrogen peroxide (H$_2$O$_2$ 30%) oxidation	1h, 4h, 16h	15% of PC degraded after 4 h, 16% of PC degraded after 16 h	No	Quenching with solid sodium bisulphite and neutralisation with concentrated hydrochloric acid	TA 97a, TA98, TA 100 and TA 102 (+/-S9)	—	—	Barek et al., 1998
	—	—	Sodium hypochlorite	1h, 2h, 16 h	Complete	No			Non-mutagenic residues		

			(NaOCl 5%) oxidation								
	—	—	Fenton's reagent (30%) oxidation	30 min	Complete	No			Non-mutagenic residues		
Asparaginase	200 I.U. Asparaginase, 1.6 mg mannitol	5% D-glucose	Hydrogen peroxide (H₂O₂ 30%) oxidation	1h, 4h, 16h	Complete	No	Quenching with solid sodium bisulphite and neutralisation with concentrated hydrochloric acid	TA 97a, TA98, TA 100 and TA 102 (+/-S9)	Non-mutagenic residues	—	Barek et al., 1998
	—	—	Sodium hypochlorite (NaOCl 5%) oxidation	1h, 2h, 16 h	Complete	No			Non-mutagenic residues		
	—	—	Fenton's reagent (30%) oxidation	30 min	Complete	No			Non-mutagenic residues		

a= Complete degradation means ≥ 98% PC elimination within the detection limits of the HPLC protocols used.

b= Complete degradation determined using HPLC coupled with UV spectrophotometry; UV Spectrophotometry and Differential pulse voltammetry

c= Ames test with strain Salmonella typhimurium as standard/modified plate incorporation or pre-incubation

HPLC = High performance liquid chromatography

GC-MS = Gas chromatography coupled with mass spectrometry

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Appendix 2

Identification of phototransformation products of thalidomide and mixture toxicity assessment: An experimental and quantitative structural activity relationships (QSAR) approach

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ABSTRACT

The fate of thalidomide (TD) was investigated after irradiation with a medium-pressure Hg-lamp. The primary elimination of TD was monitored and structures of photo-transformation products (PTPs) were assessed by LC−UV−FL−MS/MS. Environmentally relevant properties of TD and its PTPs as well as hydrolysis products (HTPs) were predicted using in silico QSAR models. Mutagenicity of TD and its PTPs was investigated in the Ames microplate format (MPF) aqua assay (Xenometrix, AG). Furthermore, a modified lumines-cent bacteria test (kinetic luminescent bacteria test (kinetic LBT)), using the luminescent bacteria species Vibrio fischeri, was applied for the initial screening of environmental toxicity. Additionally, toxicity of phthalimide, one of the identified PTPs, was investigated separately in the kinetic LBT.

The UV irradiation eliminated TD itself without complete mineralization and led to the formation of several PTPs. TD and its PTPs did not exhibit mutagenic response in the Salmonella typhimurium strains TA 98, and TA 100 with and without metabolic activation. In contrast, QSAR analysis of PTPs and HTPs provided evidence for mutagenicity, genotoxicity and carcinogenicity using additional endpoints in silico software. QSAR analysis of different ecotoxicological endpoints, such as acute toxicity towards V. fischeri, provided positive alerts for several identified PTPs and HTPs. This was partially confirmed by the results of the kinetic LBT, in which a steady increase of acute and chronic toxicity during the UV-treatment procedure was observed for the photolytic mixtures at the highest tested con-centration. Moreover, the number of PTPs within the reaction mixture that might be responsible for the toxification of TD during UV-treatment was successfully narrowed down by correlating the formation kinetics of PTPs with QSAR predictions and experi-mental toxicity data. Beyond that, further analysis of the commercially available PTP

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phthalimide indicated that transformation of TD into phthalimide was not the cause for the toxification of TD during UV-treatment.

These results provide a path for toxicological assessment of complex chemical mixtures and in detail show the toxic potential of TD and its PTPs as well as its HTPs. This deserves further attention as UV irradiation might not always be a green technology, because it might pose a toxicological risk for the environment in general and specifically for water compartments.

1.   Introduction

When pharmaceuticals are released into the environment, they can be transformed through many abiotic and biotic processes that can contribute to their degradation and elimination or lead to the formation of transformation products (TPs) (Fatta-Kassinos et al., 2011; Khaleel et al., 2013). Therefore, the removal of pharmaceuticals and their TPs provides a new challenge to treatment systems for drinking water, wastewater and water reuse. Ultraviolet (UV) light treatment is an established method for water disinfection and sterilization (Canonica et al., 2008), It is also in discussion as a technology for wastewater purification (Liberti and Notarnicola, 1999; Meneses et al., 2010). However, photodegradation can lead to phototransformation products (PTPs) which can have more toxic effects than the parent compound investigated for different toxicological endpoints (Vasquez et al., 2013; Wang and Lin, 2012). Therefore, it is important to gather more information about environmental properties of pharmaceuticals and their TPs and to consider this information in environmental risk assessment.

In the early 1960s, Thalidomide (TD) was withdrawn from the market due to its teratogenic effects when given in early pregnancy. In 1998, its use is being revived since the FDA approved TD for the treatment of erythema nodusum leprosum associated with leprosy (Sweetman, 2009). Recently, TD is expected to be a promising drug in the treatment of a number of inflammatory and cancers diseases (Bosch et al., 2008; Sweetman, 2009). Consequently, a potential increased influx of TD into the aquatic environment has to be expected. According to our best knowledge, no study until now has detected TD in the aquatic environment. For sure as a human pharmaceutical the toxic nature of TD has been well investigated, but studies of the toxic effects of TPs are limited in general and even more for TD. In 1994, McBride proposed that TD also may be a human germ cell mutagen based on clinical observations (McBride and Read, 1994). However, Ashby et al. had provided evidence that TD neither exhibited mutagenic responses in different *Salmonella typhimurium* strains (with and without metabolic activation), nor induced chromosome aberration or micronucleus formation *in vivo* and *in vitro* (Ashby et al., 1997). The non-genotoxic properties of the compound were confirmed further by studies from Teo et al. (2000). According to the best knowledge of the authors, there is no information available in published literature regarding the toxicity of TD towards environmental bacteria. The same applies to most of the previously known hydrolytic products (HTPs) and PTPs.

TD is sensitive to hydrolytic decomposition leading to formation of twelve HTPs (Schumacher et al., 1965) (Supplementary material Table S1). The exact metabolic route and fate of thalidomide is unknown, although it appears to undergo non-enzymatic hydrolysis in plasma (Sweetman, 2009). Only the three HTPs which contain the intact phthalimide moiety showed teratogenic activity (Meise et al., 1973).

TD undergoes photolysis using xenon lamp and UV lamp without complete mineralization. New PTPs are formed during photolytic process, including phthalimide (Mahmoud et al., 2013). Phthalimide is classified as a high production volume chemical and it is a degradation intermediate formed from many products. Although phthalimide is readily biodegradable, it was detected in concentrations less than 5 μg/L in the effluent of the wastewater treatment plant of a production site in Japan (OECD, 2005). Phthalimide undergoes hydrolysis in water to ammonia and phthalic acid which is readily biodegradable and also one of the HTPs of TD (Lu et al., 2002).

Generally, experimental toxicity testing of TPs is difficult as many of them are not available commercially. Computer models based on quantitative structure activity relationship (QSAR) are important tools to solve and overcome this problem (European Commission, 2003). Once structure elucidation of any TPs is performed, these structures can be investigated in QSAR programs in order to predict the toxic potential of TPs at different toxicological endpoints and other environmental parameters (Escher et al., 2009).

The aim of this work was to characterize TD and it PTPs after photolysis and monitor their toxicity experimentally in combination with *in silico* QSAR models. The mutagenicity was investigated using the Ames Microplate format (MPF) assay. Moreover, a modified luminescent bacteria test with *Vibrio fischeri* (kinetic luminescent bacteria test, kinetic LBT) was used for an initial screening of microbial toxicity of TD and its PTPs (Menz et al., 2013). Furthermore, phthalimide, one of the identified PTPs of TD, was assessed separately in the kinetic LBT due to the contradiction between different *in silico* software regarding the predicted phthalimide toxicity against *V. Fischeri*.

2.   Experimental

2.1.   Chemicals

All the chemicals used in this study were of analytical grade. Acetonitrile and Methanol (HiPerSolv CHROMANORM, LC-MS grade, BDH Prolabo), and formic acid were purchased from

VWR International GmbH (Darmstadt, Germany). TD (CAS number 50-35-1, 98.7% purity) was obtained from chemical point (Deisenhofen, Germany). Phthalimide (CAS Number 85-41-6, PESTANAL® analytical standard 99.9% purity), 3,5-Dichlorophenol (CAS Number 591-35-5, 97% purity) and Chloramphenicol (CAS Number 56-75-7, 98% purity) were obtained from Sigma—Aldrich GmbH (Steinheim, Germany).

2.2. Photodegradation

Photodegradation experiments were performed with a TQ 150 W medium-pressure mercury lamp (UV Consulting Peschl, Mainz). The irradiation experiments of 10 mg/L of TD were conducted in four different rector sizes: 800 ml (PR1), 110 ml (PR2), 1.4 ml Hellma® suprasil quartz cuvette (type 104 B-QS) (PR3) and 3 ml Brand® UV-cuvette Macro (PR4). The irradiation experiments of 47 mg/L of TD were conducted in PR1 and PR2. The specified test solution volumes 800 ml, 110 ml, 1.4 ml, and 3 ml were transferred into the PR1, PR2, PR3 and PR4, respectively.

Ultrapure water was used to prepare all test solutions. In PR1 and PR2, the photodegradation mixture was stirred with a magnetic stirrer during the photoreaction and the temperature was maintained by a circulating cooler (WKL230, LAUDA, Berlin) between 18 and 20 °C. TD samples were taken at different reaction times from the photoreactor at defined times (2, 4, 8, 16, 32, 64 and 128 min) for the analysis of the TD concentration by LC-UV-FL-ion- trap-MS/MS. Dissolved organic carbon (DOC) was monitored during irradiation experiments of 47 mg/L of TD according to European standard procedure DIN EN 1484 with a total organic carbon analyzer (TOC-Vcpn, Shimadzu GmbH, Duisburg, Germany). Toxicity tests performed for photolysis samples of 47 mg/L of TD withdrawn from PR1.

A saturated aqueous solution of TD was freshly prepared before the photodegradation experiments by stirring 50 mg of TD in 1L of water following by sonication and filtration through 0.2 μm membrane filter. The final concentration of this solution (determined by LC-UV and DOC) was approximately 47—47.4 mg/L (pH was 5.6).

2.3. Monitoring of primary elimination and structure elucidation of PTPs by LC—UV—VIS/FL and LC—ion-trap MS/MS

In order to monitor the changes of TD in the samples, HPLC—UV—VIS/FL and LC—ESI-MS/MS (ion trap) was used to measure the primary elimination of TD. A stock solution of TD (100 mg/L) was prepared in methanol. Standard solutions were prepared by further dilution with ultrapure water to reach the concentration range of linearity. Triplicate TD injections were made for each concentration and chromatographed under the specified conditions described below. The peak area values were plotted against corresponding concentrations.

LC—UV quantification was performed on Prominence HPLC apparatus (Shimadzu, Duisburg, Germany). Further, ion-trap LC—MS/MS quantification, detection and identification of the TD and PTPs was performed on Agilent Technologies 1100 HPLC series (Agilent Technologies, Böblingen, Germany)

tandem mass spectrometer Bruker Daltonic Esquire 6000 plus ion-trap mass spectrometer equipped with atmospheric pressure electrospray ionization interface (Bruker Daltonic GmbH, Bremen, Germany) (Supplementary material (Text S1)). Chromatographic separation was performed on an RP-18 column (CC 70/3 NUCLEODUR 100-3 C18 ec, Macherey and Nagel, Düren, Germany) protected by a CC 8/4 HYPERSIL 100-3 C18 ec, guard column. Gradient system 0.1% formic acid in water (solution A) and 100% acetonitrile (solution B) were used by applying the following linear gradient: 0 min 5% B, 5 min 5% B, 20 min 40% B, 23 min 40% B, 26 min 5% B, 30 min 5% B. The flow rate was set at 0.7 mL min^{-1} and the oven temperature was set to 30 °C. Total run time was 30 min.

The mass spectrometer was operated in positive polarity. A more detailed description of the mass spectrometer parameters can be found elsewhere (Mahmoud et al., 2013).

2.4. In silico prediction of toxicity

TD, its PTPs and its HTPs were assessed by a set of in silico predictions for toxicity. A set of different programs for predicting toxicity was applied in order to take into account that the available programs might have individual strengths because of different algorithms and training sets. The set of available programs used were the Case Ultra V.1.4.5.1 (MultiCASE Inc.) (Saiakhov et al., 2013), the Oasis Catalogic software V.5.11.6 TB from Laboratory of Mathematical Chemistry, University Bourgas, Bulgaria (Laboratory of Mathematical Chemistry, 2012) and Leadscope software V.3.0.11-1 with training sets from 2012 SAR Genetox Database provided by Leadscope (Roberts et al., 2000). 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.

The ecotoxicity, genotoxicity and mutagenicity of TD, the identified PTPs and the previously identified 12 HTPs were evaluated using the set of programs specified (Supplementary material (Text S2).

2.5. Mutagenicity and initial microbial toxicity testing of single substances and photolytic mixtures

The mutagenicity of TD and its photolytic mixtures were determined by experimental testing using the Ames microplate format (MPF) aqua assay (Xenometrix, AG, Switzerland). In addition, the kinetic LBT was used to evaluate the acute and chronic toxicity to the environmental bacteria species V. fischeri. Samples from photodegradation experiments were sterile filtered, and stored at −150 °C for a maximum timespan of 7 days. Every toxicity experiment was conducted in two independent repetitions, including the UV-treatment procedure. The pH was adjusted to 7.0 ± 0.2 before testing.

2.5.1. Ames MPF 98/100 aqua assay
2.5.1.1. Materials. Ames MPF 98/100 Aqua test kit containing exposure medium, reversion indicator medium, growth medium, Aroclor 1254-induced rat liver homogenate (S9), positive controls: 4-nitroquinoline-N-oxide (4-NQO) and 2-nitrofluorene (2-NF) and 2-aminoanthracene (2-AA) as well
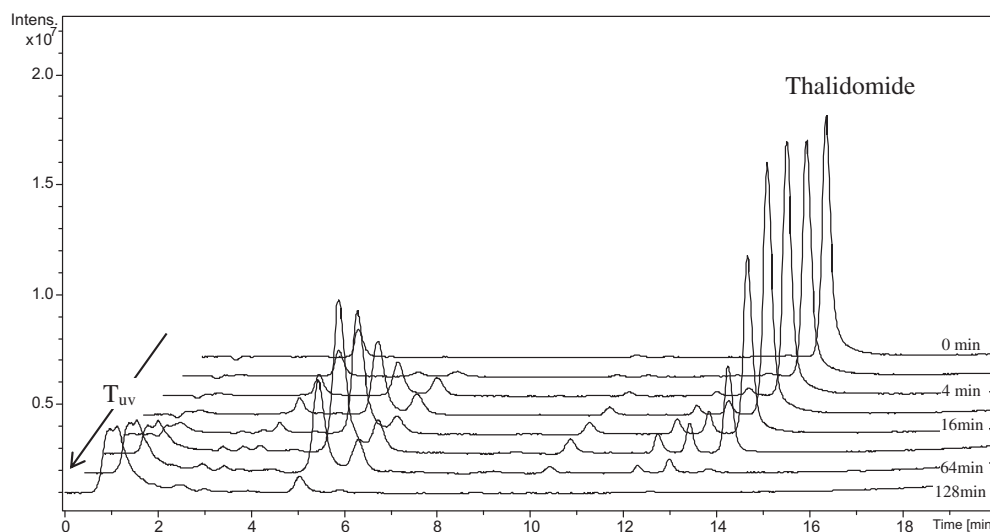
Fig. 1 – Total ion chromatograms (TICs) of thalidomide samples collected at different time points (0, 2, 4, 8, 16, 32, 64, and 128 min) of UV exposure in 800 ml photoreactor (PR1) using LC-ESI-MS in positive mode (initial concentration of thalidomide = 47 mg/L; Tuv = irradiation time with UV lamp).

as bacterial strains *S. typhimurium* TA 98 and TA 100 were supplied by Xenomtrix AG.

2.5.1.2. Method. In brief an overnight culture was grown until the OD600 reached ≥2.0. In a 24-well plate, the bacteria were exposed the photolytic mixtures, collected at 0, 2, 4, 8, 16, 32, 64 and 128 min of UV exposure, in the presence or absence of metabolic activation (± S9). The final test concentration of TD at time point 0 min was 35 mg/L. After exposure for 90 min (at 37 °C) while shaking (250 rpm) the exposure mixture was diluted with reversion indicator medium, transferred into 384-well plates and incubate at 37 °C for 48 h. During this time, the pH dependent reversion indicator dye would change from purple to yellow in the presence of bacterial growth. The result was colorimetrically scored by eye to give the number of revertants (yellow colored wells). As positive controls a mixture of 4-NQO and 2-NF (+S9) and 2-AA (-S9) were used like described in the test kit. Before the testing of mutagenicity, the cytotoxicity of TD and its PTPs were assessed to dismiss the possibility of false 'negative' mutagenicity results.

2.5.1.3. Analysis. The results were considered positive when the response was ≥2 fold increase in the number of revertants over that of the baseline number of revertants (the mean revertants of the negative control plus 1 SD). The statistical significance determined by ANOVA (Holm-Sidak method, overall significance level $p \leq 0.01$) was also used to assist in the determination of positive results.

2.5.2.   Kinetic luminescent bacteria test (kinetic LBT)
The kinetic LBT allows for the combined analysis of acute and chronic toxicity towards the luminescent bacteria species *V. fischeri*. A more detailed description and assessment of the kinetic LBT can be found elsewhere (Menz et al., 2013).

2.5.2.1. Materials. The freeze-dried luminescent bacteria (*V. fischeri* NRRL-B-11177) for the LBT were purchased from Hach-Lange GmbH, Düsseldorf.

2.5.2.2. Method. For the testing of single substances, saturated stock solutions of TD and phthalimide were prepared freshly and the final concentration was determined by DOC-Analysis. Subsequently, serial dilutions were prepared for the analysis of concentration–response relationship. Prior to testing, samples were supplemented with NaCl [2% (w/v)].

An overnight culture of *V. fischeri* was prepared in SSWC media (supplemented seawater complete media, DIN, 2009) and grown at 20 °C for 22–24 h. Turbidity of the bacteria suspension was measured according to DIN EN ISO 7027:2000–04 and the overnight culture was diluted with SSWC media to an initial turbidity of 20 formazin turbidity units (FTU). The luminescent bacteria suspension was transferred to the wells of a 96-well plate and an initial measurement of luminescence and optical density ($\lambda = 578$ nm) was conducted. Subsequently, the samples were added and a kinetic measurement of luminescence as well as optical density was carried out for 24 h at 15 °C.

In each experiment, 4.5 mg/L 3,5-Dichloropenol and 4.5 mg/L Chloramphenicol were used as positive controls for acute toxicity and chronic inhibition, respectively.

2.5.2.3. Analysis. The raw data was normalized to percent inhibition in relation to the negative controls. This was conducted for three different endpoints that are: acute luminescence inhibition after 30 min (acute LI), chronic luminescence inhibition after 24 h (chronic LI) and growth inhibition after 14 h (GI). The acute luminescence inhibition (acute LI) was calculated according to EN ISO 11348 (DIN, 2009). A more detailed description of data analysis and the calculations done is available in the Supplementary material (Text S3).
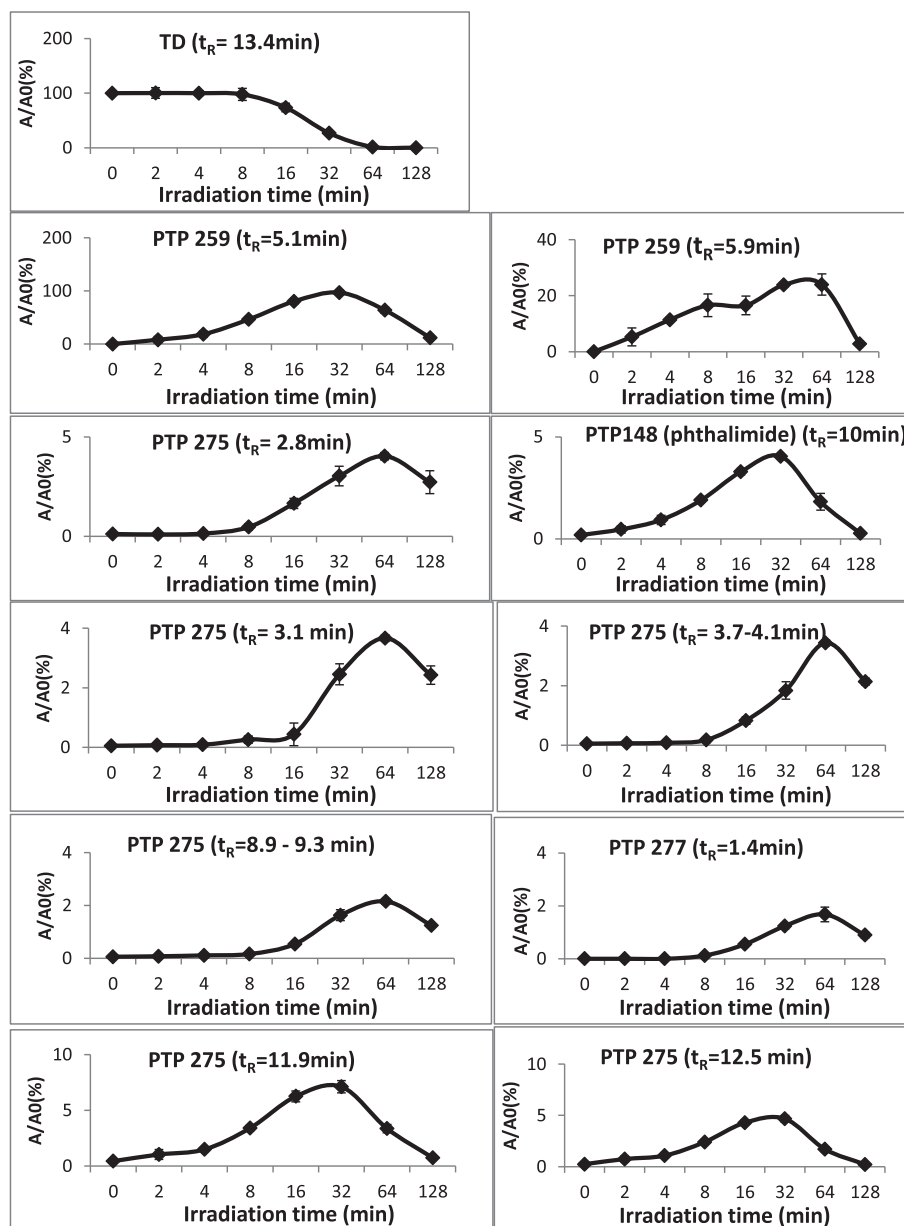
Fig. 2 – Comparison of the relative peak area (%) of the phototransformation products (PTPs) formed and decreased during photolytic process of 47 mg/L TD in 800 ml photoreactor (PR1)using LC-ESI-MS ($n = 2$). (t_R = retention time; A/A0 as A is the area of the PTP at the specified irradiation time point and A0 is the area of thalidomide (TD) at 0min).

3.   Results and discussion

3.1.   Photodegradation

The concentration of TD decreased during the irradiation process using the Hg lamp. The elimination of 10 mg/L TD in PR3 and PR4 was faster than PR1 and PR2. TD was completely degraded after 8 min in PR3 and PR4 and after 64 min in PR1 and PR2. The increase in elimination of TD in PR3 and PR4 might be due to the elevation in temperature of the cuvette within the photodegradation process, as no cooling and stirring is done for this cuvette (Neamţu and Frimmel, 2006). Therefore, further photodegradation experiment of 47 mg/L

TD was performed in PR1 and PR2 as the photodegradation solution is stirred, under controlled temperature, and larger photodegradation sample volume can be provided.

The photodegradation process of 47 mg/L TD was accompanied by a DOC loss of 15% and 18.4% after 128 min in PR1 and PR2, respectively (Supplementary material, Figure S1). The pH was decreased from 5.6 (0 min of photolysis) to 3.8 (128 min of photolysis). LC-MS revealed that new PTPs were formed (Fig. 1). Because no isolation of pure compounds was feasible, quantification of the PTPs was impossible. Therefore, the area ratio (A/A$_0$ as A is the area of the PTP and A$_0$ is the area of TD at 0 min) of the PTPs was plotted against the sampling time (Fig. 2 and Fig. 3). It is apparent from Fig. 2 that some of the PTP peaks increased
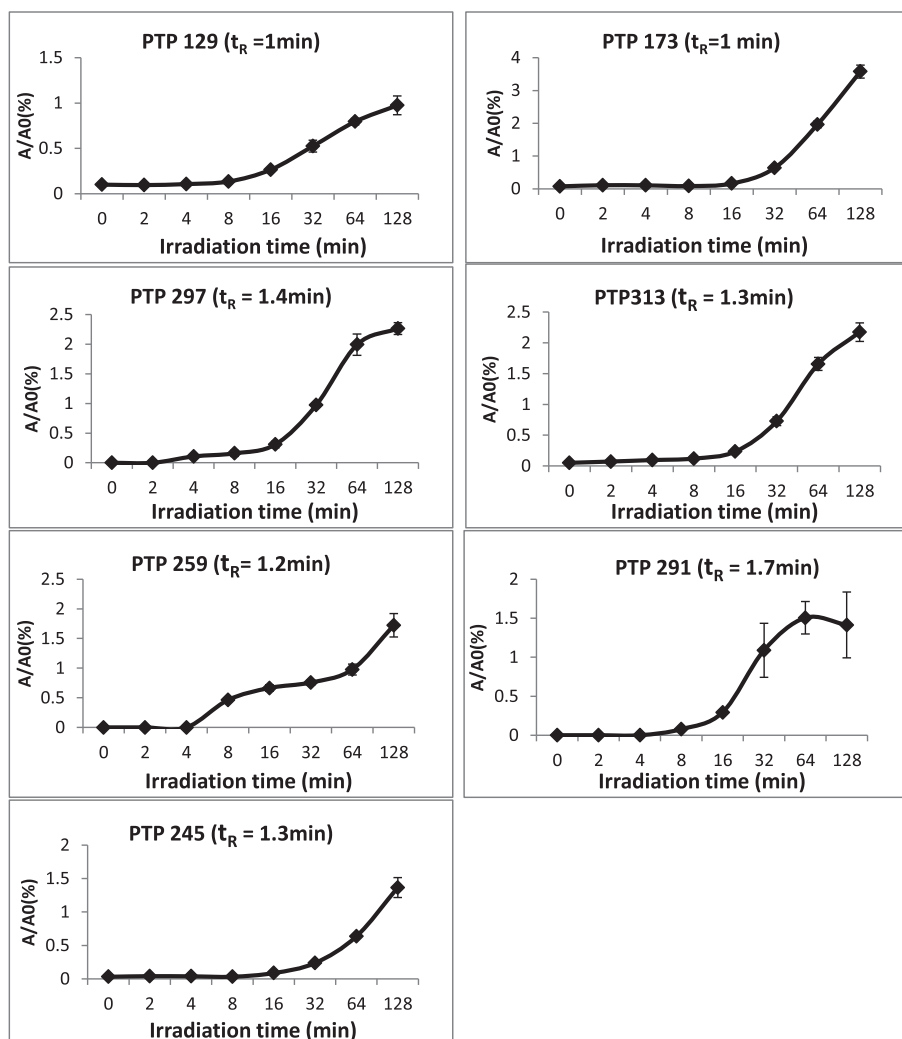
Fig. 3 − Comparison of the relative peak area (%) of the phototransformation products (PTPs) increased during photolytic process of 47 mg/L TD in 800 ml photoreactor (PR1) using LC-ESI-MS (n = 2). (t_R = retention time; A/A0 as A is the area of the PTP at the specified irradiation time point and A0 is the area of thalidomide (TD) at 0min).

with irradiation time until 32 min and then began to decrease. While others PTP peaks were formed at 16 min then increased until 128 min (Fig. 3).

3.2. Identification of phototransformation products (PTPs)

The PTPs generated during photolysis studies are considered as potential environmental pollutants. Thus, identification of the most relevant PTPs is important to predict the environmental impact of original compound. For this reason, LC-UV−MS/MS analyses based on accurate mass measures was performed during the photolysis assays. The chromatographic behavior demonstrated that some of the PTPs formed by photolysis were of higher polarity than TD itself. Structures of the five main observed PTPs were reported previously (Mahmoud et al., 2013). The photodegradation samples of 10 mg/L and 47 mg/L TD were subjected for further investigation of PTPs. These samples were analyzed by the Auto MS^n mode, where PTPs with highest peak intensity were isolated

and fragmented up to MS^3 in order to gain more structural information.

The same PTPs are formed in the photodegradation samples of 10 mg/L and 47 mg/L TD. The total ion chromatogram (TIC) in LC-MS showed a peak at 1.3 min which has several very polar PTPs with the following m/z 129.1, 173.1, 245, 259.1, 277.1, 297.1, 313.1, and 291.1 (Fig. 1). The extracted ion chromatograms and the postulated structures of all these PTPs and their smiles codes are listed in the supplementary material (Figure S2 and Table S2). The chemical structures of m/z 259.1 (t_R = 1.2 min) and m/z 245 (t_R = 1.3 min) could not be proposed, even though MS^2 and MS^3 spectra could be obtained. These very polar PTPs can be due to further photolysis of the other PTPs which were eliminated after 32 min of photolysis. The compounds eluting at 1.3 min are extremely polar as demonstrated by their very short retention time. Trials were performed to elute these polar compounds later by changing the gradient elution to begin with 0.5% ACN instead of 5%. However, these polar compounds peak still eluted early.

Several peaks were detected with the same nominal mass of m/z 275 (PTPs_275) but different retention times (supplementary materials Figure S2). A peak with additional 16 Da was observed for the PTPs_275 compared to m/z 259 of TD and its isomers. This is likely due to hydroxylation of TD or its isomers. In most cases these PTPs_275 also exhibited similar MS^2 fragmentation pathways, indicating formation of constitutional isomers (Table 1). However, on the basis of the MS fragmentation, the identification of the exact position of the hydroxyl group was not feasible. Hydroxylation of TD occurs during biological metabolism (Eriksson et al., 1998; Meyring et al., 1999). Four of the predicted structures are also reported as human metabolites (Eriksson et al., 1998;

Nakamura et al., 2006). These metabolites are 5′-hydroxythalidomide (PTP 275_1), N-hydroxythalidomide (PTP 275_3), 5-hydroxythalidomide (PTP 275_4), and 4-hydroxythalidomide (PTP 275_5) (supplementary material (Table S2)). It has been reported that at least one of the hydroxylated metabolites (PTP 275_1) has moderate anti-angiogenic activity at high concentrations (Price et al., 2002).

Moreover, there is a peak with the nominal mass of m/z 291 which has additional 32 Da compared to m/z 259 of TD and its isomers. This is likely to be due to formation of dihydroxy derivatives of TD and its isomers. Also, dihydroxylation of TD occurs during biological metabolism. Three of the predicted structures are also reported as human metabolites (Eriksson et al., 1998; Nakamura et al., 2006). These metabolites are 5,5′-Dihydroxythalidomide (PTP 291_1), 4,5-Dihydroxythalidomide (PTP 291_2), and 5,6-Dihydroxythalidomide (PTP 291_4) (Supplementary material Table S2).

The PTP_277 (nominal mass 277 m/z) has an additional 18 Da compared to m/z 259 of TD and its isomers. This is likely due to saturation of double bond and hydroxylation of TD isomers. The PTP_297 (nominal mass 297 m/z) can be due to splitting of one of the equivalent amide bonds of the phthalimide ring and glutaramide ring accompanied by reduction of the ketone moiety of the phthalimide ring to hydroxyl group. The PTP 129 (nominal mass 129 m/z) proposed to be α- amino glutaramide. The PTP_173 (nominal mass 173 m/z) proposed to be due to cleavage in the phthalimide ring.

3.3. In silico toxicity predicted parameters

The obtained results of the predicted activity of the test chemicals from the QSAR modules were expressed in different ways depending on the software.

For Case Ultra software, the predicted activities of tested chemicals are expressed as "positive" and "marginally positive" which means that one or more positive alerts for the predicted activity were found for the test chemical. "Inconclusive (orange)" means that because both positive and deactivating alerts were found in the same molecule and the system cannot draw a firm conclusion. "Inconclusive (Black)" means that because a significant portion of the test chemical is covered by unknown structural fragments and the system cannot draw a firm conclusion. "Negative" means that no positive alert was detected in the molecule and "out of domain" means structural fragments unknown to the model were found in the molecule and that for this reason the molecule is excluded from the chemical space of the training set of the model used. For Oasis Catalogic software, the predicted activity of the test chemicals in the three acute V. fischeri modules are expressed as mg/L for half maximal inhibitory concentration (IC$_{50}$) and in Salmonella Catalogic module are expressed as "mutagenic" or "not mutagenic". For Leadscope software, the predicted activity of the test chemicals is expressed as "positive", "negative" and "not in domain".

3.3.1. In silico toxicity predicted parameters of hydrolysis products (HTPs)
The HTPs included in the analysis have been reported (Schumacher et al., 1965) (Supplementary material (Table S1)).

Table 1 – Chromatographic and mass spectrometric parameters for TD and its PTPs analysis in LC/MS–MS (ESI (+); (relative intensity, %).

Compound	t_R (min)	Main precursor ion (m/z)	Product ions (m/z)
PTP129	1	129.1	84.1(100)
PTP173	1	173.1	155(100), 127(89.4), 128(24.7), 155.9(21.2), 84.1(10.5)
PTP259	1.2	259.1	240.9(100), 212.9(83.7), 173(58.6), 230.9(30.1), 213.9(19.8), 194.9(10.6)
PTP245	1.3	245.0	198.9(100), 226.9(68.4), 155(22.9), 173 (19.3), 224.9(12.6), 128 (10.2).
PTP277	1.4	277.1	259(100), 259.9(36.2),257(19.2), 230(14.4), 241 (13), 255(12.3), 242 (10.5), 201.9(10)
PTP297	1.4	297.1	279(100), 251 (29.6), 232.9(22.6), 172.9(14.2),
PTP313	1.4	313.1	173(100), 295(60.8), 293(19.8), 297(18.6), 141(16.2), 291(13.0), 296(11.9)
PTP291	1.7	291.1	273(100), 274(60.3), 246(56.4), 263(41.2), 190.9(30.8), 275(22.9), 149(19.5), 247(15.8), 179.9(13.6), 219(13.3),201.9(10.5), 276(10)
PTP275	2.8	275.1	257(100), 230 (43), 174.9 (37), 257.9(22.2)
PTP275	3.1	275.1	257.9(100), 257(44.6), 230(24.9), 247(12.6), 259(12.6)
PTP275	3.7	275.1	257(100), 174.9 (43.9), 230 (42.2), 258(28.6), 247(13.3)
PTP275	4.1	275.1	257.9(100), 257(75.5), 229.9(42), 247(19.9), 258.9(18.4), 174.9(15.6), 202(10.7)
PTP259	5.1	259.0	241.0(100), 213.9(33.6), 241.9 (15.4), 231.0(13.3), 159.0 (12.8).
PTP259	5.9	259.1	241.0(100), 214(24.3), 231.0(19.4), 159.0 (14.6), 241.9(11.3).
PTP275	8.9 & 9.3	275	257(100), 229.9(45.5), 174.9(43.5), 202.9(16.6), 258(16.4), 228.9(14.1), 163.9(11.8), 202(11), 247(10.8), 213.9(10.1)
PTP148	10	148.0	130 (100)
PTP275	11.9	275.1	247.0 (100), 84.2(53.6), 201.9(12.4), 248(11.8), 230(10.6)
PTP275	12.5	275.1	247.0 (100), 84.1 (58.7).
Thalidomide	13.4	259.1	231.1 (100), 84.1(63.2)

t_R: retention time.

PTP275_9 (Positive alert)
Alert ID 160: C2-c(:cH):c-C2=O
Statistical significance = 100%

PTP275_9 (Positive alert)
Alert ID 716: c:cH:c:c-C2=O
Statistical significance = 100%

PTP275_9 (Positive alert)
Alert ID 809: C2-c:c(-C2):cH:c:cH
Statistical significance = 100%

PTP275_9 (Positive alert)
Alert ID 982: C2-c(:c):cH:c-OH
Statistical significance = 99%

PTP 313_4 (Positive alert)
Alert ID 827: C2-c:c:c:cH
Statistical significance = 100%

PTP 313_4 (Deactivating alert)
Alert ID 1153: OH-c:c:c-C2=O
Statistical significance = 98%

Fig. 4 — The predicted structural moieties responsible for the positive alerts and deactivating alert in PTP275_9 and PTP313_4 in Salmonella t. 5-strains model using Case Ultra software are highlighted.

For genotoxicity and mutagenicity, the results in the supplementary material (Table S3) show that there is no positive predicted mutagenic effect in Ames tests using Salmonella Catalogic (Oasis Catalogic), and Bacterial Mutagenesis (Leadscope), Mutagenicity Ames (Case Ultra) and Salmonella t. 5-strains (Case Ultra). However, an inconclusive (orange) effect was predicted for TD, HTP1, HTP2 and HTP4 in the Mutagenicity in Salmonella t. 5-strains module. Some positive alerts for some HTPs were predicted in Human Carcinogenicity, Aneuploidy in Yeast, Micronucleus Formation *in vivo* composite, and Micronucleus Formation *in vivo* Mouse whereas TD activity was predicted as negative or inconclusive. In a similar manner, TD was not in domain but some of the HTPs were positive in *In vitro* chromosome aberration and *In vivo* micronucleus (Supplementary material (Table S4)).

For ecotoxicity, there are some positive alerts obtained for the TD and some of its HTPs in different modules (supplementary material (Table S5 and Table S4). Data from Table S5 shows that there are some contradictions in the prediction of bacterial toxicity such as HTP8 in the 3 Acute tox V. *fischeri* models give lower IC_{50} compared to TD, whereas the Microtox

Toxicity to Environmental Bacteria give negative results for HTP8.

3.3.2.   In silico toxicity predicted parameters of phototransformation products (PTPs)
For genotoxicity and mutagenicity, the results in supplementary material (Table S6 and Table S7) show that there is no positive predicted mutagenic effect in Ames tests using Salmonella Catalogic (Oasis Catalogic), and Bacterial mutagenesis (Leadscope). On the other hand, some positive alerts were predicted for some PTPs in Mutagenicity in Salmonella t. 5-strains (Case Ultra) whereas TD activity predicted as inconclusive (orange), and some inconclusive alerts were predicted for some PTPs in Mutagenicity Ames (Case Ultra) while TD activity was predicted as negative. In Salmonella t. 5-strains (Case Ultra), there are several positive alerts responsible for the predicted positive or marginally positive toxicity. These alerts are Alert ID 160 (PTP275_9, PTP148, and PTP259_2), Alert ID 716, 809 and 982 (PTP275_9), and Alert ID: 827 (PTP 313_4 and PTP 313_9). Moreover, PTP 313_4 and PTP 313_9 are predicted as marginally active due to the presence of a deactivating alert (Alert ID 1153) (Fig. 4). Although these structural moieties responsible for this positive alerts are present in some other PTPs but these PTPs activities are predicted inconclusive due to presence of some other moieties responsible for deactivation or unknown structural fragments.

In all genotoxicity and mutagenicity QSAR modules predicted by Case Ultra (Supplementary material (Table S7)), the predicted TD activity was negative or inconclusive except in mouse lymphoma module TD activity was positive. On the other hand, some of the PTPs have a positive alert in these genotoxicity and mutagenicity QSAR modules predicted by Case Ultra. In similar manner, TD was not in domain but some of the PTPs were positive in *In vitro* chromosome aberration and *In vivo* micronucleus (supplementary material (Table S7)). From the medical point of view, one striking observation to emerge from this QSAR is the predicted genotoxicity for these PTPs which are human metabolites as well as transformation products such as PTP_275_1. In detail, PTP_275_1 (5-hydroxy thalidomide) is predicted to be positive in Micronucleus Formation *in vivo* composite (A7S) and *In vivo* micronucleus (IVMN). This 5-hydroxyl metabolite was detected in human plasma (Ando et al., 2002; Eriksson et al., 1998; Lu et al., 2004; Luzzio et al., 2005) and after metabolism in human liver microsomes, though at much lower levels than in mice and rabbits by Chung and colleagues (Chung, 2004; Lu et al., 2004). Since the 5-hydroxy thalidomide was recently proposed as a possible anti-angiogenic compound (Noguchi et al., 2005), this possibly increased genotoxicity of the 5-hydroxy thalidomide should be taken into account, when developing this substance for the use in humans.

For ecotoxicity, there are some positive alerts obtained for the TD and some of its PTPs in different modules (supplementary material (Table S7 and Table S8)). In Microtox Toxicity to Environmental Bacteria (*V. fischeri*), Bioconcentration for Cyprinus Carpio and Gold Fish Toxicity modules, the predicted activities of TD and some of the PTPs were positive. In contrary, in the Rainbow Trout Toxicity module, TD predicted activity was negative and some of the PTPs are inconclusive.

In the three acute *V. fischeri* modules at 5 min, 15 min and 30 min, the following PTPS (PTP259_1, PTP259_2, PTP148, PTP291_3, PTP291_9, PTP291_13, PTP275_5, PTP275_6, PTP275_8) have lower predicted IC_{50} than TD (Supplementary material Table S8).

3.4. Toxicity testing

3.4.1. Ames MPF 98/100 aqua assay

Cytoxicity testing of the TD and its TP confirmed that the growth of both test strains was not affected by the mixtures taken at any time point. Further, TD and its PTPs formed at different time points proved negative for mutagenicity in both strains (Table 2).

According to the QSAR predictions for the modules pertaining to mutagenicity, there were some positive and marginally positive alerts for some PTPs. However, given that the experimental results were negative, it cannot be excluded that perhaps the concentration of these PTPs may be too low to express a mutagenic effect or the possibility of antagonistic interactions of mixture components or that these positive alerts may be for strains other than TA 98 and 100 since the QSAR modules cover more strains. One should also consider that the Ames MPF 98/100 Aqua test is another variation of the standard Ames test. Though this test is capable of detecting strong mutagens, it is less sensitive in the case of weak mutagens (Escobar et al., 2013). Nevertheless, the experimental data should not be ignored since QSAR is an estimation method and these estimations can be poor, even for well evaluated models (European Commission, 2003). They can give guidance but not a final proof.

3.4.2. Kinetic luminescent bacteria test

3.4.2.1. Bacterial toxicity of TD during UV-treatment. Application of the undiluted photolytic mixtures, leading to a final sample dilution in the test of 1:2, demonstrated a significant increase of toxicity in relation to the untreated sample for the

reaction mixtures obtained after 16, 32, 64 and 128 min of irradiation, regarding the endpoint acute luminescence inhibition (Fig. 5). The analysis of chronic luminescence inhibition and growth inhibition showed a significant toxification for the samples taken after 32, 64, and 128 min of irradiation (Fig. 5). The final sample dilution of 1:50 showed no significant inhibition (data not shown).

3.4.2.2. Toxicity of untreated TD and phthalimide. After DOC analysis of the saturated stock solution, a maximum concentration of TD in the kinetic LBT of 23 mg/L was estimated. At this concentration, only the luminescence emission after 24 h (chronic LI) was significantly inhibited (15% inhibition). The endpoints of the acute LI and GI were not affected. According to measured DOC, the highest concentration of phthalimide applied to the test was 230 mg/L. At this concentration, a maximum chronic LI of 78% was observed. The endpoints acute LI and GI showed a comparatively lower inhibition than the endpoint of the chronic LI, but exhibiting maximum inhibition values of 21% and 31%, respectively. EC_{10} and EC_{50} values of TD and phthalimide, including 95% confidence intervals, are presented in Table 3. Regarding the most sensitive endpoint in both cases, i.e. chronic LI, there was no significant difference between the EC_{10} of phthalimide and the EC_{10} of the parent compound or its HTPs, probably because hydrolysis will most likely occur in the setting including bigger time, i.e. the chronic test. QSAR predictions from different models give contradictory results regarding the

Fig. 5 − **Kinetic luminescent bacteria test − Toxicity of Thalidomide reaction mixtures during UV-treatment for the endpoints acute Luminescence inhibition after 30 min (acute LI), chronic Luminescence inhibition after 24 h (chronic LI) and Growth inhibition after 14 h (GI) Photolysis was conducted with an initial thalidomide concentration of 47 mg/L. Photolytic mixtures were applied to the kinetic LBT in final dilutions of 1:2. 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 control were identified by ANOVA following post hoc multiple comparisons (Holm-Sidak method, P < 0.050).**

Table 2 − Mutagenicity results of Ames MPF assay of thalidomide and its PTPs formed at different time points after photolysis with *Salmonella typhimurium* TA 98 and TA 100 in the absence and presence of S9 mix.

Time (min)	Number of revertants			
	TA98		TA100	
	−S9	+S9	−S9	+S9
NC	1 ± 1	2 ± 1	7 ± 3	3 ± 2
0	1 ± 1	1 ± 1	7 ± 3	4 ± 2
2	1 ± 1	1 ± 1	5 ± 3	3 ± 2
4	1 ± 1	2 ± 1	9 ± 4	5 ± 1
8	1 ± 1	2 ± 1	8 ± 3	4 ± 2
16	1 ± 1	2 ± 1	6 ± 3	3 ± 1
32	2 ± 1	1 ± 1	9 ± 5	5 ± 3
64	2 ± 1	2 ± 1	7 ± 3	5 ± 3
128	3 ± 2	2 ± 1	8 ± 2	4 ± 1
PC	42 ± 3[a]	48 ± 0[a]	47 ± 2[a]	48 ± 0[a]

Positive results are presented in **bold italics**. PC = positive controls, NC = negative control.

[a] Represents $p \leq 0.01$. Testing was done in triplicates with 2 independent repetitions.

endpoint acute LI: while Oasis Catalogic predicts a lower IC_{50} for phthalimide, indicating a higher acute toxicity compared to TD, Case Ultra showed a positive alert for Microtox activity for TD, but a negative alert for Microtox activity in case of phthalimide (Supplementary material, Table S8). Moreover, the photolytic mixtures, especially those obtained after 64 and 128 min, showed a major increase of acute and chronic inhibition (Fig. 5). In contrast, phthalimide (PTP_148) occurrence was already decreasing after 32 min of irradiation (Fig. 3). In summary, it can be concluded that the formation of phthalimide might not be the only cause for the drastic toxification of TD during UV-treatment. This leads to the assumption that other PTPs/HTPs within the photolytic mixture, possibly in combination with the parent compound and phthalimide, might be responsible for the observed effects. Phthalimide toxicity is not environmentally relevant as high exposure level is needed to exert toxic effects and as phthalimide and its hydrolysis product 'phthalic acid' are readily biodegradable.

3.5. Identification of PTPs with microbial toxicity

It was stated above that the toxicity in the luminescent bacteria test was increasing during the photolytic process especially after 16 min of irradiation, reaching the maximum after 128 min of irradiation (Fig. 5). Therefore it is important to correlate the kinetic luminescent bacteria test results with the QSAR prediction for these PTPs formed and that increased in intensity at these time points. The acute luminescence inhibition (acute LI) of the kinetic LBT was compared with the following QSAR modules: Microtox Toxicity to Environmental Bacteria (Case Ultra) and Acute Toxicity Vibrio Fischeri 5min/15min/30min (Oasis Catalogic).

There is a significant increase in toxicity after 16 min of photolysis that was postulated to be related to the PTPs increased after 16 min (Fig. 3). When looking closely at the TIC in LC-MS, it can be seen that the peak intensity at 1.3 min is increased after 16 min of photolysis (Fig. 1). Therefore, it can be presumed that these PTPs (Fig. 3) might be responsible for the observed toxicity. Surprisingly, the PTPs (accounting for $\geq 4\%$ of the initial TD area at any sampling time) increased with irradiation time until 32 min and then began to decrease while toxicity increased, i.e. the high intensity PTPs were not responsible for this significant increase in toxicity. However, the QSAR results give a positive alert for some of them (see Section 3.3.2). As mentioned above the mixtures of the PTPs and the residual parent compound could be another reason for the changings of the toxicity during the treatment period. Earlier studies using different groups of pharmaceuticals e.g.

antibiotics or beta-blockers showed different effects like growth or luminescence inhibition and immobilization of *Daphnia magna* (Christensen et al., 2007; Cleuvers, 2004; Escher et al., 2006). Therefore not only one PTP might be responsible for the toxicity.

According to the kinetics of PTPs formation, PTP129, PTP173, PTP297, PTP313, PTP259, PTP291, and PTP245 are possible candidates that might be responsible for the toxification of TD during UV-treatment. Comparing the predicted QSAR results, it can be seen that PTP291_3, PTP291_9, and PTP291_13 have lower predicted IC_{50} than TD using the three acute *V. fischeri* modules (Table S8). Moreover, positive alerts for PTP291_1, PTP291_4, PTP291_5, PTP291_6, PTP291_7, PTP291_10, and PTP291_12 have been predicted in Microtox Toxicity Environmental Bacteria module. The positive alerts responsible for the predicted toxicity were alert Id 175 (present in PTP291_1, PTP291_4, PTP291_5, PTP291_6, PTP291_7, PTP291_10, and PTP291_12) and alert Id 214 (present in PTP291_10, and PTP291_12) (Fig. 6). Therefore, it can be concluded that PTP291_1, PTP291_4, PTP291_5, PTP291_6, PTP291_7, PTP291_10, and PTP291_12 might be responsible for the increase of toxicity in the luminescent bacteria test.

Of note is that the structural moieties responsible for the positive alerts (Fig. 6) are present in some other PTPs but these PTPs activities are predicted inconclusive or negative. These inconclusive or negative predictions from the Microtox Toxicity to Environmental Bacteria module (Case Ultra) are due to many reasons (Supplementary material (Text S4)).

4. Conclusion

Although photolysis was able to remove TD within the photoreactor, numerous PTPs were formed. Our study has proven that the mixture of PTPs was more toxic than the parent compound as evidenced by the increasing acute and chronic toxicity towards *V. fischeri*. Furthermore, the number of PTPs within the photolytic mixture that might be responsible for the toxification of TD during UV-treatment was successfully narrowed down by combining *in silico* methods and conventional experimental testing, including analysis of the mutagenic potential and the bioluminescence and growth inhibition to *V. fischeri*.

No mutagenic potential of the photolytic mixtures was detected with the Ames test. In contrast the QSAR predictions provided indication that various PTPs and HTPs might have genotoxic potential.

Nevertheless an elevated risk to the environment and human health resulting from the various PTPs and HTPs

Table 3 – EC_{10} and EC_{50} values with 95% confidence intervals (in brackets) of thalidomide and phthalimide in the kinetic LBT.

Substance	Tested range [mg/L]	Acute LI		Chronic LI		GI	
		EC_{10} [mg/L]	EC_{50} [mg/L]	EC_{10} [mg/L]	EC_{50} [mg/L]	EC_{10} [mg/L]	EC_{50} [mg/L]
Thalidomide	0.2–23	n.d.	n.d.	16.5 (0.1–40.0)	n.d.	n.d.	n.d.
Phthalimide	2.5–230	70.6 (0.2–323.6)	n.d.	23.7 (14.9–33.4)	100.7 (88.2–113.2)	69.4 (21.1–136.9)	n.d.

n.d.: not determinable because of low water solubility.
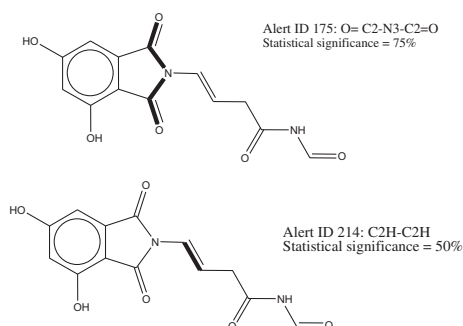
Fig. 6 – The predicted structural moieties responsible for the positive alerts in the phototransformation product (PTP291_10) by Microtox Toxicity to Environmental Bacteria model using Case Ultra software are highlighted. Alert ID number is provided by the software database. The structural moieties responsible for the alerts are presented in bold.

cannot be completely excluded regarding to our initial toxicity data. Therefore, further investigations need to be carried out in the future.

At the moment, there is no risk for public health and the environment, when taking into account that TD was not detected in the aquatic environment until now. Nevertheless, TD and its PTPs may become environmentally relevant in future because of the expected increased consumption. These results emphasize that not only the removal of parent pollutants is important but also the elimination of the PTPs from waste water should be considered.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.11.014.

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1   **Supplementary materials**

2

3   **Identification of phototransformation products of Thalidomide and mixture**

4   **toxicity assessment: an experimental and quantitative structural activity**

5   **relationships (QSAR) approach**

6

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Text S1

23   Structural identification of the photoproducts was based on the analysis of the total ion

24   chromatogram (TIC) and the corresponding mass spectrum. For PTP peaks, depending on the

25   peak intensity of each PTP, up to MS^3 spectra were generated using the Auto MS^n mode in order

26   to have structural information on the PTPs and to make structural elucidation. Therefore, the

27   precursor ion was fragmented first. The two most abundant product ions were then selected and

28   fragmented again if peak intensity was high enough. Furthermore, the formation kinetics of the

29   PTPs were monitored during the photodegradation of 47 mg/L TD in PR1 in order to correlate

30   them with QSAR predictions and data from toxicity experiments.

Text S2

32   The ecotoxicity, genotoxicity and mutagenicity of TD, the identified PTPs and the 12

33   HTPs were evaluated using the set of programs specified. Case Ultra was used to predict

34   ecotoxicity using the following QSAR models: Microtox Toxicity to Environmental Bacteria

35   (AUA), Bioconcentration for *Cyprinus Carpio* (BCF), Gold Fish Toxicity (AUG), and Rainbow

36   Trout Toxicity (AUE). Genotoxicity, mutagenicity and carcinogenicity was predicted with Case

37   Ultra using the following QSAR models: Human Carcinogenicity (A0J), Aneuploidy in Yeast

38   (A6A), mutagenicity in *Salmonella typhimurium* 5-strains (A7B) (including the strains TA97,

39   TA98, TA100, TA1535, TA1536, TA1537, TA1538), Micronucleus Formation *in vivo* composite

40   (A7S), Micronucleus Formation *in vivo* Mouse (A7T), Chromosome Aberrations *in vitro*

41   composite (A7U), Chromosome Aberrations *in vitro* CHO cells (A7V), Rat Carcinogenicity

42   (A0D), Mouse Lymphoma (ML), Mouse Carcinogenicity (A08), Mutagenicity Ames (A2H)

43   (Salmonella Ames mutagenicity updated from NTP, Genetox, FDA and others. It consists the *S.*

44   *typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535, TA1536, TA1537, TA1538

45   using a different training set compared with A7B), Unscheduled DNA Synthesis (UDS) Induction

46   (A64).

47   The Oasis Catalogic software was used to predict acute toxicity towards *V. fischeri* after 5

48   min, 15 min and 30 min exposure (Acute Toxicity Vibrio Fischeri 5min/15min/30min v.01). In

49   addition to that, the Oasis Catalogic software predicts mutagenicity based on bacterial

50   mutagenicity (module mutagenicity v.04) in *S. typhimurium* (Salmonella Catalogic model (SC)).

51   The Leadscope software predicts genotoxicity and mutagenicity using the following four QSAR

52   modules: *In vitro* chromosome aberration (IVCA), Mammalian mutagenesis (MM), *In vivo*

53   micronucleus (IVMN), Bacterial mutagenesis (BM).

54 **Text S3**

55   A modified formula (1) was used for the calculation of the chronic luminescence

56 inhibition (chronic LI), which has been described before by Backhaus et al. (Backhaus et al.,

57 1997):

58 $LI_{24h} = 100\ (I_{NC} - I_t)\ /\ I_{NC}$ (1)

59 LI_{24h} = luminescence inhibition after 24 h (%); I_{NC} = average light intensity of the negative

60 controls after 24 h in relative luminescence units (RLU); I_t = light intensity of the test culture

61 after 24 h (RLU).

62   The measured optical density after 14 h was used for calculation of the growth inhibition

63 (GI) according to formula (2):

64 $GI_{14h} = 100\ (OD_{NC} - OD_t)\ /\ (OD_{NC} - OD_0)$ (2)

65 GI_{14h} = growth inhibition after 14 h (%); OD_t = optical density of the test culture after 14 h; OD_{NC}

66 = average optical density of the negative controls after 14 h; OD_0 = average optical density of the

67 negative controls after sample addition.

68   According to Menz et al. (Menz et al., 2013) the following thresholds were applied for the

69 identification of significant inhibition values: acute LI = 20% inhibition, chronic LI = 15%

70 inhibition, GI = 20% inhibition. In case of significant inhibition, analysis of concentration-

71 response relationships was performed by non-linear, logistic regression applying the function

72 "Four Parameter Logistic Curve"(3) to the normalized inhibition values.

73 $y = min + (max - min)\ /\ (1 + (x\ /\ EC_{50})^{-Hillslope})$ (3)

74 y = inhibition in %; min = bottom of the curve; max = top of the curve; Hillslope = slope of the

75 curve at its midpoint; EC_{50} = x value for the curve point that is midway between the max and min

76 parameters (half-maximal effective concentration). Because of the low water solubility of TD and

77 phthalimide, only partial concentration-response curves could be obtained. Therefore, logistic

78 regression was conducted under the assumption that higher concentrations would reach a plateau

79 with a total inhibition (max = 100%). After fitting the data to the curve, EC_{10} was derived from

80 the given plot equation using Formula (4).

81 $EC_{10} = EC_{50} (1/9)^{1/Hillslope}$ (4)

82 Significant changes of inhibition during photolysis were identified by One Way ANOVA,

83 following post hoc multiple comparisons (Holm-Sidak method, overall significance level = 0.05),

84 in which the untreated sample after 0 min of irradiation was defined as the control group.

85 Non-linear regressions and analysis of variance (ANOVA) were performed with the statistical

86 software SigmaPlot 12 (Systat Software, USA).

87 **References:**

88 Backhaus, T., Froehner, K., Altenburger, R., Grimme, L.H., 1997. Toxicity testing with Vibrio

89 Fischeri: A comparison between the long term (24 h) and the short term (30 min) bioassay.

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92 characterization of an automated method for the combined assessment of acute and chronic

93 effects. Chemosphere 93 (6), 990–996.

94   **Text S4**

95   Of note is that the structural moieties responsible for the positive alert (Figure 6Figure 6)

96   are present in some other PTPs but these PTPs activities are predicted inconclusive or negative.

97   These inconclusive or negative predictions from the Microtox Toxicity to Environmental Bacteria

98   module (Case Ultra) are due to many reasons: 1. the presence of some other moiety responsible

99   for deactivation; 2. a significant portion of the test chemical is covered by unknown structural

100   fragments; 3. if multiple positive alerts were found then the prediction is made using the alert

101   with highest statistical significance even if the resulting activity based on the corresponding

102   compounds from the training set for this alert might be low; 4. If none of the positive alerts

103   contain any QSAR then the average activity of the alert is used as the predicted activity; 5. If a

104   positive alert contains a QSAR then the activity is calculated using the QSAR equation. For

105   example, PTP291_11 and PTP291_13 are predicted as negative in Microtox Toxicity to

106   Environmental Bacteria module although there are 3 positive alerts (alert Ids 112, 175, and 214)

107   predict in the molecule (supplementary material (Figure S 3)). Alert 112 obviously overrules

108   alerts 175 and 214, but refers to a low activity based on the training set examples containing this

109   alert.

110
111
112
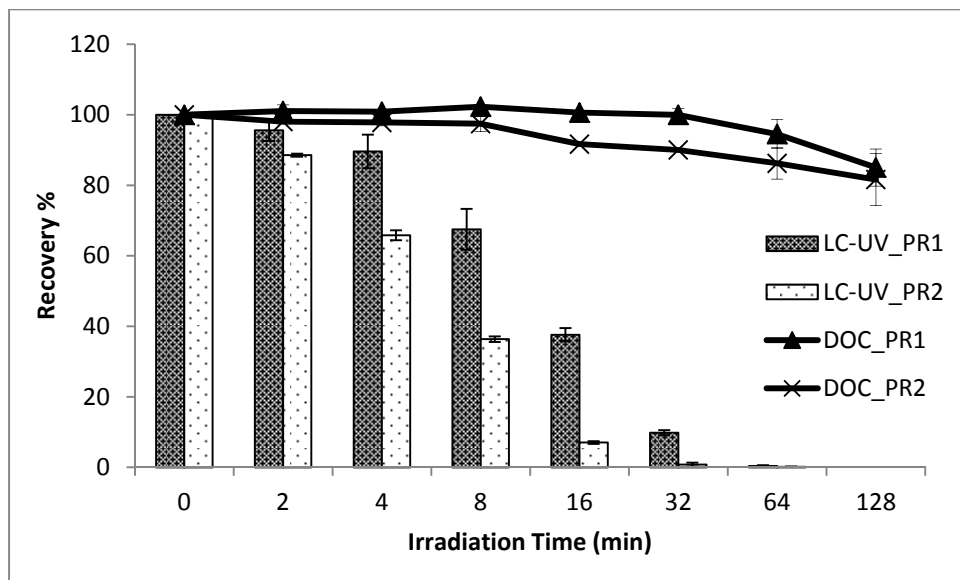
113
Figure S 1 Photodegradation of thalidomide (47 mg/ L) in two different rector volumes PR1
(800ml photoreactor) and PR2 (110 ml photoreactor) during UV-irradiation (n = 2). (DOC=
dissolved organic carbon)

117

118
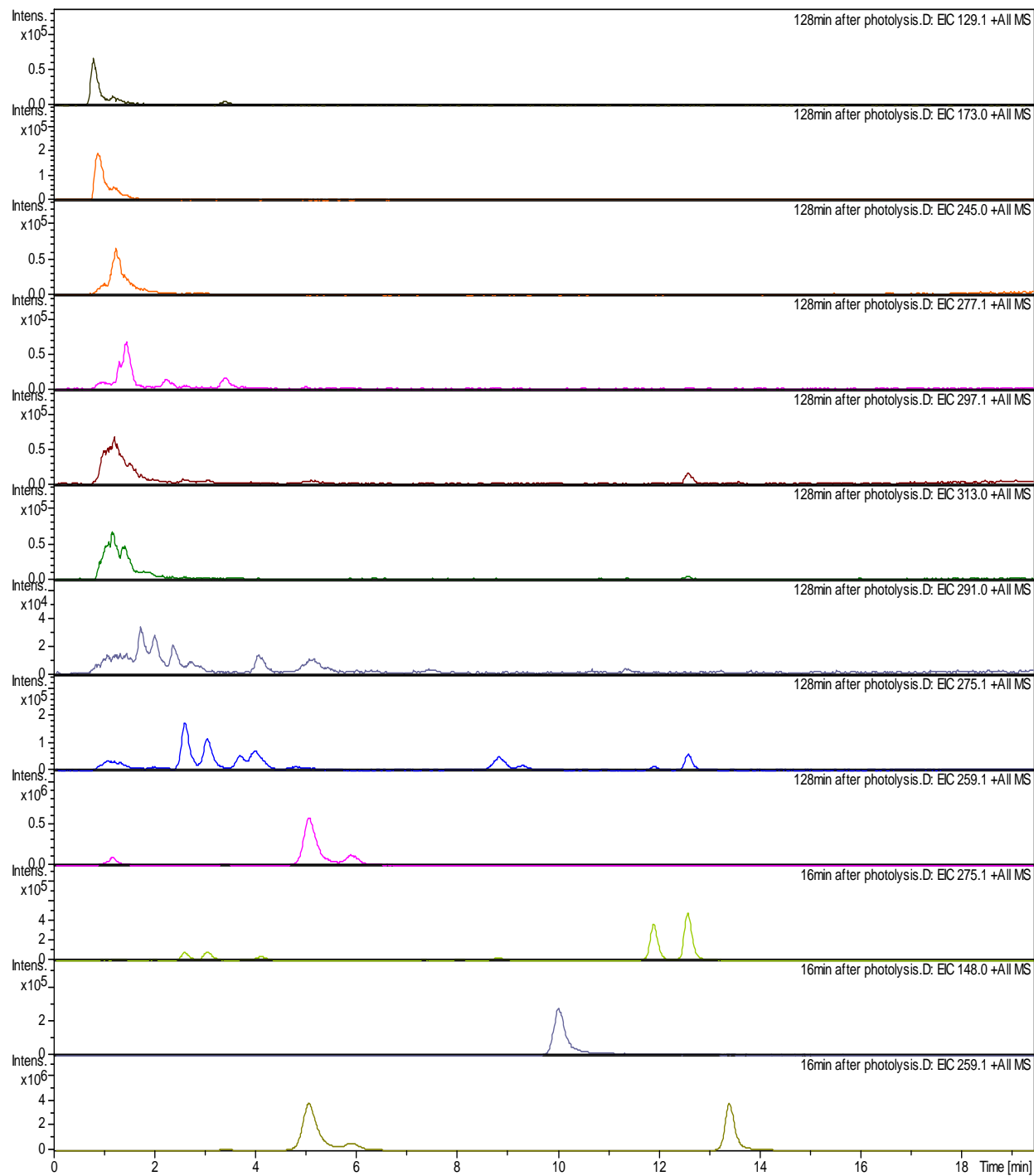
Figure S 2. Extracted ion chromatograms of the identified PTPs by LC-MS.

119

120
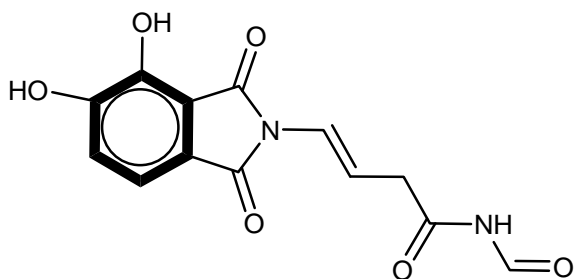
Alert ID 112: c:c:c:c:cH
Statistical significance = 95%
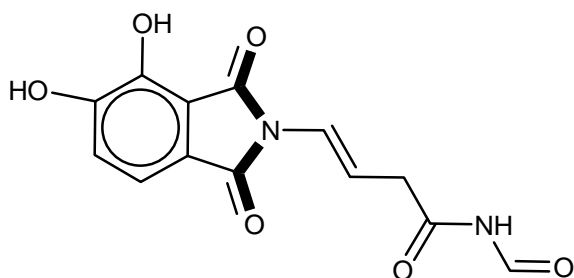
Alert ID 175: O=C2-N3-C2=O
Statistical significance = 75%
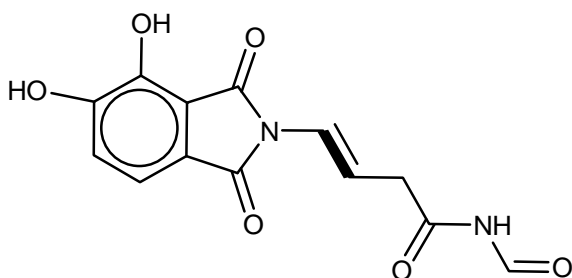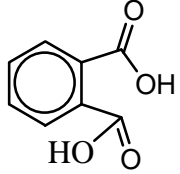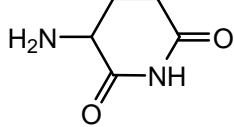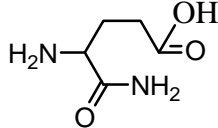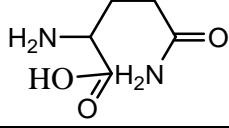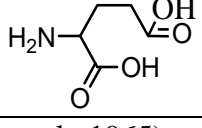
Alert ID 214: C2H-C2H
Statistical significance = 50%

121
122
123 **Figure S 3** The structural moiety responsible for the positive alert in PTP 291_11 by Microtox
124 Toxicity to Environmental Bacteria model using Case Ultra software. Alert ID number is
125 provided by the software database. The structural moieties responsible for the alerts are presented
126 in bold.

127   **Table S 1** The structures of the previously reported HTPs*

Name	Structure	Chemical Formula	Smiles
Thalidomide		$C_{13}H_{10}N_2O_4$	O=C(N2C3CCC(NC3=O)=O)c1ccccc1C2=O
HTP_1		$C_{13}H_{12}N_2O_5$	O=C(N2C(C(N)=O)CCC(O)=O)c1ccccc1C2=O
HTP_2		$C_{13}H_{12}N_2O_5$	OC(C(CCC(N)=O)N2C(c1ccccc1C2=O)=O)=O
HTP_3		$C_{13}H_{12}N_2O_5$	O=C(O)c1ccccc1C(NC2CCC(NC2=O)=O)=O
HTP_4		$C_{13}H_{11}NO_6$	O=C(N2C(C(O)=O)CCC(O)=O)c1ccccc1C2=O
HTP_5		$C_{13}H_{14}N_2O_6$	OC(CCC(C(N)=O)NC(c1c(C(O)=O)cccc1)=O)=O
HTP_6		$C_{13}H_{14}N_2O_6$	OC(c1ccccc1C(NC(C(O)=O)CCC(N)=O)=O)=O
HTP_7		$C_{13}H_{13}NO_7$	OC(c1ccccc1C(NC(C(O)=O)CCC(O)=O)=O)=O

HTP_8		$C_8H_6O_4$	O=C(O)c1ccccc1C(O)=O
HTP_9		$C_5H_8N_2O_2$	NC1CCC(NC1=O)=O
HTP_10		$C_5H_{10}N_2O_3$	OC(CCC(C(N)=O)N)=O
HTP_11		$C_5H_{10}N_2O_3$	OC(C(CCC(N)=O)N)=O
HTP_12		$C_5H_9NO_4$	NC(C(O)=O)CCC(O)=O

128   *(Schumacher et al., 1965).

129

130   **Table S 2** The postulated structures of the identified PTPs
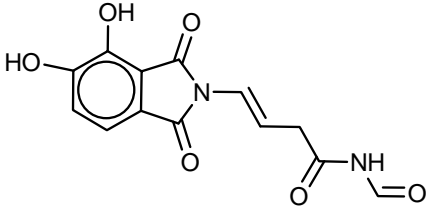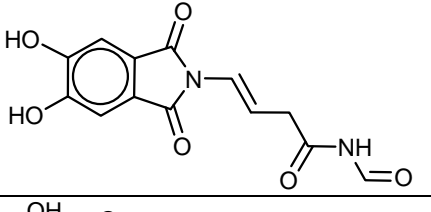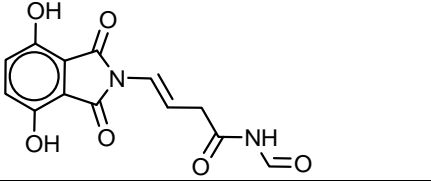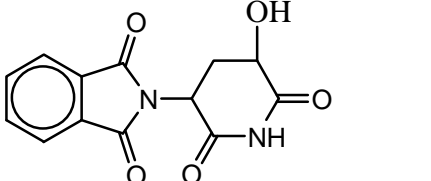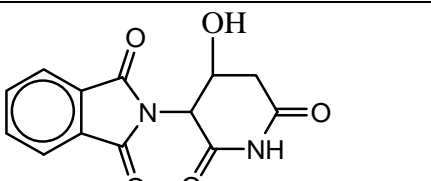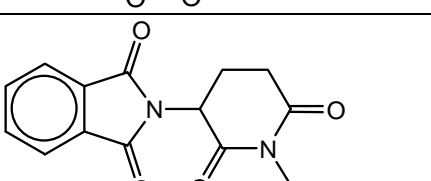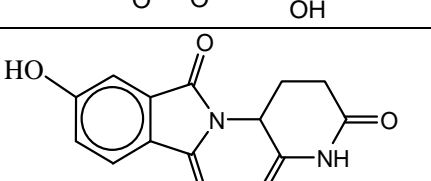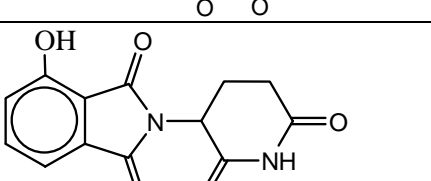
Name	Structure	Molecular Formula	Smiles
Thalidomide		$C_{13}H_{10}N_2O_4$	O=C(N2C3CCC(NC3=O)=O)c1ccccc1C2=O
PTP129_1		$C_5H_8N_2O_2$	NC1CCC(NC1=O)=O
PTP 173_1		$C_6H_8N_2O_4$	NC(/C(CCC(O)=O)=N/C=O)=O
PTP 173_2		$C_6H_8N_2O_4$	OC(/C(CCC(N)=O)=N/C=O)=O
PTP 173_3		$C_6H_8N_2O_4$	OC(NC1CCC(NC1=O)=O)=O
PTP277_1		$C_{13}H_{12}N_2O_5$	O=CNC(C(CCO)N(C(C2=C1C=CC=C2)=O)C1=O)=O
PTP277_2		$C_{13}H_{12}N_2O_5$	O=C(N2CC(O)CC(NC=O)=O)c1ccccc1C2=O
PTP297_1		$C_{13}H_{16}N_2O_6$	NC(C(CCC(O)=O)NC(O)C1=C(C(O)=O)C=CC=C1)=O
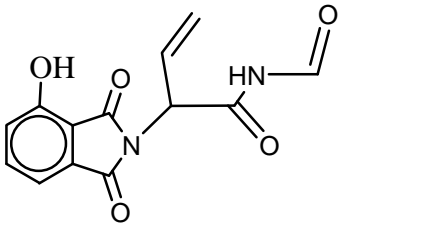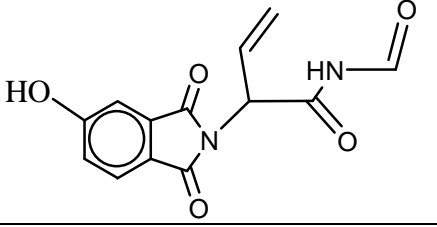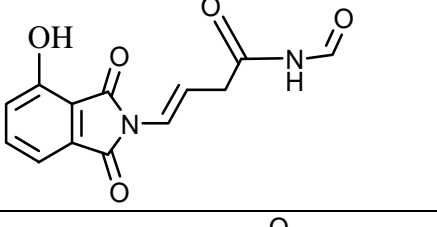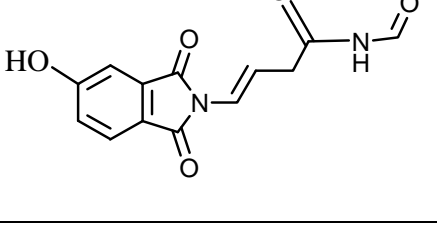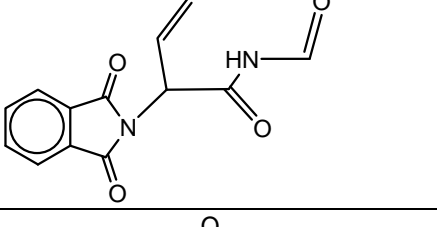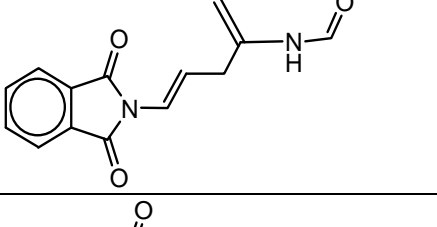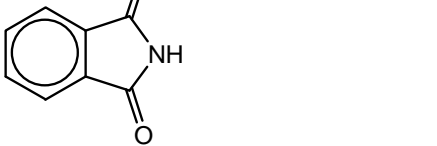
PTP297_2		$C_{13}H_{16}N_2O_6$	OC(C(CCC(N)=O)NC(O)C1=C(C(O)=O)C=CC=C1)=O
PTP313_1		$C_{13}H_{16}N_2O_7$	NC(C(CCC(O)=O)NC(O)C1=C(C(O)=O)C(O)=CC=C1)=O
PTP313_2		$C_{13}H_{16}N_2O_7$	NC(C(CCC(O)=O)NC(O)C1=C(C(O)=O)C=C(O)C=C1)=O
PTP313_3		$C_{13}H_{16}N_2O_7$	NC(C(CCC(O)=O)NC(O)C1=C(C(O)=O)C=CC(O)=C1)=O
PTP313_4		$C_{13}H_{16}N_2O_7$	NC(C(CCC(O)=O)NC(O)C1=C(C(O)=O)C=CC=C1O)=O
PTP313_5		$C_{13}H_{16}N_2O_7$	NC(C(CCC(O)=O)N(O)C(O)C1=C(C(O)=O)C=CC=C1)=O

PTP313_6		$C_{13}H_{16}N_2O_7$	OC(C(CCC(N)=O)NC(O)C1=C(C(O)=O)C(O)=CC=C1)=O
PTP313_7		$C_{13}H_{16}N_2O_7$	OC(C(CCC(N)=O)NC(O)C1=C(C(O)=O)C=C(O)C=C1)=O
PTP313_8		$C_{13}H_{16}N_2O_7$	OC(C(CCC(N)=O)NC(O)C1=C(C(O)=O)C=CC(O)=C1)=O
PTP313_9		$C_{13}H_{16}N_2O_7$	OC(C(CCC(N)=O)NC(O)C1=C(C(O)=O)C=CC=C1O)=O
PTP313_10		$C_{13}H_{16}N_2O_7$	OC(C(CCC(N)=O)N(O)C(O)C1=C(C(O)=O)C=CC=C1)=O
PTP291_1		$C_{13}H_{10}N_2O_6$	O=C(N2C3CC(O)C(NC3=O)=O)c1cc(O)ccc1C2=O
PTP291_2		$C_{13}H_{10}N_2O_6$	O=C(N2C3CCC(NC3=O)=O)c1c(O)c(O)ccc1C2=O

PTP291_3		$C_{13}H_{10}N_2O_6$	O=C(N2C3CCC(NC3=O)=O)c1c(O)ccc(O)c1C2=O
PTP291_4		$C_{13}H_{10}N_2O_6$	O=C(N2C3CCC(NC3=O)=O)c1cc(O)c(O)cc1C2=O
PTP291_5		$C_{13}H_{10}N_2O_6$	O=C(N2C3CCC(NC3=O)=O)c1c(O)cc(O)cc1C2=O
PTP291_6		$C_{13}H_{10}N_2O_6$	O=C(N2C(C=C)C(NC=O)=O)c1cc(O)cc(O)c1C2=O
PTP291_7		$C_{13}H_{10}N_2O_6$	O=C(N2C(C=C)C(NC=O)=O)c1cc(O)c(O)cc1C2=O
PTP291_8		$C_{13}H_{10}N_2O_6$	O=C(N2C(C=C)C(NC=O)=O)c1ccc(O)c(O)c1C2=O
PTP291_9		$C_{13}H_{10}N_2O_6$	O=C(N2C(C=C)C(NC=O)=O)c1c(O)ccc(O)c1C2=O
PTP291_10		$C_{13}H_{10}N_2O_6$	O=C(c1c(C2=O)c(O)cc(O)c1)N2/C=C/CC(NC=O)=O

PTP291_11		C$_{13}$H$_{10}$N$_2$O$_6$	O=C(c1c(C2=O)c(O)c(O)cc1)N2/C=C/CC(NC=O)=O
PTP291_12		C$_{13}$H$_{10}$N$_2$O$_6$	O=C(c1c(C2=O)cc(O)c(O)c1)N2/C=C/CC(NC=O)=O
PTP291_13		C$_{13}$H$_{10}$N$_2$O$_6$	O=C(c1c(C2=O)c(O)ccc1O)N2/C=C/CC(NC=O)=O
PTP275_1		C$_{13}$H$_{10}$N$_2$O$_5$	O=C(N2C3CC(O)C(NC3=O)=O)c1ccccc1C2=O
PTP275_2		C$_{13}$H$_{10}$N$_2$O$_5$	O=C(N2C3C(O)CC(NC3=O)=O)c1ccccc1C2=O
PTP275_3		C$_{13}$H$_{10}$N$_2$O$_5$	O=C(N2C3CCC(N(O)C3=O)=O)c1ccccc1C2=O
PTP275_4		C$_{13}$H$_{10}$N$_2$O$_5$	O=C(N2C3CCC(NC3=O)=O)c1ccc(O)cc1C2=O
PTP275_5		C$_{13}$H$_{10}$N$_2$O$_5$	O=C(N2C3CCC(NC3=O)=O)c1cccc(O)c1C2=O

PTP275_6		$C_{13}H_{10}N_2O_5$	O=C(N2C(C=C)C(NC=O)=O)c1cccc(O)c1C2=O
PTP275_7		$C_{13}H_{10}N_2O_5$	O=C(N2C(C=C)C(NC=O)=O)c1ccc(O)cc1C2=O
PTP275_8		$C_{13}H_{10}N_2O_5$	O=C(N(/C=C/CC(NC=O)=O)C2=O)C1=C2C(O)=CC=C1
PTP275_9		$C_{13}H_{10}N_2O_5$	O=C(N(/C=C/CC(NC=O)=O)C2=O)C1=C2C=C(O)C=C1
PTP259_1		$C_{13}H_{10}N_2O_4$	O=C(N2C(C=C)C(NC=O)=O)c1ccccc1C2=O
PTP259_1		$C_{13}H_{10}N_2O_4$	O=C(N2/C=C/CC(NC=O)=O)c1ccccc1C2=O
PTP148 (phthalimide)		$C_8H_5NO_2$	O=C(N2)c1ccccc1C2=O

131
132

133 **Table S 3**. Predicted mutagenic activity of TD and its HTPs calculated with Salmonella t. 5-
134 strains (A7B, Case Ultra), Mutagenicity Ames (A2H, Case Ultra), Salmonella Catalogic (SC,
135 Oasis Catalogic), and Bacterial mutagenesis (BM, Leadscope).

Compounds	QSAR models*			
	A7B	A2H	SC	BM
Thalidomide	IN(O)	-	-	-
HTP1	IN(O)	-	-	-
HTP2	IN(O)	-	-	-
HTP3	-	-	-	-
HTP4	IN(O)	-	-	-
HTP5	-	-	-	-
HTP6	-	-	-	-
HTP7	-	-	-	-
HTP8	-	-	-	-
HTP9	-	-	-	-
HTP10	-	-	-	-
HTP11	-	-	-	-
HTP12	-	-	-	-

136 * negative (-), inconclusive orange (IN(O)).
137

Table S 4. Predicted QSAR toxicity of TD and its HTPs calculated with the following QSAR modules: Human Carcinogenicity (A0J), Aneuploidy in Yeast (A6A), Micronucleus Formation in vivo composite (A7S), Micronucleus Formation in vivo Mouse (A7T), Chromosome Aberrations in vitro composite (A7U), Chromosome Aberrations in vitro CHO cells (A7V), Rat Carcinogenicity (A0D), Mouse Lymphoma (ML), Mouse Carcinogenicity (A08), UDS Induction (A64), In vitro chromosome aberration (IVCA), Mammalian mutagenesis (MM), In vivo micronucleus (IVMN), Bioconcentration for Cyprinus Carpio (BCF), Gold Fish Toxicity (AUG), and Rainbow Trout Toxicity (AUE).

	QSAR genotoxicity and mutagenicity modules													QSAR ecotoxicity modules		
	AOJ	A6A	A7S	A7T	A7U	A7V	A0D	ML	A08	A64	IVCA	MM	IVMN	BCF	AUG	AUE
Thalidomide	-	IN	IN(O)	IN(O)	-	-	-	+	-	IN(O)	OD	+	OD	+	+	-
HTP1	-	IN	+	IN(O)	-	-	-	IN(O)	OD	OD	OD	-	OD	+	IN	-
HTP2	-	IN	+	IN(O)	-	-	-	IN(O)	OD	OD	-	-	+	IN	IN	-
HTP3	+	+	IN(O)	IN(O)	-	-	IN(O)	IN(O)	-	IN(O)	+	+	-	+	OD	OD
HTP4	-	+	+	IN(O)	-	-	-	IN(O)	-	OD	-	-	+	+	IN	-
HTP5	IN(O)	+	+	+	-	-	IN(O)	IN(O)	OD	-	-	-	+	+	OD	OD
HTP6	IN(O)	+	+	+	-	-	IN(O)	IN(O)	OD	-	-	-	+	+	OD	OD
HTP7	IN(O)	+	+	+	-	-	IN(O)	IN(O)	-	-	-	-	+	+	OD	OD
HTP8	-	+	-	-	-	-	-	+	-	-	-	-	-	+	OD	-
HTP9	-	+	IN(O)	IN(O)	-	-	-	+	-	IN	+	OD	+	+	OD	OD
HTP10	-	+	+	+	-	-	-	-	-	-	-	-	-	+	OD	OD
HTP11	-	+	+	+	-	-	-	-	-	-	-	-	-	IN	OD	OD
HTP12	-	+	+	+	-	-	-	-	-	-	-	-	-	+	OD	OD

*Positive (+), negative (-), inconclusive (IN), inconclusive orange (IN(O)), out of domain (OD)

Table S 5. Predicted bacterial toxicity of TD and its HTPs calculated with four acute toxicity models for *Vibrio fischeri* [Microtox Toxicity to Environmental Bacteria (AUA, Case Ultra) and three acute toxicity *Vibrio fischeri* models (Oasis Catalogic)]

Compounds	QSAR Models			
	AUA*	Acute tox 5min**	Acute tox 15min**	Acute tox 30min**
Thalidomide	+	4772	8452	22076
HTP1	IN(O)	5888	10655	28370
HTP2	+	5638	10384	27387
HTP3	-	23243	45640	149035
HTP4	IN(O)	**1610**	**2653**	**5838**
HTP5	-	29239	58092	194387
HTP6	-	29626	58545	196472
HTP7	-	7874	14337	39511
HTP8	-	**259**	**366**	**670**
HTP9	-	92675	190620	867736
HTP10	-	133202	269951	1277380
HTP11	-	14338732	42238272	3,95E+08
HTP12	-	3895611	10493339	80871480

*Positive (+), negative (-), inconclusive orange (IN(O)).
**PTPs with lower IC 50 (mg/L) than Thalidomide are presented in colored bold (orange is marginally lower and red is strongly lower).

Table S 6. Predicted mutagenic activity of TD and its PTPs calculated with Salmonella t. 5-strains (A7B, Case Ultra), Mutagenicity Ames (A2H, Case Ultra), Salmonella Catalogic (SC, Oasis Catalogic), and Bacterial mutagenesis (BM, Leadscope).

Compounds	QSAR models*			
	A7B	A2H	SC	BM
Thalidomide	IN(O)	-	-	-
PTP129_1	-	-	-	-
PTP 173_1	-	-	-	-
PTP 173_2	-	-	-	-
PTP 173_3	-	-	-	-
PTP277_1	IN(O)	-	-	-
PTP277_2	IN(O)	-	-	-
PTP297_1	-	-	-	OD
PTP297_2	-	-	-	-
PTP313_1	-	-	-	-
PTP313_2	-	-	-	-
PTP313_3	-	-	-	-
PTP313_4	+ (M)	IN(O)	-	-
PTP313_5	-	-	-	-
PTP313_6	-	-	-	-
PTP313_7	-	-	-	-
PTP313_8	-	-	-	-
PTP313_9	+ (M)	IN(O)	-	-
PTP313_10	-	-	-	-
PTP291_1	IN(O)	-	-	-
PTP291_2	-	IN(O)	-	-
PTP291_3	-	IN(O)	-	-
PTP291_4	-	-	-	-
PTP291_5	-	-	-	-
PTP291_6	-	-	-	-
PTP291_7	-	-	-	-
PTP291_8	-	IN(O)	-	-
PTP291_9	-	IN(O)	-	+
PTP291_10	-	-	-	-
PTP291_11	-	IN(O)	-	-
PTP291_12	-	-	-	-
PTP291_13	-	IN(O)	-	-
PTP275_1	IN(O)	-	-	-
PTP275_2	IN(O)	-	-	-
PTP275_3	IN(O)	-	-	-
PTP275_4	IN(O)	-	-	-
PTP275_5	-	IN(O)	-	-
PTP275_6	-	IN(O)	-	-
PTP275_7	IN(O)	-	-	-
PTP275_8	-	IN	-	-
PTP275_9	+	-	-	-
PTP148	+	-	-	-
PTP259_1	IN(O)	-	-	-
PTP259_2	+	-	-	-

*Positive (+), marginally positive (+(M)), negative (-), inconclusive (IN), inconclusive orange (IN(O)), out of domain (OD)

Table S 7. Predicted QSAR toxicity of TD and its PTPs calculated with the following QSAR modules: Human Carcinogenicity (A0J), Aneuploidy in Yeast (A6A), Micronucleus Formation in vivo composite (A7S), Micronucleus Formation in vivo Mouse (A7T), Chromosome Aberrations in vitro composite (A7U), Chromosome Aberrations in vitro CHO cells (A7V), Rat Carcinogenicity (A0D), Mouse Lymphoma (ML), Mouse Carcinogenicity (A08), UDS Induction (A64), Invitro chromosome aberration (IVCA), Mammalian mutagenesis (MM), In vivo micronucleus (IVMN), Bioconcentrationfor Cyprinus Carpio (BCF), Gold Fish Toxicity (AUG), and Rainbow Trout Toxicity (AUE).

	QSAR genotoxicity and mutagenicity													QSAR ecotoxicity models		
	AOJ	A6A	A7S	A7T	A7U	A7V	A0D	ML	A08	A64	IVCA	MM	IVMN	BCF	AUG	AUE
Thalidomide	-	IN	IN(O)	IN(O)	-	-	-	+	-	IN(O)	OD	+	OD	+	+	-
PTP129_1	-	+	IN(O)	IN(O)	-	-	-	+	-	IN(O)	+	OD	+	+	OD	OD
PTP 173_1	IN	IN	+	+	-	OD	OD	-	OD	OD	OD	-	OD	IN	OD	IN
PTP 173_2	OD	IN	IN(O)	IN(O)	-	OD	OD	-	OD	OD	OD	OD	OD	IN	OD	IN
PTP 173_3	+	+	IN(O)	IN(O)	-	-	-	IN(O)	OD	IN(O)	+	+	OD	+	OD	OD
PTP277_1	-	IN	-	-	-	-	-	+	OD	OD	+	+	+	IN	IN	OD
PTP277_2	-	IN	-	+	+(M)	-	IN	+	OD	OD	OD	-	OD	+	IN	OD
PTP297_1	OD	IN	+	IN(O)	-	-	OD	IN(O)	OD	OD	OD	OD	OD	IN	OD	IN
PTP297_2	OD	IN	+	IN(O)	-	-	-	IN	OD	OD	-	-	-	IN	OD	IN
PTP313_1	IN	IN	+	IN(O)	-	+	OD	IN(O)	OD	OD	-	-	-	IN	OD	IN
PTP313_2	OD	IN	+	IN(O)	IN(O)	IN(O)	OD	IN(O)	IN	OD	-	-	-	IN	OD	IN
PTP313_3	OD	IN	+	+	-	+(M)	OD	IN(O)	OD	OD	-	-	-	IN	OD	IN
PTP313_4	IN	IN	+	+	-	+	OD	-	OD	OD	-	-	-	IN	OD	OD
PTP313_5	OD	IN	+	IN(O)	OD	OD	OD	IN(O)	OD	OD	OD	+	-	IN	OD	IN
PTP313_6	IN	IN	+	IN(O)	-	+	-	IN(O)	OD	OD	-	-	+	IN	OD	IN
PTP313_7	OD	IN	+	IN(O)	IN(O)	IN(O)	-	IN(O)	IN	OD	-	-	-	IN	OD	IN
PTP313_8	OD	IN	+	+	-	+(M)	-	IN(O)	OD	OD	-	OD	+	IN	OD	IN
PTP313_9	IN	IN	+	+	-	+	-	-	OD	OD	-	-	+	IN	OD	OD
PTP313_10	OD	IN	+	IN(O)	OD	OD	OD	IN(O)	OD	OD	OD	OD	OD	IN	OD	IN
PTP291_1	-	OD	+	IN(O)	-	IN(O)	-	IN(O)	+	OD	+	+	+	IN	IN	-
PTP291_2	+(M)	IN	IN(O)	IN(O)	-	+	IN(O)	+	-	IN(O)	+	+	-	+	+	-
PTP291_3	+(M)	IN	IN(O)	IN(O)	+	+	IN(O)	+	-	IN(O)	+	+	+	+	+	-
PTP291_4	-	IN	IN(O)	IN(O)	-	-	-	+	+	IN(O)	+	+	-	+	+	-
PTP291_5	-	IN	IN(O)	IN(O)	+	+	-	+	+	IN(O)	+	+	+	+	+	-
PTP291_6	-	OD	+	IN(O)	+	+	-	IN(O)	IN	OD	OD	+	OD	IN	IN	OD

Continue **Table S 7**

	QSAR genotoxicity and mutagenicity													QSAR ecotoxicity models		
	AOJ	A6A	A7S	A7T	A7U	A7V	A0D	ML	A08	A64	IVCA	MM	IVMN	BCF	AUG	AUE
PTP291_7	-	OD	+	IN(O)	-	-	-	IN(O)	IN	IN	OD	+	OD	IN(O)	IN	OD
PTP291_8	+(M)	OD	+	IN(O)	-	+	IN(O)	IN(O)	OD	OD	OD	+	OD	IN	IN	OD
PTP291_9	+(M)	OD	+	IN(O)	+	+	IN(O)	IN(O)	OD	OD	+	+	+	IN	IN	OD
PTP291_10	-	OD	+	+	+	+	OD	+	IN	OD	-	+	+	IN	IN	OD
PTP291_11	+(M)	OD	+	+	-	+	+	+	IN	OD	+	+	+	IN	IN	OD
PTP291_12	-	OD	-	+	-	-	OD	+	IN	IN	+	+	+	IN	IN	OD
PTP291_13	+(M)	OD	-	+	+	+	+	+	IN	OD	+	+	+	IN	IN	OD
PTP275_1	-	IN	+	+(M)	-	-	-	IN(O)	-	OD	+	+	+	IN	IN	-
PTP275_2	-	IN	+	IN(O)	-	-	-	+	-	OD	OD	+	OD	IN	IN	-
PTP275_3	-	IN	-	-	-	-	-	+	OD	+	OD	+	OD	IN	+	-
PTP275_4	-	IN	IN(O)	IN(O)	-	IN(O)	-	+	+	IN(O)	+	+	+	+	+	-
PTP275_5	+(M)	IN	IN(O)	IN(O)	-	+	-	+	-	IN(O)	+	+	+	+	+	-
PTP275_6	+(M)	IN	+	IN(O)	-	+	-	IN(O)	OD	OD	+	+	+	IN	IN	OD
PTP275_7	-	OD	+	IN(O)	-	IN(O)	-	IN(O)	IN	OD	OD	+	OD	IN	IN	OD
PTP275_8	+(M)	IN	-	+	-	+	OD	+	IN	OD	-	+	+	IN	IN	OD
PTP275_9	-	OD	-	+	-	IN(O)	OD	+	IN	OD	-	+	+	IN	IN	OD
PTP148	-	+	+	+	-	-	+	IN(O)	-	-	+	+	+	+	-	-
PTP259_1	-	IN	+	IN(O)	-	-	-	IN(O)	OD	OD	OD	+	OD	IN	IN	OD
PTP259_2	-	IN	-	+	-	-	OD	+	IN	OD	OD	+	OD	IN	IN	OD

*Positive (+), marginally positive (+(M)), negative (-), inconclusive (IN), inconclusive orange (IN(O)), out of domain (OD)

Table S 8. Predicted bacterial toxicity of TD and its PTPs calculated with four acute toxicity models for *Vibrio fischeri* [Microtox Toxicity to Environmental Bacteria (AUA, Case Ultra) and three acute toxicity vibrio *fischeri* models (Oasis Catalogic)]

Compounds	QSAR Models			
	AUA*	Acute tox 5min**	Acute tox 15min**	Acute tox 30min**
Thalidomide	+	**4772**	**8452**	**22076**
PTP129_1	-	92675	190620	867726
PTP 173_1	OD	32582	65946	243584
PTP 173_2	OD	30131	62966	228602
PTP 173_3	OD	1121726	2835106	17687038
PTP277_1	+	61705	135798	508310
PTP277_2	+	62610	136972	514351
PTP297_1	OD	143405	319377	1348079
PTP297_2	OD	2227087	6199375	38976000
PTP313_1	OD	165682	370706	1586430
PTP313_2	OD	375217	906459	4361442
PTP313_3	OD	369000	897552	4302719
PTP313_4	OD	383116	917690	4435801
PTP313_5	OD	711124	1753250	9301782
PTP313_6	OD	2622604	7277318	46582816
PTP313_7	OD	5684938	17340104	123596776
PTP313_8	OD	5797778	17542722	125583680
PTP313_9	OD	6180744	18218648	132273640
PTP313_10	OD	674733	1699652	8913655
PTP291_1	+	258017	629915	2885552
PTP291_2	IN	7429	13692	37419
PTP291_3	IN	**1655**	**2708**	**5948**
PTP291_4	+	33939	69973	238877
PTP291_5	+	7461	13728	37555
PTP291_6	+	7065	13064	35406
PTP291_7	+	32233	66704	225745
PTP291_8	IN	7053	13051	35354
PTP291_9	IN	**1548**	**2559**	**5553**
PTP291_10	+	7067	13130	35559
PTP291_11	-	7176	13248	36000
PTP291_12	+	32144	66917	226158
PTP291_13	-	**1572**	**2594**	**5643**
PTP275_1	+	97158	219631	880525
PTP275_2	+	14066	27275	82536
PTP275_3	+	47002	100010	360895
PTP275_4	+	12743	24356	72723
PTP275_5	+	**2797**	**4774**	**11419**
PTP275_6	+	**2665**	**4560**	**10820**
PTP275_7	+	12108	23224	68751
PTP275_8	+	**2679**	**4596**	**10909**
PTP275_9	+	12139	23371	69171
PTP148 (phthalimide)	-	**146**	**200**	**343**
PTP259_1	+	**4541**	**8067**	**20896**
PTP259_2	+	**4516**	**8079**	**20888**

*Positive (+), negative (-), inconclusive (IN), out of domain (OD)

**PTPs with lower IC 50 (mg/L) than Thalidomide are presented in colored bold. (orange is marginally lower and red is strongly lower).

Appendix 3

Paper III

Anju Priya Toolaram, Tarek Haddad, Christoph Leder, Klaus Kümmerer <u>Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a combination of experimental and *in-silico* testing</u>, *(submitted)*

1 **Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a**

2 **combination of experimental and *in-silico* testing**

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22   **Abstract**

23   Ciprofloxacin (CIP) is a broad-spectrum antibiotic that can be found in the environment in µg/L

24   concentration. Its photolysis results in many transformation products of mostly unknown

25   toxicity.

26   In this study, CIP was subjected to UV photolysis and the transformation products (TPs) formed

27   were identified. Quantitative Structure-Activity Relationships (QSARs) were used to predict

28   selected genotoxicity endpoints of the TPs. Further, CIP and its irradiated mixtures were

29   assessed in a battery of genotoxicity and cytotoxicity *in vitro* assays. The combination index

30   (CI) analysis of residual CIP in the irradiated mixtures was performed for the umu assay.

31   CIP achieved primary elimination after 128 min of irradiation but was not completely

32   mineralized. Nine photo-TPs of CIP were identified. The irradiated mixtures of TPs and CIP

33   were neither mutagenic in the Ames test nor genotoxic in the *in vitro* micronucleus (MN) test.

34   The irradiated mixtures were *umuC* inducing but genotoxicity decreased with increasing

35   irradiation time. The CI analysis revealed that the irradiated mixtures and the corresponding

36   CIP concentration in the mixtures shared similar *umuC* potentials. QSAR predictions suggested

37   that the TPs may be capable of inducing chromosome aberration and mammalian mutation.

38   Unlike CIP, some TPs were predicted to cause bacterial mutation and MN *in vivo*. However,

39   the experimental testing for a few genotoxic endpoints did not show significant genotoxic

40   activity for the TPs present as a component of the whole mixture analysis when compared to

41   the genotoxicity of CIP itself. Therefore, it may be possible that the genotoxicity of

42   ciprofloxacin-enriched water can be reduced by degrading the parent compound. However,

43   more genotoxic endpoints need to be investigated to fully confirm this.

44

1.0 Introduction

Ciprofloxacin (CIP) is a broad-spectrum second-generation fluoroquinolone (FQ) antimicrobial drug found in µg/L concentration range in the aquatic environment (Hartmann et al., 1998; Hartmann et al., 1999; Martins et al., 2008). The mode of action (MOA) of CIP involves the binding of the quinolone moiety to the bacterial deoxyribonucleic acid (DNA) gyrase, leading to the stabilization of the cleavable complex (Albertini et al., 1995; Clerch et al., 1996). The cleavable complex is necessary to control DNA-topology for DNA-replication and cell multiplication. The stabilization of the cleavable complex prevents the enzyme turnover thereby inhibiting the resealing of DNA strand breaks (Albertini et al., 1995; Clerch et al., 1996). Clerch et al. (1992), proposed that the formation of intra- and inter- strand adducts could arrest DNA replication and induce the SOS system as well as produced lesions that induces excision repair. Hence, mutagenicity of quinolones in the Ames test strains required a functional uvrB gene for excision repair and a pKM101 plasmid which has the mucAB genes analogous to the *umuDC* operon of *E.coli* that allows for error prone DNA repair (Albertini et al., 1995; Clerch et al., 1996). The induction of the SOS repair response system may enhance bacterial survival and could eventually lead to antimicrobial resistance (Cirz et al., 2005; Dörr et al., 2009). CIP has been reported to induce the SOS repair response system at concentration as low as 0.005 mg/L (Hartmann et al., 1998; Power and Phillips, 1993). Further, Hartmann et al. (1998) found that the main source of *umuC* genotoxicity in their hospital wastewater came from the presence of CIP.

Quinolones bind differently to eukaryotic topoisomerase II mainly because of the difference in structural DNA and therefore the genotoxic potential is lower in eukaryotic organisms than prokaryotic organisms (Shen et al., 1989; Clerch et al., 1992; Albertini et al., 1995). The MOA of CIP in eukaryotic organisms is believed to be the same as in bacteria with stabilization of the cleavable complex leading to DNA strand breaks (Lynch et al., 2003). If these DNA strand

4

89 breaks are not repaired, they can lead to clastogenicity and/or cytotoxicity (Lynch et al., 2003).

90 Curry et al. (1996) suggested that the inhibition of the enzyme can result in incomplete

91 chromosome separation and chromosome 'stickiness'. Although the stabilization of the

92 cleavable complex plays an important role in detecting DNA damage at all stages of cell cycle,

93 it is likely that other quinolone-topoisomerase II mediated mechanisms maybe responsible for

94 genotoxicity of FQs (Curry et al., 1996). Evidence of DNA damage using *in vitro* assays showed

95 micronucleus (MN) formation, chromosome aberration (CA), unscheduled DNA synthesis,

96 induction of HPRT mutation cells and thymidine kinase (TK) mutation (Bredberg et al., 1989;

97 Albertini et al., 1995; Chételat et al., 1996; Curry et al., 1996; Gibson et al., 1998; Lynch et al.,

98 2003; Garcia-Käufer et al., 2012).

99 Environmental monitoring of FQs in a study of the Glatt Valley in Switzerland showed that

100 their concentrations were significantly reduced from raw sewage concentration to wastewater

101 effluent and drastically reduced downstream in the Glatt River (Giger et al., 2003). The lower

102 concentration of FQs found in the sewage effluent was attributed to the sorption process in the

103 wastewater treatment plant (WWTP) (Giger et al., 2003). Further improvement of FQ removal

104 from wastewater e.g. by membrane filtration could require WWTP to have longer hydraulic

105 retention time and this may actually contribute to the establishment of resistant species in the

106 sewage effluent (Manaia et al., 2010). Generally, the conditions in WWTPs could promote

107 horizontal gene transfer processes that can foster the passage of plasmids and transpose

108 encoding antibiotic resistance (Manaia et al., 2010). In fact, Manaia et al. (2010) found that an

109 estimated 1-5% of the total enterobacteria species were CIP resistant in the treated wastewater

110 from domestic WWTPs. Therefore, it is necessary to investigate possible methods of reducing

111 the CIP concentration and toxicity prior to its introduction into WWTP.

112 In the aquatic environment, the fate of CIP is governed by several mechanisms such as

113 photodegradation, adsorption and biotransformation (Cardoza et al., 2005). CIP was reported

5

114 as not readily biodegradable and therefore this is not expected to be the major removal pathway

115 (Al-Ahmad et al., 1999; Kümmerer et al., 2000). Photodegradation can be a possible method of

116 removal of CIP prior to and upon its release to the environment. Several authors have shown

117 that CIP can be photodegraded both with ultraviolet (UV) and simulated sunlight (Xenon lamp)

118 often leading to the formation of transformation products (TPs) with structures that retained the

119 core quinolone molecule but had alterations, substitutions and/or deletion of its substituents

120 (Chételat et al., 1996; Sánchez et al., 2005; Vasconcelos et al., 2009; Paul et al., 2010; Garcia-

121 Käufer et al., 2012; Haddad and Kümmerer 2014). Several *in vitro* genotoxicity assays have

122 shown that the irradiated mixtures containing TPs and CIP may be mutagenic in the Ames test

123 and genotoxic to several cell lines including mouse lymphoma, human hepatic carcinoma cells

124 (HepG2) and human T lymphocyte cells (Jurkat cells) (Chételat et al., 1996; Sánchez et al.,

125 2005; Garcia-Käufer et al., 2012). However, Paul et al. (2010) showed that UV irradiation can

126 attenuate the cytotoxicity of CIP. Even though CIP is a known *umuC* inducer, none of these

127 studies have monitored the changes in genotoxicity of treated CIP using this test. However,

128 quantitative structure activity relationships (QSAR) predictions have shown that some TPs may

129 be capable of inducing the *umuC* gene at lower concentrations than CIP (Li et al., 2014).

130 Therefore in this study we monitored the genotoxicity and cytotoxicity of CIP and its mixture

131 of TPs after UV irradiation as both a whole mixture analysis using a battery of genotoxicity

132 assays and an individual TP analysis combined with *in silico* predictions using QSAR models.

133 Hence, the aim of the study was to determine the genotoxicity of the UV mixtures and to

134 understand the possible genotoxic role of the stable TPs.

135 **2.0 Materials and Methods**

136 **2.1 Photodegradation and mineralisation monitoring**

137   Photodegradation of CIP (CAS RN: 85721-33-1; from Sigma–Aldrich) was performed in a 1L

138   immersion-type reactor (UV-Consulting Peschl) using a 150 W medium-pressure mercury lamp

139   (TQ 150, UV-Consulting Peschl). The reactor was filled with CIP dissolved as 20 mg/L in

140   Millipore water (pH 7) and the irradiation was performed for 128 minutes. The initial CIP

141   concentration was selected based on the detection limits for non-purgeable organic carbon

142   (NPOC) measurements using a Shimadzu TOC-5000 analyzer (Haddad and Kümmerer, 2014).

143   Further information on experimental-setup can be found elsewhere (Haddad and Kümmerer,

144   2014). For analytical and toxicological analyses, aliquots of irradiated samples were collected

145   at several time intervals.

146   **2.2 Liquid chromatography analysis**

147   Detection, identification and quantification, of CIP and its TPs were performed on Agilent

148   Technologies 1100 HPLC series connected to a mass spectrometer Bruker Daltonics Esquire

149   6000 [Plus], which is equipped with an atmospheric pressure electrospray ionization (AP-ESI)

150   source. Chromatographic Separation was performed on a RP18 EC 125 mm x 4 mm, 5µm

151   Nucleodur reverse phase column (Macherey-Nagel). Additionally, the accurate masses of CIP

152   and its TPs were measured by LTQ-Orbitrap XL mass spectrometer interfaced with a heated

153   electrospray ionization (H-ESI) source (Thermo Scientific). All used LC instruments,

154   chromatographic parameters and mass spectrometer settings have been detailed elsewhere

155   (Haddad and Kümmerer, 2014).

156   **2.3 QSAR Predictions**

157   Structure illustrations were performed with MarvinSketch 5.8.0. using simplified molecular

158   input line entry specification (SMILES) codes. These SMILES codes allowed the introduction

159   of molecular formula into various computer based QSAR models for predicting the effects on

160   a number of genotoxicity endpoints.

161 *In silico* toxicity predictions of CIP and its TPs were performed using a set of QSAR software

162 each with different algorithms and training sets. The software used included Case Ultra

163 V.1.4.6.6 (MultiCASE Inc.) (Saiakhov et al., 2013), and Leadscope software V.3.0.11-1 with

164 training sets from 2012 SAR Genetox Database (Leadscope) (Roberts et al., 2000). Also Oasis

165 Catalogic software predicted mutagenicity based on bacterial mutagenicity (module

166 mutagenicity v.04) in *S. typhimurium* (*Salmonella* Catalogic model, SC) from Laboratory of

167 Mathematical Chemistry, University Bourgas, Bulgaria. For the endpoint "bacterial

168 mutagenicity", a combination of statistical (Case Ultra and Leadscope) and rule-based (Oasis

169 Catalogic) models was applied according to suggestions of the ICH guidelines M7 (ICH, 2014).

170 All *in silico* models used validated database and training sets (Roberts et al., 2000; Chakravarti

171 et al., 2012; Saiakhov et al., 2013). Further information on each model can be seen in

172 Supplementary (Table S1). These models also have been applied in other works (Mahmoud et

173 al., 2014; Rastogi et al., 2014).

174

175 **2.4 Genotoxicity testing**

176 Prior to testing, the samples were kept at 4°C for 24 h to reduce the presence of short lived

177 reactive oxygen species (ROS) that can affect the bioassays (Vasquez et al., 2013) and to ensure

178 that mostly the stable transformation products are considered in the mixtures. Then the samples

179 were sterile filtered (0.2 µm) and frozen in aliquots at -150°C. All tests were performed at least

180 twice with 3 replicates per bacterial test and 2 replicates for the *in vitro* MN test. Sample pH

181 was measured and adjusted to pH7.0 ± 0.2 prior to performing bioassays.

182 **2.4.1 Bacterial mutagenicity - Ames-Test**

183 *2.4.1.1. Test organism: Salmonella typhimurium* TA100 and TA98 from the Ames MPF 98/100

184 Aqua test kit (Xenometrix AG).

185 *2.4.1.2. Method:* the Ames test was preformed based on the Ames MPF 98/100 Aqua test

186 manual (Xenometrix AG) and used a microplate format that was adapted from the fluctuation

187 assay.

188 Before the testing of mutagenicity, the cytotoxicity of samples was assessed to exclude the

189 possibility of false 'negative' mutagenicity results. A detailed description on this test procedure

190 with and without metabolic activation is provided in the supplementary (Text S1).

191 *2.4.1.3. Analysis:* Classification as positive for mutagenicity followed a > 2 fold increase in the

192 number of revertants over that of the baseline number of revertants (the mean revertants of the

193 negative control plus standard deviation (SD)). The statistical significance determined by

194 ANOVA (Holm-Sidak method, overall significance level $p \leq 0.05$) was also used to assist in

195 the determination of positive results.

196 **2.4.2 Bacterial genotoxicity: Umu Test**

197 *2.4.2.1 Test organism: Salmonella typhimurium* TA1535 psk 1002 was bought from Leibniz

198 Institute DSMZ- German Collection of Microorganisms and Cell cultures.

199 *2.4.2.2. Method:* The umu test was preformed according to ISO 13829 (ISO, 2000). Further

200 details of the test procedure is given in Supplementary (Text S1).

201 *2.4.2.3. Calculation and Analysis:* The calculation of growth (G) and induction ratio (IR) were

202 performed according to ISO 13829. However, classification as positive for *umuC* induction was

203 taken as IR $\geq$ 2 and G $\geq$ 0.5. The statistical significance determined by ANOVA (Holm-Sidak

204 method, overall significance level $p \leq 0.05$) was also used to assist in the determination of

205 positive results.

206 **2.4.3. Mammalian genotoxicity:** *In vitro* **micronucleus assay using flow cytometry**

207   *2.4.3.1. Cell line:* Chinese hamster ovary cells (CHO-K1) were bought from American Type

208   Culture Collection (ATCC). These cells had a doubling rate of 16-18 hours.

209   *2.4.3.2. Method:* The *in vitro* MN test was designed and executed using the guidelines of the *In*

210   *vitro* MicroFlow Kit (Litron Laboratories) and Bryce et al. (2010). The details of the *in vitro*

211   MN test and the cell staining procedure can be found in the Supplementary (Text S1).

212   *2.4.3.3. Analysis:* Flow cytometry analysis was performed using BD Biosciences FACSCalibur

213   according to the gating and settings recommended by the *In vitro* MicroFlow Kit protocol.

214   20,000 nucleated cells per samples were analysed for MN formation, and cytotoxicity (EMA+

215   and relative survival). The validity criteria for the test were defined as suggested by Bryce et

216   al. (2010). Samples were classified as positive when MN frequency ≥ 3 SD of the mean negative

217   control value. Samples were determined to be cytotoxic if there was 50% reduction in relative

218   survival. The statistical significance was determined by ANOVA (Holm-Sidak method, overall

219   significance level $p \leq 0.05$).

220   **2.5 Comparison of the *umuC* induction between CIP and its irradiated mixtures**

221   Under the assumption that the TPs may exhibit similar activity as CIP since the quinolone core

222   was retained in the identified TPs, the relationship between CIP and its irradiated mixtures was

223   investigated in the umu assay using both non-linear regression curves and combination index

224   (CI) computation. A dose-response curve was constructed for induction ratio of CIP from the

225   non-cytotoxic to the marginally cytotoxic range using Four Parameter Logistic Curve function

226   (Sigmaplot 12) with equation:

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

228   Where min = bottom of the curve; max = top of the curve; slope = slope of the curve at its

229   midpoint; EC50 = x value for the curve point that is midway between the max and min

230 parameters. This curve was used to predict the CIP concentrations that would cause similar

231 effect as those observed in the irradiated mixtures. The residual CIP concentrations found in

232 the irradiated mixtures after treatment time at 2 min, 4 min, 8 min and 16 min after 1:400,

233 1:2000 and 1:4000 dilutions also were plotted to show their relationship to CIP dose-response

234 curve.

235 The combination index has been well defined and used to quantify drug-drug interactions in

236 mixtures (Chou and Talalay, 1984; Kortenkamp et al., 1999; Zhao et al., 2010). In this paper,

237 the combination index is used and calculated similar to Zhao et al. (2010) and using the

238 guidelines of The Danish Veterinary and Food Administration (2003). Based on our results (see

239 section 3.2.2.), we assumed that CIP may be the main component in the irradiated mixtures that

240 was responsible for the induction of the *umuC* gene. Therefore, we used the following equation

241 from the Danish Veterinary and Food Administration (2003):

242
$$CI = \frac{d_1}{D_1} = \frac{CIPmix}{CIPpred} \qquad (2)$$

243 In this case, d_1 is the dose of the residual CIP in the mixture (CIP mix) and D_1 is the dose of

244 CIP that would produce the same effect (CIP predicted). Genotoxicity was the endpoint

245 investigated and this is limited by cytotoxicity. Therefore, instead of a median toxicity

246 parameter such as EC_{50}, the CI was calculated for each effect observed by the mixture that has

247 an IR $\geq$ 2 and G $\geq$ 0.5 to provide a better comparison within the non-cytotoxic but genotoxic

248 range. A graph was then plotted of the computed CI versus the irradiation time. According to

249 LC-MS and NPOC data, mixtures obtained from 2 min to 16 min of photolysis contained most

250 of the TPs (Figure 1). Therefore, these mixtures were selected for the CI analysis.

251 **2.6 Statistical Software**

252 All statistical analysis and graphs were processed using Microsoft Excel 2010 (Microsoft

253 Corporation) and Sigmaplot 12.0 (Systat Software, Inc).

11

3.0 Results and Discussion

3.1 CIP and its photolytic products

According to LC-MS/MS analysis CIP under UV photolysis gradually underwent primary elimination and transformation with time. After an irradiation time of 4 min, 50% of the initial CIP concentration was already eliminated, and at 46 min >99% of parent compound was eliminated (Figure 1). The relative peak areas of all TPs exhibited a similar trend, that is, all peaks increased significantly, and then decreased rapidly with increasing irradiation time (Figure 1). After 64 min of irradiation, all identified TPs as well as CIP were not detectable. However, complete mineralization of CIP did not occur as only 70% of total NPOC was eliminated at the end of the photolysis (Figure 1).

Nine known stable TPs of CIP were found which have been previously published (Haddad and Kümmerer, 2014; Table 1). The details of their formation and elucidation are detailed elsewhere (Haddad and Kümmerer, 2014). Table 1 shows the TPs identified with their proposed structures, and their predicted genotoxicological properties from QSAR analyses. All of the proposed TP structures retained the quinolone molecule and the cyclopropyl ligand of CIP but the transformation occurs mostly to the piperazine moiety.

The proposed structure of TP5 is the human metabolite desethylenciprofloxacin, that is known to have a lesser microbial activity than CIP (Shah, 1991). Using our analytical settings, TP5 occurred at < 1% of the area mass of CIP. Only three TPs, namely TP 3, 6 and 8, occurred at $\geq$10% of the initial CIP peak area in the mixture which is a criterion of the U.S. Food and Drug Administration (FDA) to identify relevant TPs for further assessment ({FDA, 1998). Rastogi et al. (2014) also recommended that TPs with relative abundance $\geq$2% (A/A0 $\geq$ 2%) should be considered since they may possess high activity at low concentration. In our case that would include additionally TPs 7 and 9. However, the use of the relative peak area may not be the best

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278   way for determining the relevant TPs in the mixture as the measured concentration may be low

279   because of low analytical sensitivity due to low ionisation rates in mass spectrometry or low

280   molar extinction coefficient in case of UV-vis detection. This method only provides a relative

281   change in concentration of the identified TPs as pure standards of the TPs are not available for

282   absolute quantification of actual TP concentration. Notwithstanding, currently this provides a

283   means of identifying possible relevant TPs from analytical data and therefore emphasizes the

284   necessity of using whole mixture toxicity in conjunction with QSAR.

285

286   **3.2. Genotoxicity characteristics of CIP and its mixtures of photo-TPs**

287   A battery of *in vitro* genotoxicity assays was used for the initial characterization of the

288   genotoxicity of the photolytic mixtures. In the selected battery of assays, at least one bioassay,

289   namely the umu test is known to be able to detect the bacterial genotoxicity of CIP (Hartmann

290   et al., 1998).

291   **3.2.1. Bacterial mutagenicity**

292   The Ames test using the frameshift strain TA98 and the base substitution strain TA100 were

293   negative for mutagenicity in CIP and its photolytic mixtures (Table 2). Chételat et al. (1996)

294   showed that even with concomitant irradiation and mutagenicity testing, there was a slight but

295   insignificant increase in strain TA 100 revertants. They also have determined that the

296   photoproducts did not enhance the gyrase-mediated genotoxicity but the notable increase in

297   strain TA104 revertants was most probably from short lived ROS (Chételat et al., 1996). QSAR

298   modelling revealed a few TPs were predicted to have Salmonella mutagenicity (Table 1).

299   However, for strain TA 100 and 98, we observed no mutagenicity and this may be as a result

300   of the low concentration of the relevant TPs or mixture interactions or simply that the strains

301   tested here were not suitable to demonstrate mutagenic mechanism of action predicted by

13

302   QSAR analyses. CIP requires strains with both UVR excision repair and an error-prone repair

303   system for detection of bacterial mutagenicity (Clerch et al., 1992) and this may also hold for

304   the TPs. In the case of Case Ultra, the inconclusive prediction had both positive and negative

305   alerts in TP5. However, the positive alert of TP5 was also found in CIP structure (Table S2).

306   Therefore, using classification rule 4 of the ICH M7 guideline (ICH, 2014), one can propose

307   that TP5 may not be mutagenic in strain TA100 or TA98 since CIP was not mutagenic in these

308   strains. In the rule based model of Oasis, three TPs namely TP 7, 8 and 9 were predicted positive

309   in the Ames test (Table 1). However, in the Leadscope model based on both statistical and rule

310   based criteria, none of the TPs were predicted to cause bacterial mutagenicity (Table 1). QSAR

311   estimations by different models all have their respective weaknesses and strengths and therefore

312   predictions may be different. Regardless, experimentally, these photolytic mixtures of TPs and

313   CIP were not mutagenic to strains TA100 and TA98. However, the Leadscope model predicted

314   that like CIP, all TPs may cause *in vitro* mammalian mutation (Table 1) and this was not tested

315   for here.

316   **3.2.2. Bacterial genotoxicity**

317   Even though cytotoxicity was not explicitly explored in this research, relative growth inhibition

318   testing with *S. typhiumurium* TA 1535 psk 1002 in the umu test revealed that bacterial growth

319   was less affected when exposed to mixtures produced after photolysis. Even though CIP was

320   present at lower concentrations in these mixtures and therefore cytotoxicity is expected to be

321   lower, it cannot be excluded that the presence of the various TPs did not enhance the toxicity

322   of the mixtures. Similarly, Paul et al. (2010), found that cytotoxicity of photolytic and

323   photocatalytic treated CIP solution to *E.coli* K12 correlated with the residual CIP

324   concentrations in the solutions and therefore concluded that the TPs did not significantly

325   influence the overall cytotoxicity. In fact, the authors postulated that transformation of the

326   piperazine moiety and the fluoride ion would diminish the antimicrobial potency of CIP. In our

327   case, the TPs were mostly altered at these two positions often resulting in a deflourination

328   and/or breakage of the piperazine moiety (Table 1). Sukul and Spiteller, (2007) further

329   suggested that dealkylated TPs have a much lower antimicrobial potency than deflourinated

330   TPs.

331   The irradiated mixtures were positive for *umuC* induction without any metabolic activation

332   following a similar diminishing pattern as in the primary elimination of CIP (Figure 1, 2). As

333   such, it was hypothesized that CIP may be the main contributor to the levels of *umuC* induction

334   in the photolytic samples. CIP was also tested with metabolic activation to reveal that the S9

335   mix led to a detoxification of the samples (Figure 2). The observed minimum genotoxic

336   concentration (MGC) of CIP was 0.004 mg/L (-S9) and 0.025 mg/L (+S9). Since the use of

337   metabolic activation resulted in lower genotoxic potentials of the mixtures, the relationship

338   between CIP and its irradiated mixtures was investigated without the use of metabolic

339   activation.

340   The CI of the measured CIP concentration in the treated mixtures (residual CIP) relative to the

341   predicted CIP concentration based on the single-substance toxicodynamics was computed and

342   illustrated in Figure 3a and 3b. In such a graph, the CI of 1 indicated that the predicted CIP

343   concentration and its residual concentration in the treated mixture producing the same level of

344   *umuC* induction, are identical. In this particular case, the CI is estimated to be solely based on

345   CIP and therefore a CI significantly <1 would involve possible additive or synergistic effects

346   from TPs. If the CI is significantly >1, antagonistic interactions were possible within the

347   mixture. In this case, none of the CI was significantly <1. The analysis revealed that the mixture

348   genotoxicity were not greater than the parent compound (Figure 3). The 1:2000 dilution of

349   mixtures for 4 min and 8 min had CIs relatively >1 for their effective concentrations. However,

350   this could be attributed to errors in prediction of CIP concentration from the curve fitting model

351   for concentrations that corresponds to the same measured effect in the irradiated mixture. Figure

352 3a shows that the corresponding concentration and effect for these time points were within the

353 standard deviations of the measured effects of CIP. Nevertheless, the CI analysis revealed that

354 the SOS repair response induction potential of the irradiated mixtures containing the TPs and

355 CIP was not greater than CIP itself.

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

357 when there was the addition of bulky groups in position 1 (cyclopropyl ligand of CIP), a

358 negative charge or bulkier group at position 7 (piperazine moiety of CIP) as well as small

359 electronegative species in position 6 (fluoride ion of CIP), position 3 (carboxylic group of CIP)

360 and position 8 of the molecule. Due to the TPs retaining the quinolone molecule that binds to

361 the DNA gyrase, their mechanism of genotoxicity is assumed to be similar to CIP. However,

362 given the alterations in the substituents of the TPs, their genotoxic potential should vary.

363 Further, it cannot be excluded that chemical modifications of the FQ scaffold may lead to novel

364 mechanisms of genotoxicity or other toxicity. Li et al. (2014) did QSAR predictions for umu

365 test with several known TPs and concluded that some TPs were predicted to show stronger

366 genotoxicity than CIP. The authors have predicted the LOEC of two of the TPs identified in

367 this work namely, TP3 (as P5) and TP5 (as P1) (Li et al., 2014). TP3 had a LOEC of 3.23 nM

368 (pLOEC = 8.49 M) while TP5 had a LOEC of 2.09 nM (pLOEC = 8.68 M), both of these LOEC

369 were lower than CIP with a LOEC of 13.40 nM (pLOEC = 7.85 M) (Li et al., 2014). Since the

370 predicted LOECs for these two TPs suggested that they have greater genotoxic potential than

371 CIP, it could be assumed that they may contribute to an increased genotoxicity in the umu test.

372 However, our irradiated samples containing these TPs did not demonstrate this. In actuality, the

373 presence of any of the TPs did not enhance the genotoxicity after photolysis probably because

374 of their interaction whether antagonistic or synergistic in the mixtures and/or they did not occur

375 at concentrations that can cause an observable effect. The loss of the fluoride ion and alteration

376   of the piperazine ring could influence cell penetration and DNA binding and ultimately geno-

377   and cytotoxicity of the TPs.

378   Several authors have noted that there is a correlation between the genotoxicity of SOS

379   chromotest and the mutagenicity observed in strain TA 102 of Ames test possibility because

380   the SOS response system is induced in both tests (Power and Phillips, 1993; Albertini et al.,

381   1995). Since CIP induced the SOS repair response system that would compensate DNA

382   damage, it is possible that bacteria can become resistant to CIP (Mamber et al., 1993; Power

383   and Phillips, 1993; Clerch et al., 1992; Yim et al., 2011). Experimentally, our results suggested

384   that it is very likely that under these conditions the mixture of TPs did not exhibit a mechanism

385   of genotoxicity different from that of CIP and the TPs did not contributed significantly to *umuC*

386   induction or bacterial cytotoxicity at the present concentrations. The retention of the quinolone

387   moiety would suggest that the TPs have an intrinsic ability to bind to DNA and possibly induce

388   the SOS repair response system. However, the lipophilicity, cell penetration and DNA binding

389   affinity may be altered because of the changes in the substituents. Nonetheless, in our case, the

390   photolytic mixtures may not pose a threat towards enhancing bacterial resistance stemming

391   from induction of the SOS repair response system since they did not increase the induction of

392   *umuC* gene. Further, the mixtures of TPs were not more cytotoxic than CIP and therefore would

393   not cause added selection pressure to microbial communities that would favour resistance

394   mutants. The *umuC* inducing effect of CIP was observed below the MGC of CIP which is at

395   environmentally relevant concentrations (µg/L), even within the photolytic mixtures tested.

396   Therefore, photolysis is an effective method to reduce the umuC potential of CIP as an

397   environmental contaminant, as long as CIP is completely removed from the mixture or present

398   at concentrations below the MGC. Haddad and Kümmerer (2014) have also identified similar

399   TPs after simulated sunlight photolysis. Thus, it may be possible that the mixtures generated

400   from natural irradiation could also result in compounds with less potency than CIP. However,

401 bearing in mind that kinetics of formation and concentration of the TPs may be different for

402 direct sunlight photolysis; it is still possible that other mixture interactions could occur that may

403 influence bacterial genotoxicity and cytotoxicity. Further, natural photolysis could lead to the

404 formation of short lived ROS that may play an important role in environmental genotoxicity.

405 **3.2.3. Mammalian genotoxicity**

406 As reiterated here, quinolones are only genotoxic in eukaryotic test systems at concentrations

407 that are 100-1000 fold higher than the effective concentrations in prokaryotic test systems

408 (Table 3, Bredberg et al., 1989; Albertini et al., 1995). The lower affinity of CIP to the

409 topoisomerase in eukaryotic organisms may imply that there is a lower genetic risk to plants,

410 animals and humans from their residues in the environment (Kümmerer et al., 2000). Our

411 experiments revealed that CHO-K1 cells treated with CIP at concentrations 1 mg/L (5% CIP

412 v/v) to 4 mg/L (20% CIP v/v) had relative survival rates of ≤60 % and induced

413 apoptosis/necrosis (EMA+ ≥5 %) while producing no significant MN induction. As such,

414 cytotoxicity and genotoxicity of the photolysis samples was investigated at 2.5 v/v. Garcia-

415 Käufer et al. (2012) used the WST-1 assay to measure the viability of HepG2 cells to UV

416 photolytic samples and found that the samples were not cytotoxic to this cell line. Cytotoxicity

417 testing of our photolytic mixtures with the CHO-K1 cells showed that there were slight but

418 insignificant reductions on the relative survival and no significant effect on apoptosis/necrosis

419 (EMA+) after exposure (Table 3).

420 MN formation was found in CHO-K5 cells, V79 cells, Hep G2 cells and mouse lymphoma

421 L5178Y TK$^{+/-}$ treated with CIP (Albertini et al., 1995; Curry et al., 1996; Lynch et al., 2003;

422 Garcia-Käufer et al., 2012). QSAR modelling predicted that like CIP, the TPs would be positive

423 for mammalian mutation involving mouse lymphoma mutation assay and for *in vitro*

424 chromosome aberration in cell lines such as Chinese hamster lung (CHL) cells and human

425 peripheral blood lymphocytes (HPBL) but with uncertainty in CHO cell lines (model A7V) due

426 to the presence of unknown fragments (Table 1). Gibson et al. (1998) found a statistically

427 significant increase in MN formation in CHO-K1 cells but reasoned that this is of no biological

428 significance since the MN counts occurred within the deviations of their historical negative

429 control. Similarly, we found no significant effect on MN formation in CHO-K1 cells exposed

430 to not only CIP but also its irradiated mixtures (Table 3). This is contrary to many

431 photogenotoxicity *in vitro* studies performed on a number of cell lines where CIP was shown

432 to induce MN, chromosome aberration and/or other DNA damage (Chételat et al., 1996;

433 Sánchez et al., 2005; Garcia-Käufer et al., 2012). All of these authors have postulated that the

434 increase in photogenotoxicity observed may be due to the presence of short lived ROS and not

435 by the TPs (Chételat et al., 1996; Sánchez et al., 2005; Garcia-Käufer et al., 2012). In this study

436 precautions were taken to ensure the influence of ROS were negligible and we had used CHO-

437 K1 cell lines in which CIP itself does not induce significant MN formation. However, using

438 other cell lines in which CIP is known to induce MN and using lower dilution factors should be

439 investigated before it can be excluded that the mixture of TPs are not genotoxic to mammalian

440 cells. QSAR predictions suggested that MN formation is not likely *in vivo* from exposure to

441 CIP. It is already known that *in vivo* testing has shown that CIP is not genotoxic especially at

442 the tissue concentrations achieved from the therapeutic dose (Albertini et al., 1995; Herbold et

443 al., 2001). However, Case Ultra MN *in vivo* (A7S) test for rat and mouse model predicted some

444 positive structural alerts for TPs 1, 4, 6 and 8. Since none of the positive structural alerts were

445 found in CIP, this risk should not be excluded from further evaluations of the TPs (Table S2).

446 Further, unscheduled DNA synthesis was predicted for some TPs. These TPs had either a

447 retention or substitution in the fluoride position of the CIP molecule (TP3, 5 6) or like TP9, the

448 presence of a tertiary amide group (Table S2).

449 **4.0 Conclusion**

450 The photo-TPs identified all retained the quinolone moiety but have alteration on the piperazine

451 moiety and/or loss or substitution of the fluoride ion. The retention of the quinolone moiety

452 would suggest that the TPs could bind to the DNA similarly as CIP but the alterations of the

453 substituents can affect the affinity of TPs to DNA binding and subsequently their potency. In

454 fact, QSAR predictions suggested that there were a few TPs that may be genotoxic to bacteria

455 and mammals. The battery of genotoxicity assays employed here only covered a few endpoints

456 with one cell line and a few bacterial strains. Nevertheless, it was able to demonstrate that the

457 photolytic samples were *umuC* inducing and this may most likely be because of the presence

458 of CIP. Therefore, the TPs in these irradiated mixtures did not contribute significantly to the

459 SOS repair response induction. It is possible that the effect of the TPs was masked by

460 antagonistic mixture interactions or that the concentration of TPs in the mixture was not

461 sufficient to cause any observable effect in the bioassays.

462 Therefore, we have observed that while photolysis provides a mean for the removal of CIP, the

463 genotoxicity and cytotoxicity potential of the resultant mixtures could be dependent primarily

464 on the concentration of residual CIP. The genotoxic risk of the TPs in the environment was not

465 particularly defined in this study as CIP was determined to be the main genotoxin in the

466 bioassays used. As such, this study provided only an initial risk characterization of the particular

467 mixtures generated here. The TPs that were predicted as genotoxic would require a more

468 comprehensive assessment that would include chemical analysis characterization (e.g.

469 detection, isolation, formulation), exposure analyses (including biodegradation studies) and

470 effect driven analyses for TP threshold identification to determine their environmental risks.

471

472 **5.0 Acknowledgment**

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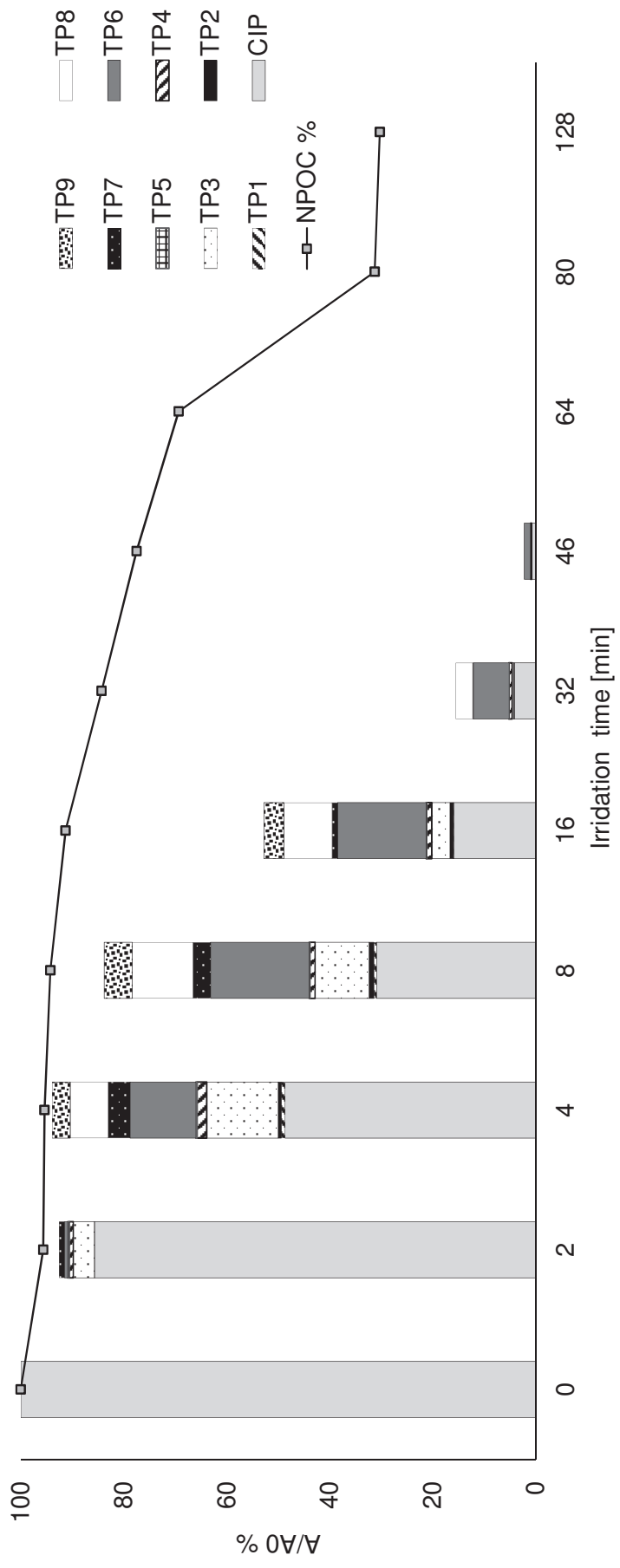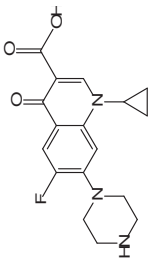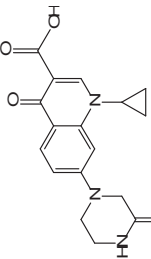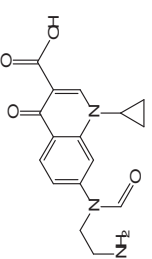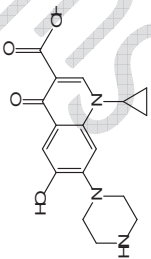
Fig 1. Time course of the relative peak area of ciprofloxacin (CIP), its transformation products (TPs) (bars), and the non-purgeable organic carbon (NPOC) (line) during the photodegradation experiment. A/A0; A is the area of TP at specified irradiation time point and A0 is the area of CIP at 0 min monitored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

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

TP ID	Retention time (min)	Structure	Molar mass (gmol⁻¹)	Case Ultra					Leadscope				Oasis
				Genotoxicity				Mutagenicity	Genotoxicity		Mutagenicity		Mutagenicity
				A		B	C	D	A	B	D	E	D
				A7U	A7V	A7S	A64	A2H					
CIP	8.5		331.35	+	§	-	+	-	+	-	-	+	-
TP1	4.6		327.34	+	§	§*	-	-	+	-	-	+	-
TP2	6.5		315.33	+	§	-	-	-	+	-	-	+	-
TP3	*6.7*		*329.36*	+	§	-	+	-	+	-	-	+	-

TP4	7.0		287.32	+	§	§*	-	-	-	+	-	+	-
TP5	7.5		305.31	+	§	-	+	§§*	-	+	-	+	-
TP6	7.8		345.36	+	§	§*	+	-	-	+	-	+	-
TP7	11.5		343.34	+	§	-	OD	-	-	+	-	+	+
TP8	12.0		315.33	+	§	§*	OD	-	-	+	-	+	+

TP9	12.5		329.36	+	+	§	-	+	-	+	-	-	+	+
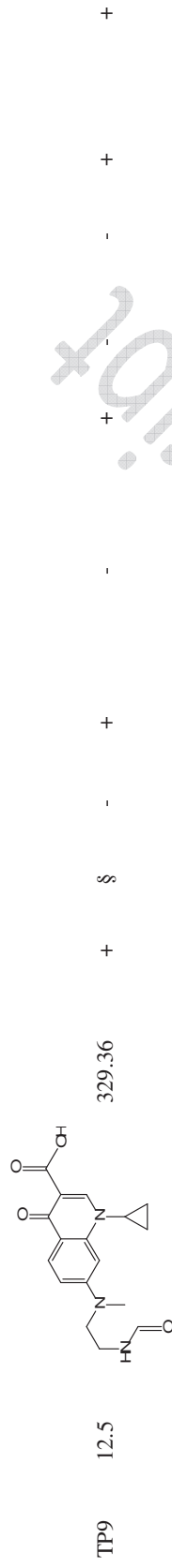
A= In vitro Chromosome Aberration; B= In vivo Micronucleus Composite; C= Other Genotoxicity Tests; D= Salmonella Mutagenicity; E= Mammalian Mutagenicity

A7S: MN in vivo; A7U: Chromosome aberration composite in vitro; A7V: Chromosome aberration in CHO cells; A64: Unscheduled DNA synthesis induction; A2H: Mutagenicity Ames *Salmonella typhimurium*

- =Negative alert for activity; + = Positive alert for activity; §= Inconclusive because of the presence of unknown structural fragments from the training set; §* = Inconclusive because of the presence of both positive and deactivating alerts in the molecule; OD= molecule fragments are out of domain

TPx in bold and italics represents the TPs that are ≥ 10% relative abundance to CIP

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

Time points	Ames[1]			
	Number of Revertants			
	TA 98		TA 100	
	-S9	+S9	-S9	+S9
NC	2 ± 2	2 ± 2	3 ± 1	1 ± 1
0	2 ± 1	1 ± 1	4 ± 2	1 ± 0
2	n.t	n.t	n.t	n.t
4	n.t	n.t	n.t	n.t
8	4 ± 1	3 ± 3	5 ± 2	2 ± 1
16	3 ± 1	4 ± 2	4 ± 2	2 ± 1
32	3 ± 1	3 ± 1	6 ± 2	1 ± 2
64	4 ± 3	2 ± 2	5 ± 1	1 ± 2
128	1 ± 1	2 ± 2	4 ± 1	1 ± 0
PC	45 ± 2*	48 ± 0*	48 ± 0*	48 ± 0*

Data presented are the mean values ± the standard deviation. n.t: not tested; PC: positive control 0.1 µg/ml 4-NQO + 2 µg/ml 2-NF (-S9) and 5 µg/ml 2-AA (+S9). NC: negative control of Millipore water; 1: samples tested at 1:2000 dilution; * represents p ≤ 0.05 of samples compared to the negative control (NC)
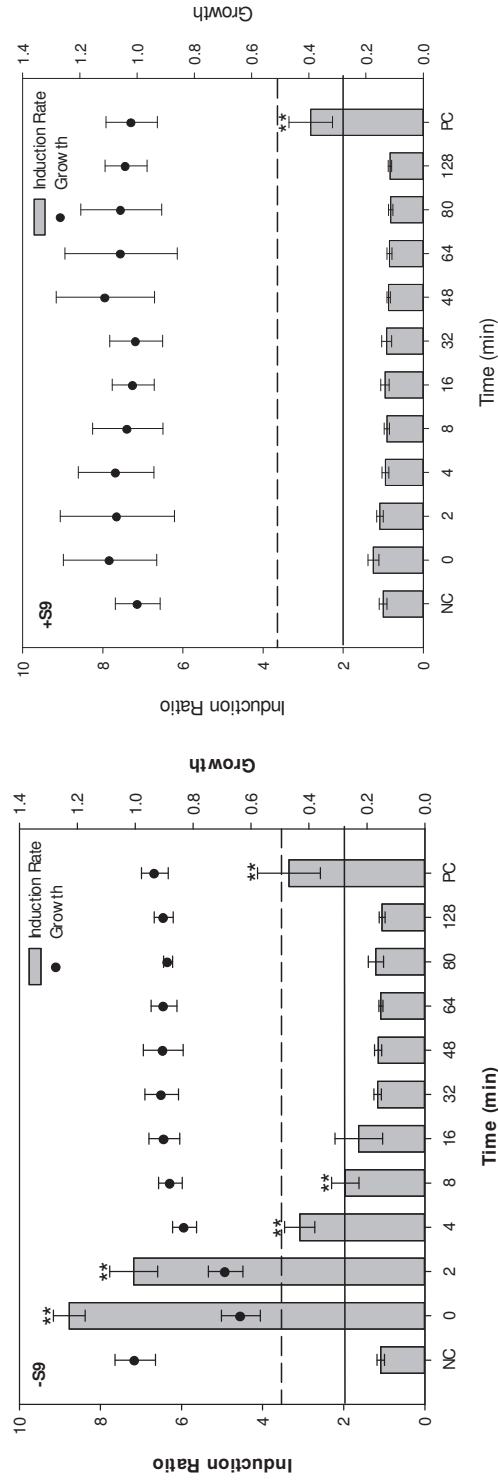
Fig 2. Umu C induction of CIP and its irradiated mixtures without metabolic activation (left) and with metabolic activation (right). Results represented are from a 1:2000 dilution of each sample. Dotted lines represent growth threshold of G≥0.5, solid line represents the induction ratio threshold of IR ≥ 2. ** represents p ≤ 0.01 of samples compared to the negative control (NC). NC = Millipore water as sample, T0 = CIP at 0.01 mg/L. PCs are the positive controls of 4-nitroquinoline-1-oxide (4-NQO) without S9 and 2-aminoanthracene (2-AA) in the presence of S9.
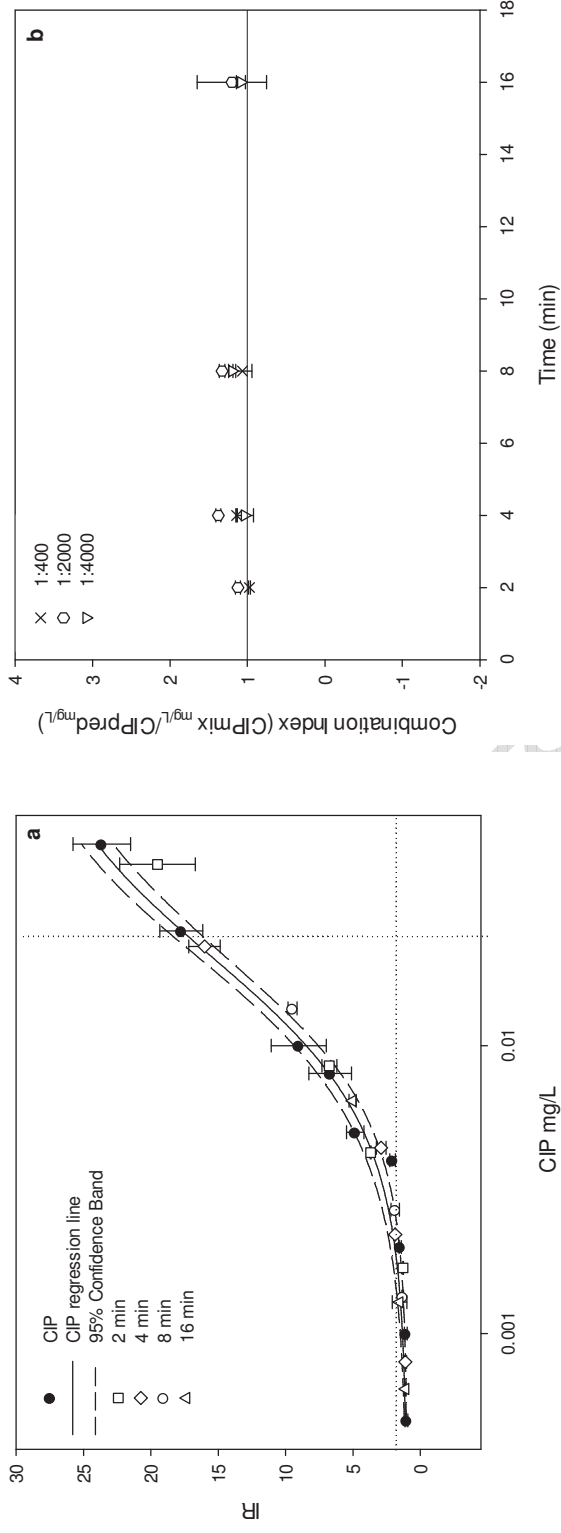
Fig 3: (a) Dose-response relationship of CIP and effect of residual CIP in its irradiated mixtures in the Umu C test. Dotted lines represent the threshold of IR≥2 and G≥0.5. (b) Combination index of residual CIP in irradiated mixtures and predicted CIP to cause similar effects of IR≥2 and G≥0.5 versus irradiation time.

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

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

Data presented are the mean values ± the standard deviation. MMC: 0.1 μg/ml mitomycin C; VB: 0.01 μg/ml vinblastine sulphate; NC: negative control of Millipore water; 1: samples tested at 2.5% v/v; * represents $p \leq 0.05$ of samples compared to the negative control (NC)

Supplementary Data

for

Evaluation of genotoxicity of Ciprofloxacin and its photo transformation products by a combination of experimental and *in-silico* testing

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Text S1: Genotoxicity testing procedure

Genotoxicity testing

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

Bacterial mutagenicity: Ames MPF 98/100 Aqua

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

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

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

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

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

Bacterial genotoxicity: Umu Test

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

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

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

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

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

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

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

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

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

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

Briefly, the cell staining and lysis protocol includes placing the 24 well plate on ice for 20 minutes after the 48 h exposure time. After that the solution was aspirated and 300 µl of EMA solution was added to each well. The plate was exposed to fluorescence light for 30 min to undergo photoactivation of the dye. Then the EMA dye was aspirated and the cells were washed with 1ml of cold buffer solution. 500 µl of complete lysis solution 1 was added to each well and

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

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

Table S1: Description of the applied QSAR models

Toxicity	QSAR software	Model	Description	Endpoint	References
Genotoxicity	CASE Ultra v.1.4.6.6 (MultiCASE Inc.)	A7U Chromosome Aberration *In vitro* composite	model develop from FDA databases	*In vitro* Chromosome abberation	Chakravarti et al., 2012; Saiakhov et al., 2013
		A7V Chromosome Aberration *In vitro* CHO cells	model develop from FDA databases		
		A7S Micronucleus *In vivo* composite	model develop for rat and mice data from FDA databases	*In vitro* MN formation	
		A64 UDS Induction	model developed for unscheduled DNA induction with primary rat liver, human peripheral blood lymphocytes and fibroblast	Unschedule DNA synthesis	
Mutagenicity		A2H Salmonella Ames mutagenicity	model developed *S. typhimurium* strains (+/- S9) as a composite from NTP, GENETOX, USEPA and FDA database	Bacterial mutagenicity	
Genotoxicity	Leadscope V.3.0.11-1	*In vitro* Chromosome Aberration average model	model develop with CHO, CHL, HPBL and other mammalian cell culture from 2012 Genetox Database from Leadscope	*In vitro* Chromosome abberation	Roberts et al., 2000
		Micronucleus *in vivo* composite model	model develop with rat and mice data from 2012 Genetox Database from Leadscope	*In vitro* MN formation	
Mutagenicity		Salmonella composite	model develop with data from *S. typhimurium* TA 97, TA 98, TA 100, TA1535, TA 1536, TA 1537, TA 1538 from 2012 Genetox Database from Leadscope	Bacterial mutagenicity	
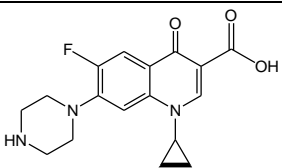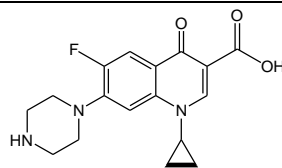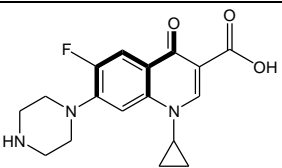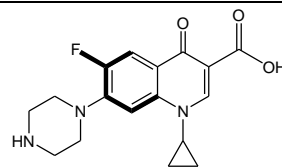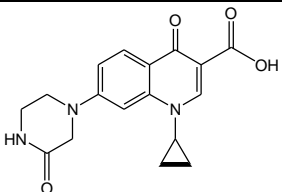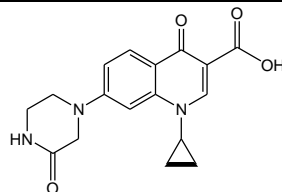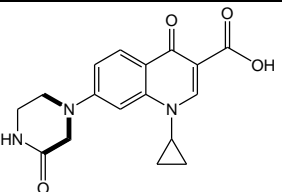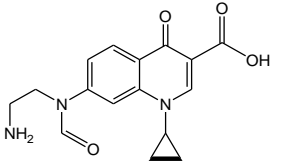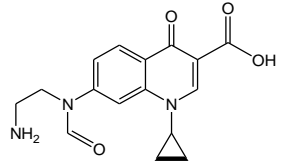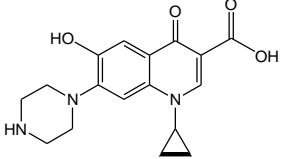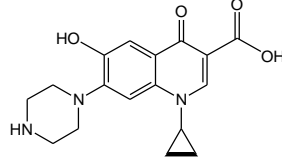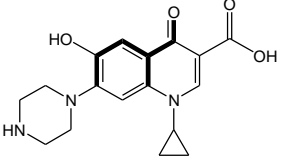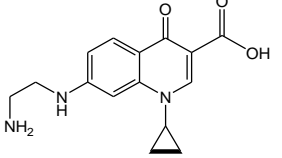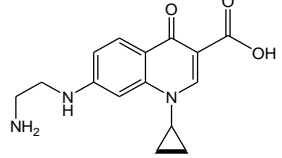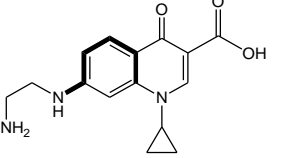
	Mammalian mutation *in vitro*	model develop for mammalian mutation including mouse lymphoma mutation gene mutation assays at the thymidine kinase (tk) locus using L5178Y cells in culture from 2012 Genetox Database from Leadscope	Mammalian mutagenicity	
Mutagenicity Oasis	Mutagenicity v .04	model developed with data from *S. typhimurium*+/-S9 using NTP database	Bacterial mutagenicity	Laboratory of Mathematical Chemistry, 2014

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

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

TP5

TP6

TP7

TP8

TP9

Bold lines represent positive structural alerts

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Appendix 4

1 **Genotoxicity and cytotoxicity characterization of mixtures generated from photolysis of**

2 **the ß-blockers Atenolol and Metoprolol using a combination of experimental and (Q)SAR**

3 **approaches**

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Abstract

ß-blockers are known to exert a wide spectrum of toxicity but little is known about the toxicity of their transformation products (TPs) formed in wastewater treatment plants (WWTPs) and advanced oxidation processes. This study investigated the toxicity of UV photolysis mixtures of Atenolol (ATL) and Metoprolol (MTL), respectively, on a whole mixture level using a battery of *in vitro* genotoxicity and cytotoxicity assays and on an individual photo-TP level applying different software packages and approaches (*in silico* "testing battery") for *in silico* predictions.

ß-blockers were neither completely removed nor mineralized. The resulting photolysis mixtures were cytotoxic to *Vibrio fischeri* and mammalian cells but not mutagenic in the Ames test or genotoxic in the *in vitro* micronucleus and umu tests. Potentially cytotoxic TPs were proposed by relating the observed effects to the kinetics of TP occurrence, and applying *in silico* toxicity and hydrophobicity predictions. Several TPs were predicted to be genotoxic. Individual assessment of the identified TPs for all endpoints would be desirable to corroborate these predictions. Nevertheless, the use of whole mixture toxicity assessment coupled with *in silico* predictions and analytics proved to be a versatile tool to shortlist possible TPs for further assessments.

Key words

Whole mixture toxicity testing, (Quantitative) Structure activity relationships ((Q)SAR), risk characterization, micropollutants

Introduction

As a class of micropollutants, ß-blockers were detected in the ranges of 0.006-25 µg/L in raw effluent of wastewater treatment plants (WWTPs) *(1)*. Atenolol (ATL) and Metoprolol (MTL) are some of the most prescribed ß-blockers in several countries *(2-4)*. ATL has a high daily mass load in WWTPs of 316 mg/1000 inh/d while MTL has <100 mg/1000 inh/d *(1)*. The removal rates in WWTPs significantly varies with average removal rates of 38% to 24% in conventional activated sludge WWTP and 71% to 44% in membrane bio-reactor WWTPs for ATL and MTL, respectively *(1)*. Further, ATL and MTL were classified as not readily biodegradable using several standardized tests *(5-7)*.

ATL and MTL as selective β1-receptor antagonists lowers heart rate and systemic blood pressure in humans and are used in treatment of hypertension, angina pectoris and after heart attack *(8)*. Over the years, there has been a general agreement that these effects may also occur on non-targeted organisms in the environment *(9-11)*. Fish, algae, amphibians and crustaceans were all found to be sensitive to ß-blockers exhibiting specific and non-specific toxicity but at environmentally irrelevant concentrations *(2, 4, 9-13)*. More relevant as contaminants is that mixture toxicity assessment of several ß-blockers revealed that they are baseline toxicants in assays with bacteria (*Vibrio fischeri*), algae (*Desmodesmus subspicatus*) and crustaceans (*Daphnia magna*) *(2, 4, 10)*. Thus far, MTL has not shown DNA damaging potential both by repair or fragmentation but *in vivo* micronucleus (MN) formation in human lymphocytes for ATL was reported *(3, 10, 14, 15)*. Moreover, both ß-blockers showed evidence for carcinogenicity in rats and mice *(3)*.

Given the effects of ß-blockers coupled with their varying removal rates from WWTPs, several researchers have sought to investigate advanced oxidation processes (AOPs) including ozonation (O_3), ultraviolet (UV) and Xenon (Xe) photolysis that resulted in their incomplete mineralization *(16-20)*. Only little is known about the toxicological relevance of the resulting

71 transformation products (TPs) of these treatments. There are some indications that

72 photolytic/photocatalytic treatment of various ß-blockers, depending on specific settings of

73 treatment, can produce mixtures with reduced or increased toxicity when compared to the

74 toxicity of the parent compound (PC) *(16-21)*. Since ß-blockers can be toxic towards a number

75 of aquatic species, many researchers have monitored the changes in toxicity of the treated

76 mixtures using mostly acute testing with algae, crustaceans and bacteria *(16-20)*. Šojic´ et al.

77 also reported that mixtures derived from several oxidative treatments of MTL were also

78 mutagenic in the Ames test and genotoxic in the comet assay *(20)*. All of these studies have

79 investigated the effects of the whole mixtures from the treatments. Escher et al. studied the

80 toxicities of a few human metabolites of some ß-blockers but studies that have characterized

81 the toxicities of individual TPs formed from treatment processes were not found in our literature

82 review *(10)*. However, the metabolites and TPs may be a necessary inclusion for a more sound

83 risk assessment of ß-blockers. Structure activity relationships (quantitative (Q)SAR and ruled

84 based) provide an additional supportive tool that can be used to predict fate and toxicity and to

85 further identify cases for further testing *(22)*. This tool is a useful addition to the study of

86 mixtures to characterize individual TP toxicity if the chemical structures of the TPs are known.

87 Further, its combination with experimental results can provide greater depths in understanding

88 the observed effects and the physico-chemical characteristics of the compounds *(22)*.

89 Therefore, this study was designed to characterize the toxicity of mixtures derived from UV

90 photolysis of two ß-blockers, ATL and MTL, respectively. Evaluation of the influence of

91 individual photo-TPs were done by combining data from mass spectrometry, experimental

92 genotoxicity and cytotoxicity testing and predictions based on (Q)SAR.

93 **Materials and Methods**

94 **Chemicals:** Atenolol (CAS-RN: 29122-68-67) and Metoprolol tartrate (CAS-RN: 56392-17-

95 7) were purchased from Sigma-Aldrich. Acetonitrile (HiPerSolv CHROMANORM ®, LC-MS

96 grade) and formic acid were purchased from VWR International GmbH. Ultra-pure water was

97 used to prepare all test solutions.

98 **UV Photolysis and monitoring of carbon elimination:** UV treatment experiments were

99 carried out in a 1L immersion-type reactor (UV-Consulting Peschl) using a 150 W medium-

100 pressure mercury lamp (TQ 150, UV-Consulting Peschl). The reactor was filled with a solution

101 of the respective ß-blocker dissolved in ultra-pure water (ATL = 100 mg/L, MTL = 400 mg/L)

102 and irradiation was performed for 256 minutes. High initial ß-blocker concentrations were

103 selected based on water solubility limit to ensure maximum formation of stable TPs in

104 photolysis mixtures. The degree of mineralization was measured as dissolved organic carbon

105 (DOC) using a Shimadzu TOC-VCPN analyzer. Further detailed information on experimental-

106 setup can be found elsewhere *(6, 7)*.

107 **Liquid chromatography analysis:** Primary elimination of parent compounds was monitored

108 using a Shimadzu Prominence HPLC system. The formation and identification of the TPs were

109 performed using LC-ESI-IT-MS (HPLC 1100, Agilent Technologies in tandem with Esquire

110 6000[Plus], Bruker Daltonic). All LC instruments, chromatographic parameters and mass

111 spectrometer settings have been detailed elsewhere *(6-7)*.

112 **(Q)SAR Predictions:** QSAR toxicity predictions of ATL, MTL and their elucidated photo-

113 TPs were performed using a set of software each with different algorithms and training sets as

114 an "*in silico* battery". The software used included CASE Ultra V.1.5.0.1 (MultiCASE Inc.), and

115 Leadscope software V. 3.2.4-1 *(23, 24)*. Mutagenicity was predicted using two models in CASE

116 Ultra suite, namely, mutagenicity of the 7 *Salmonella typhimurium* strains (GT1_A7B) and

117 Salmonella mutagenicity (SALM2013). Oasis Catalogic software from Laboratory of

118 Mathematical Chemistry, University Bourgas, Bulgaria was used to predict mutagenicity based

119 on bacterial mutagenicity (module mutagenicity v.04) in *S. typhimurium* (Salmonella Catalogic

120 model, SC). For the endpoint "bacterial mutagenicity", a combination of statistical (Multicase

121 and Leadscope) and rule-based (Oasis Catalogic) models were applied according to suggestions

122 of the International Conference on Harmonization of Technical Requirements for Registration

123 of Pharmaceuticals for Human Use (ICH) guideline M7 *(25)*. Bacterial toxicity was predicted

124 in CASE Ultra V.1.5.0.1 using the MICROTOX toxicity to environmental bacteria module

125 (MultiCASE Inc.). Physico-chemical parameters such as octanol-water partition coefficient

126 (Log K_{ow}) and bioconcentration factor (BCF) was predicted using the EPI Suite software

127 KOWWIN v1.68 model (Environmental Protection Agency, US).

128 All the *in silico* models contained validated database and training sets *(23, 24, 26)*. Further

129 information on each model can be seen in Supplementary (Table S1). These models also have

130 successfully been applied in other work *(6, 7, 27)*.

131 **Toxicity testing:** Photolytic samples were sterile filtered (0.2 μm) and frozen in aliquots at -

132 150°C before the assay. All tests were performed at least twice with 3 replicates per bacterial

133 test and 2 replicates for *in vitro* MN test. Sample pH was measured and adjusted to pH 7.0 ±

134 0.2, if needed, prior to performing bioassays. All photolytic mixtures were tested for peroxide

135 using Merckoquant Peroxide test strips 0.5-25 ppm (VWR).

136 **Modified luminescent bacteria test (modified LBT):** The modified luminescent test *using V.*

137 *fischeri* NRRL-B-11177 (Hach-Lange GmbH, Düsseldorf) was conducted similarly to that

138 described in Menz et al. *(28)* and can be found in details in Supplementary text S1. The raw

139 data from this test were normalized to percent inhibition in relation to the negative controls.

140 This was conducted for three different endpoints which were: short-term luminescence

141 inhibition after 30 min (LI_{30min}), long-term luminescence inhibition after 24 h (LI_{24h}) and growth

142 inhibition after 14 h (GI_{14h}). Calculations and data analysis were performed using the

143 recommendation of Menz et al. *(28)*. Regardless of the measured endpoint, 20% was considered

144 as the threshold value for inhibition. Analysis of concentration-response relationships of parent

145 compounds was performed by plotting the normalized inhibition values against the respective

146 concentrations followed by a logistic regression analysis, as described in Menz et al. *(28)*.

147 **Bacterial mutagenicity- Ames MPF 98/100 Aqua:** The Ames test was preformed using a

148 microplate format that was adapted from the Ames fluctuation assay. It was done based on the

149 Ames MPF 98/100 Aqua test manual (Xenometrix AG) with *Salmonella typhimurium* TA 98

150 and TA 100.   Further details on the methods are presented in Supplementary text S1.

151 Classification as positive for mutagenicity occurred when the response was ≥ 2 fold increase in

152 the number of revertants over that of the baseline number of revertants (the mean revertants of

153 the negative control plus standard deviation (SD)). Variations between observed effects at

154 different sampling times were evaluated by one way analysis of variance (ANOVA), following

155 post hoc multiple comparisons (Dunnett's method), in which the untreated samples (0 min)

156 were defined as the control group.

157 **Bacterial genotoxicity- umu-Test:** The umu-test was preformed according to ISO 13829 *(29)*.

158 Further details of the test procedure is given in supplementary data (Text S1). The calculation

159 of growth (G) and induction ratio (IR) was performed according to ISO 13829. Classification

160 as positive for *umuC* induction was assessed when the IR > 1.5 and G ≥ 0.5. Significant changes

161 of *umuC* induction after photolysis were determined by ANOVA (Dunnett method, overall

162 significance level p$\leq$0.05) using the samples collected at 0 min irradiation as the control group.

163 **Mammalian genotoxicity-** *In vitro* **micronucleus assay using flow cytometry:** The *in vitro*

164 MN test was preformed with Chinese hamster ovary cells (CHO-K1) and was designed and

165 executed using the guidelines of the In vitro MicroFlow Kit and Bryce et al. *(30)*. The details

166 of the *in vitro* MN test and the cell staining procedure can be found in supplementary data (Text

167 S1). Flow cytometry analysis was performed using BD Biosciences FACSCalibur with data

168 acquired according to the gatings and settings recommended by the InVitro MicroFlow Kit

169 protocol. 20,000 nucleated cells per samples were analysed for MN formation, cytotoxicity

170   (EMA+ and relative survival), and cell cycle perturbation. The validity criteria for the test were

171   defined as suggested by Bryce et al. *(30)*. Samples were classified as positive when MN

172   frequency ≥ 3 SD of the mean negative control value. Samples were determined to be cytotoxic

173   if there was 50% reduction in relative survival to negative control. The statistical significance

174   determined by ANOVA (Dunnett method, overall significance level $p \leq 0.05$) using 0 min

175   irradiation as the control group was also used to assist in the determination of positive results.

176   **Statistical Software:** All statistical analysis and graphs were processed using Microsoft Excel

177   2010 (Microsoft Corporation) and Sigmaplot 12.0 (Systat Software, Inc).

178   **Results and Discussion**

179   **Photo-transformation of ATL and MTL:** Photolysis achieved better primary elimination for

180   ATL ($> 90\%$) than for MTL ($\sim 60\%$) after 256 min of UV irradiation (Fig. 1a, 2a). Both ATL

181   and MTL resulted in incomplete mineralization (Fig. 1a, 2a). Several transformation products

182   (TPs) could be identified by LC-MS analysis of the photolytic mixtures. Twelve TPs were

183   detected in MTL photolytic mixture while >30 TPs were detected in ATL photolytic mixture

184   (Table S2, S3). Most of the TPs in the mixtures from both ß-blockers begun to steadily rise in

185   their concentration at 16 min and peaked at 256 min (Fig. 1b, 2b). The proposed structures for

186   TP 134 and TP 210 were found in the photolytic mixtures of both ß-blockers (Table S2, S3).

187   The identification, structure elucidation and formation pathways of the TPs were not

188   specifically elaborated here but can be found in Rastogi et al. (6, 7).

189   **Mammalian and bacterial cytotoxicity characteristics of the photolytic mixtures:** Using

190   the modified LBT, MTL had EC_{50} for $LI_{30min} > 2000$ mg/L, LI_{24h} of 178.23 mg/L and GI_{14h} of

191   1208.84 mg/L. EC_{50} for ATL was >2000 mg/L for all endpoints in the modified LBT. The

192   photolytic mixtures were more cytotoxic than the ß-blockers (Fig. 3). A significant effect on

193   bacteria luminescence was observed in mixtures after 16 min or more irradiation of MTL and

194 after 32 min or more irradiation of ATL (Fig. 3). Photolysis can result in the formation of

195 oxidative species such as peroxide that can affect the bioassays. Our photolytic samples

196 contained < 2 mg/L peroxide which was the threshold for cytotoxicity in the modified LBT test

197 and therefore would not contribute to the observed toxicity. ATL photolytic mixtures exhibited

198 a more pronounced short-term luminescence inhibition (LI_{30min}) than long-term inhibition

199 (LI_{24h}), which was contrarily the most sensitive endpoint during exposure to MTL photolytic

200 mixtures (Fig. 3). Like the concentration of most TPs, the bacterial luminescence and growth

201 inhibition also increased with irradiation time. This correlation can suggest that exposure to

202 TPs within photolytic mixtures of ATL and MTL led to an immediately occurring and long-

203 lasting destabilization of bacterial cell integrity and consequently to a significantly reduced cell

204 multiplication. In fact, it is also quite possible that the observed effects could be from a

205 cumulative effect for several TPs.

206 Mixtures of MTL and its TPs at 16 min and 256 min of photolysis were cytotoxic to CHO-K1

207 cells (Table 2). These mixtures affected not only the relative survival of CHO-K1 cells but also

208 indicated that a significant percentage of cells underwent apoptosis and/or necrosis. Unlike the

209 MTL photolytic mixtures, no significant mammalian cytotoxicity was observed in ATL

210 photolytic mixtures.

211 **Evaluation of suggested TPs for cytotoxicity:** Identification of the possible contributors to

212 the observed whole mixture effects was performed by correlating changes in toxicity over time

213 with the formation kinetics of individual mass peaks during the treatment procedure. As a

214 further step, molecular structures of suggested TPs were subject to *in silico* analysis in order to

215 provide further evidence and to get information about the responsible structure elements.

216 QSAR toxicity predictions using CASE Ultra AUA model found several structural alerts that

217 predicted MTP 318, MTP 238, MTP 234_1, MTP 192, ATP 275, ATP 232, ATP 301_{1-6}, ATP

218 297_1 and ATP 152 to be toxic to luminescent bacteria (Table 3, S4, S5). However, the CASE

219   Ultra model AUA could not recognize two parts relating to the ethanolamine part of the ß-

220   blockers molecules that were found also in most of the TPs. As a result, both ß-blockers and

221   many of the TPs were predicted as "out of domain" using this model. These TPs that were "out

222   of domain" cannot be excluded as possible contributors to bacteria cytotoxicity. The AUA

223   model only acted as an estimator for acute bacterial luminescence toxicity. It is very possible

224   that one or more of these TPs may have been responsible for the observed bacterial cytotoxicity

225   by acting as baseline toxicants or by mixture interactions such as synergism or potentiation.

226   Toxicity assessment of the individual TPs and mixture toxicity would need to be performed to

227   characterize these individual TPs. All of the proposed structures were estimated to have low

228   bioconcentration potential (Table 3). These findings demonstrate the limitations of QSAR

229   predictions and calls for prudent use. However, if the compounds to be predicted are within the

230   applicability domain, QSARs are helpful tools to get further insights.

231   In case of ATL, short-term luminescence inhibition drastically increased from 128 min to 256

232   min, but some identified TPs (e.g. TP 318 [m/z 318.2]) were not detected in the latter 256 min

233   mixtures (Fig. 1b). Moreover, primary elimination of ATL went from ~29% at 128 min to ~2%

234   at 256 min but DOC levels did not change significantly (Fig. 1a), i.e. there was no

235   mineralization but the possible generation of unidentified TPs of unknown toxicity. This could

236   play an important role in addition to the presence of the identified TPs that are at their peaked

237   relative abundance, for the observed drastic increase in toxicity in the latter 256 min photolytic

238   mixture.

239   In both ß-blockers, LI_{30min} peaked in mixtures formed after 256 min irradiation (Fig . 3). Šojic´et

240   al. reasoned that TP 134 (called MP1) as well as formation of aliphatic intermediates formed

241   from oxidative treatment of MTL may have influenced their chronic and acute based test

242   systems *(20)*. Small aliphatic compounds were reported to affect bioluminescence in *V. fischeri*

243   after 30 min exposure much more than the long-term exposure endpoints such as growth *(31)*.

244 This could have been a contributor to the increased toxicity measured in our study in samples

245 after 256 min irradiation for both ß-blockers where TP 134 peaked in formation (Fig. 1, 2,3).

246 Četojević-Simin et al. also suggested that TP 134 (as TP2) was a possible contributor to

247 cytotoxicity of their MTL photocatalytic mixtures to several mammalian cell lines *(32)*. We

248 also found that MTL photolytic mixtures were cytotoxic to CHO-K1 cells. Therefore, TP 134

249 should be included in further toxicity assessments.

250 Several authors have suggested that the difference between the toxicity of ß-blockers to aquatic

251 organisms can be partially explained by their degree of lipophilicity and related to their nature

252 as baseline toxicants *(2, 10, 33)*. Hermes et al. determined that there can be an underestimation

253 of short term bioluminescence toxicity for lipophilic chemicals *(34)*. Actually, MTL is the more

254 lipophilic of the two ß-blockers and proved more toxic to *V. fischeri* after long term exposure

255 affecting both luminescent and growth (Table 3, 4). The incorporation of the Log K_{ow} for the

256 proposed structures of TPs may further assist in identifying TPs of possible concerns especially

257 as baseline toxicants.

258 QSAR prediction of Log K_{ow} using the proposed structure of the TPs showed that there were

259 six proposed structures MTP 192, MTP 234_{1-2}, MTP 238, MTP 254 and MTP 284_1 predicted

260 as or more lipophilic than MTL (Table 4). TP 238 and TP 252 peaked at 16 min but all other

261 TPs of MTL begun to steadily increase around 16 min and peaked in formation at 256 min,

262 congruently to the observed bacterial cytotoxicity (Fig. 2). MTP 238 has a predicted Log K_{ow}

263 higher than MTL and is expected to be more lipophilic and may be more likely to contribute to

264 both long term exposure effects of a reduction in relative survival in CHO-K1 and luminescence

265 inhibition in bacteria in mixtures generated at 16 min (Table 4, Fig. 3). Similarly, the rising

266 trend in luminescence and growth inhibition from long term exposure from mixtures of 16 min

267 irradiation and greater, could be related to the presence of the five MTPs, MTP 192, MTP 234_{1-}

268 $_2$, MTP 254 and MTP 284_1, with an increased lipophilicity and a high relative abundance in the

269   mixtures (Table 4, Fig 2,3). In fact, the proposed structure for MTP 254 was the human

270   metabolite of O-desmethylmetoprolol which was estimated to be similarly active as MTL in the

271   *V. fischeri* 30 minutes assay *(10)* and therefore like MTL could be more active during long term

272   exposure in the modified LBT.

273   Of the more lipophilic ATPs, ATP 254_{1a} and ATP 238 had a high relative abundance in the 256

274   min and 128 min irradiation mixtures and this coincided with increased luminescent and growth

275   inhibition after long term exposure (Table 3, Fig 1, 3). Further, ATP 275 and ATP 232 both

276   also more lipophilic than ATL peaked in their relative abundance in the photolytic mixtures

277   obtained from 32 min to 256 min (Table 3, Fig 1). During this time, the more chronic parameter

278   of luminescent and growth inhibition also began to increase and so ATP 275 and ATP 232 may

279   have played a role (Fig 3).

280   Generally, the more lipophilic TPs of both ß-blockers would need further investigation with

281   their standards to confirm their identity and characterize their specific and non-specific

282   toxicities. However, it is most likely that the observed toxicity is a result of one or more TPs

283   that may have acted as baseline toxicants.

284   **Genotoxicity and mutagenicity characteristics of the photolytic mixtures:** Neither the

285   parent compounds nor the photolytic mixtures of ATL and of MTL was classified as genotoxic

286   or mutagenic in the test battery applied (Table 1, 2). However, a statistically significant increase

287   in the *UmuC* gene induction was seen in photolytic mixtures of both ß-blockers derived at 256

288   min of irradiation. Even though the observed induction rate was still below the threshold value

289   for positive classification, this may indicate that one or more photo-TPs may have potential to

290   be genotoxic.

291   **Evaluation of suggested TPs for genotoxicity and mutagenicity:** Despite the non-genotoxic

292   and non-mutagenic effects of our photolytic mixtures, the (Q)SAR predictions suggested that a

293 few TPs of both MTL and ATL should be mutagenic and/genotoxic (Table 3, 4). Mixture

294 interferences (such as antagonism), the presence of relevant TPs in non-effective concentrations

295 and the use of only a few tester strains in the Ames test may have contributed to the lack of

296 observed mutagenicity or genotoxicity in the mixture during experimental testing. Further it

297 should be taken into account that (Q)SAR predictions are also not definitive toxicity assessment

298 tools but merely provides an estimate that may or not be the best for even well evaluated models

299 *(22)*. Nevertheless, the structural alerts of an aldehyde in TPs of both ß-blockers and the

300 phenylethyl alcohol of MTP 254 in model SALM2013 predicted TPs as mutagenic in Ames

301 test (Table S4, S5). Using several (Q)SAR models ("in *silico* test battery") to predict similar

302 endpoints can strengthen the predictions. Therefore although the Leadscope model predicted

303 none of the TPs from either PC to be mutagenic, the rule based Oasis Catalogic model shared

304 similar predictions with the statistical based SALM2013 model for mutagenicity in MTP 192

305 and MTP 234_1 (Table 3,4). Further, since the structural alerts found in the CASE Ultra models

306 were not part of either PC structure, it is possible that some of the TPs from both ß-blockers

307 have potential to be mutagenic to one or more of the Ames tester strains. Moreover, Leadscope

308 predicted some TPs for each substance as mutagenic to mammals and these were not predicted

309 as mutagenic in any of the Ames models used (Table 3,4).

310 Chromosome aberration (CA) formation was predicted for TPs with an addition of one or more

311 OH groups to the benzene ring (e.g. MTP 284_2, ATP 316_{1b}, ATP 281_1) or the inclusion of a

312 carbonyl group (e.g. MTP 234_2, ATP 281_1, ATP 254_{1a}) (Table S4, S5). Only the TPs ATP 281_5,

313 ATP 152 and MTP 234_2 were predicted to cause CA in both Leadscope and CASE Ultra models

314 (Table 3,4). CASE Ultra model A7S predicted ATL as MN inducing due to the structural alert

315 C-NH2 of carbamate group (Table S4). ß-blockers themselves were not active in *in vitro* tests

316 (bacterial and mammalian) for DNA damaging and repair but ATL was reported as MN

317 inducing *in vivo* (3, 10, 15). This same structural alert was featured in most of the TPs of ATL

318 predicted as MN inducing and as such may provide enough evidence to consider the TPs of

319 both ß-blocker for further *in vivo* testing (Table S4, S5). Other TPs such as ATP 152 and ATP

320 316_{1c} with more than one OH group added to the benzene ring and the longer side chain of ATP

321 152 and MTP 284_2 were also structural alerts for MN induction *in vivo* in CASE Ultra model

322 A7S.

323 A major reason for the seemingly contradictory outcomes of the *in silico* predictions and the

324 previously discussed *in vitro* experiments might be that experimental testing was conducted for

325 whole mixtures that might include the suspected mutagenic or genotoxic products only in

326 comparatively low concentrations. This can lead to the observation that a mixture is classified

327 negative even though it includes mutagenic or genotoxic components in low concentration or

328 low activity or both. Therefore, the observed increase in *umuC* induction might indicate that

329 there are some TPs with genotoxic potential formed. Therefore suspected genotoxic TPs should

330 be further assessed individually to allow a clear classification in terms of genotoxicity and

331 mutagenicity. Further, MTP 284 and MTP 254 were predicted genotoxic and mutagenic,

332 respectively, and were identified also as intermediates found in the genotoxic oxidative samples

333 of Šojic´et al. *(20)*. These two TPs along with MTP 192 and MTP 234_{1-2} would be a good

334 starting-point for individual TP genotoxicity characterization. Further ATP 275, ATP 281_{1-3}

335 ATP 316_{1c} were predicted genotoxic in all three mammalian genotoxic modules and therefore

336 could be prioritized for further mammalian genotoxicity testing.

337 **Implications of whole mixture toxicity analysis for the photolytic mixtures of ß-blockers:**

338 Our findings clearly showed that photolysis of the ß-blockers ATL and MTL can principally

339 lead to the formation of TPs with increased bacterial cytotoxicity. Moreover there was some

340 evidence that genotoxic TPs also might be formed during photolysis of the two ß-blockers. The

341 overarching repercussion from photolysis of beta ß-blockers would be that it can result in toxic

342 mixtures depending on the initial concentration of parent compound, duration of irradiation and

343 concentrations of individual TPs in the treated mixtures. Because of the limited sensitivity of

344 (bio)analytical methods, the initial identification and characterization of TPs should be

345 conducted at comparatively high concentrations to take all possible TPs into consideration. On

346 the other hand, the high complexity of resulting photolytic mixtures caused some difficulties in

347 identifying specific TPs of concern. (Q)SAR analysis has enabled the postulation of a few TPs

348 that may have contributed to the observed toxicity but the lack of their analytic standards

349 inhibited their further characterization. The same accounts for the investigation of the

350 environmental relevance of suggested toxic TPs that would demand targeted analytical

351 methods.

352 Nevertheless, since ß-blockers are baseline toxicants in mixtures exhibiting concentration

353 addition, this behavior cannot be excluded for the observed toxicity for mixtures inclusive of

354 their metabolites and TPs as some of them were estimated to be lipophilic. This is an area that

355 would need further investigation in understanding the potential risks of treating these

356 pharmaceuticals. If such a case is considered, it may be likely that photolysis treatment of ß-

357 blockers at much lower and more relevant concentrations could also produce toxic mixtures.

358 Rastogi et al. listed six more TPs after photolysis of MTL at 10 mg/L and most of these TPs

359 also increased with irradiation time *(6)*. While whole mixture toxicity analysis have provided

360 sufficient evidence of toxic effects from TPs formed during UV photolysis treatment,

361 characterization of individual TPs is necessary to define their effect mechanisms and respective

362 toxicodynamics. Further, toxicity assessment would have to extend beyond acute exposure to

363 more chronic testing systems as we have shown that long term exposure endpoints can be

364 equally or more sensitive to toxicity changes after any treatment process. Finally, the

365 environmental occurrence of suggested toxic TPs must be determined quantitatively to allow a

366 scientifically sound risk evaluation.

Supporting Information Available

Addition information is available on: i) Toxicity testing methodology, ii) Description of the QSAR models, iii) Structures of TPs, and iv) Structural alerts for all predicted genotoxic and bacterial cytotoxic TPs using the CASE Ultra models.

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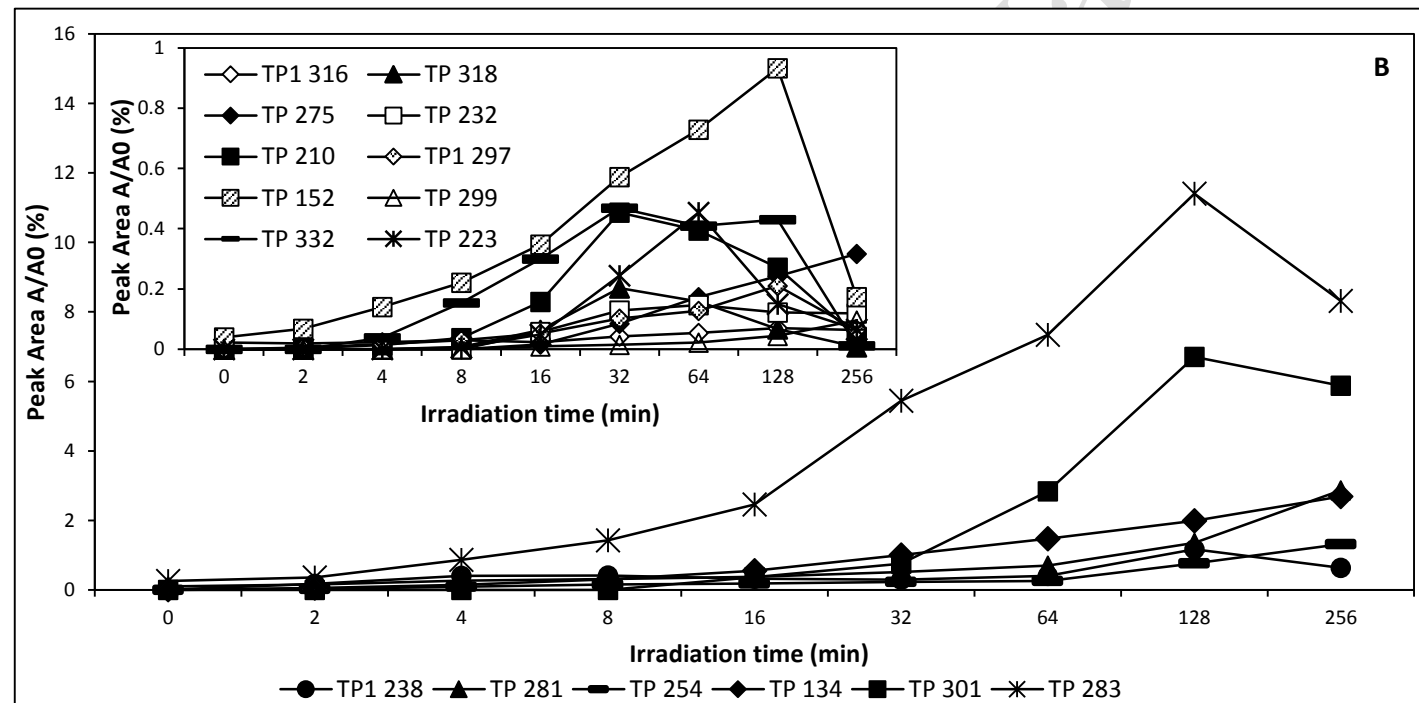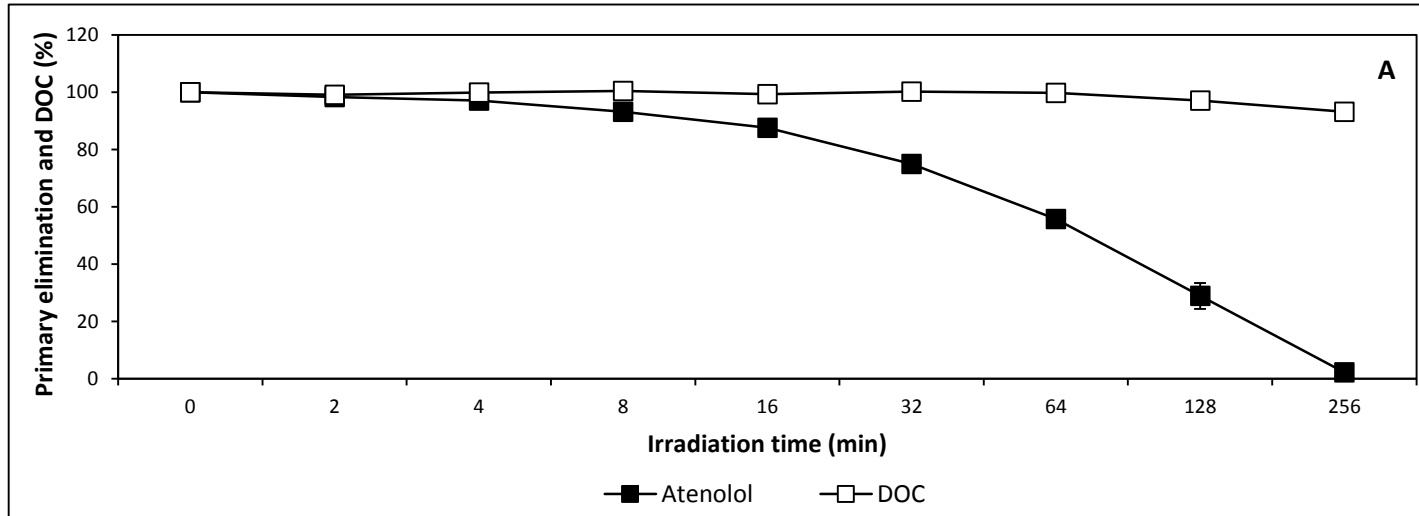
Figure 1: (a) Primary elimination of 100 mg/L Atenolol (ATL) and percentage Dissolved organic carbon (DOC). (b) Kinetic of formation of photo-transformation products of ATL (ATPs) *inset plot*: kinetic of formation of ATPs with peak area ratio < 1.
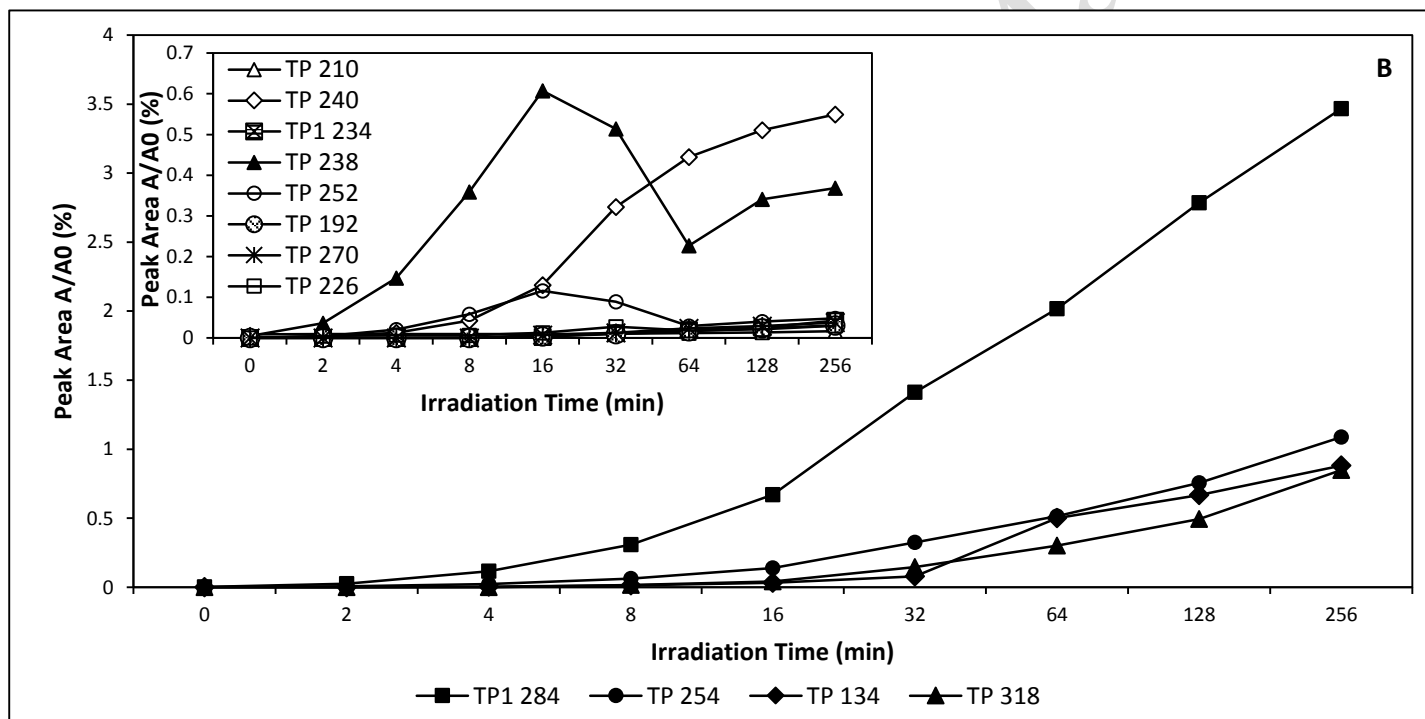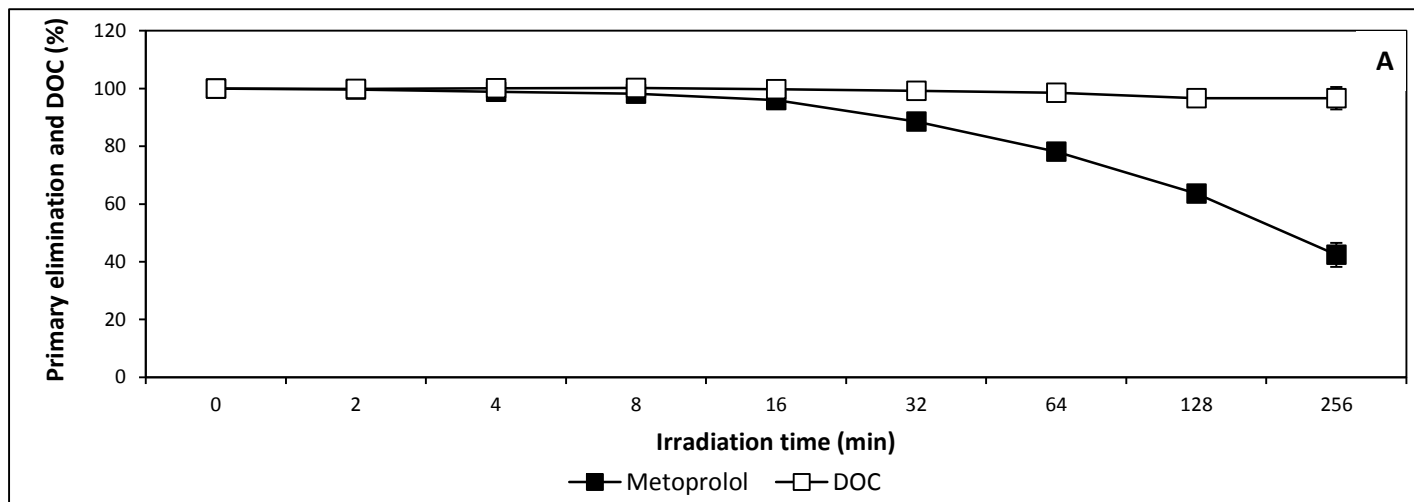
Figure 2: (a) Primary elimination of 400 mg/L Metoprolol (MTL) and percentage Dissolved organic carbon (DOC). (b) Kinetic of formation of photo-transformation products of MTL (MTPs) *inset plot*: kinetic of formation of MTPs with peak area ratio < 1.

Table 1: Genotoxicity characteristics of the mixture derived from the photolysis of 100 mg/L Atenolol (ATL).

Treatment	Dilution Factor	Ames Number of Revertants TA 98 -S9	+S9	TA 100 -S9	+S9	Umu Growth -S9	+S9	Induction Ratio -S9	+S9	*In vitro* Micronucleus (MN) Relative survival (%)	EMA+ (%)	Hypodiploid (%)	MN (%)
Millipore water	1.35	2±1	1±1	7±2	3±1								
	1.5					1.00±0.08	0.99±0.04	1.00±0.11	1.00±0.12				
	10									100±7.21	0.34±0.10	0.20±0.02	1.72±0.57
ATL 0 min	1.35	3±2	3±1	5±2	6±6								
	1.5					1.18±0.04	1.07±0.03	0.79±0.08	0.81±0.13				
	10									104.68±0.22	0.33±0.08	0.22±0.07	2.28±1.03
8 min	1.35												
	1.5					1.17±0.07	1.09±0.05	0.83±0.06	0.70±0.05				
	10												
16 min	1.35	1±1	2±2	4±1	1±1								
	1.5					1.09±0.03	1.03±0.01	0.98±0.02	0.78±0.03				
	10									89.30±0.57	0.57±0.03	0.27±0.01	2.21±0.12
32 min	1.35	2±1	1±1	8±3	3±2								
	1.5					1.05±0.04	1.01±0.03	0.97±0.09	0.80±0.09				
	10									69.48±4.53	1.54±0.35	0.38±0.06	1.77±0.04
	20									123.04±6.73	0.22±0.02	0.24±0.07	1.55±0.59

Time	Conc.												
64 min	1.35	3±2	3±1	5±2	3±1								
	1.5					1.06±0.08	1.00±0.05	0.99±0.12	0.73±0.05				
	10									103.15±10.24	0.40±0.25	0.34±0.02	1.99±0.16
128 min	1.35	2±1	2±1	8±1	3±2								
	1.5					0.96±0.09	1.00±0.04	1.05±0.05	0.82±0.16				
	10									103.21±3.26	0.36±0.10	037±0.30	1.48±0.01
256 min	1.35	3±2	1±1	9±2	2±1								
	1.5					0.90±0.05	0.97±0.02	1.37±0.01*	0.99±0.14				
	10									70.51±35.6	5.79±7.5	0.24±0.07	1.93±0.16
	20									116.10±2.67	0.19±0.07	0.28±0.10	1.35±0.01

*$p \leq 0.05$, **Bold and italic** indicates statistical significant values that can be classified as positive based on test criteria defined in Materials and Methods.

Table 2: Genotoxicity characteristics of the mixture derived from the photolysis of 400 mg/L Metoprolol (MTL).

Treatment	Dilution Factor	Ames Number of Revertants TA 98 -S9	+S9	TA 100 -S9	+S9	Umu Growth -S9	+S9	Induction Ratio -S9	+S9	*In vitro* Micronucleus (MN) Relative survival (%)	EMA+ (%)	Hypo-diploid (%)	MN (%)
Millipore water	1.35	1±1	2±2	5±3	4±3								
	1.5					1.00±0.08	0.99±0.04	1.00±0.11	1.00±0.12				
	10									100±7.21	0.34±0.10	0.20±0.02	1.72±0.57
MTL 0 min	1.35	1±1	2±2	4±2	2±2								
	1.5					1.06±0.02	1.05±0.03	0.93±0.09	0.76±0.07				
	40									110.45±0.49	0.34±0.06	0.16±0.01	1.83±0.71
8 min	1.35												
	1.5					1.01±0.05	1.02±0.03	0.95±0.09	0.79±0.07				
	40									109.11±15.067	0.47±0.16	0.18±0.08	1.92±0.80
16 min	1.35	2±2	1±1	7±3	5±3								
	1.5					0.96±0.03	1.02±0.04	1.01±0.02	0.70±0.12				
	40									*46.32±4.02**	*5.25±1.37**	0.28±0.12	4.27±2.00
	80									109.60±3.31	0.18±0.00	0.19±0.01	1.70±0.36
32 min	1.35	1±1	1±1	5±3	5±5								

	1.5					0.97±0.02	1.04±0.03	1.04±0.04	0.85±0.15				
	40									111.04±2.05	0.32±0.02	0.18±0.02	2.30±0.42
64 min	1.35	2±1	1±2	5±2	5±3								
	1.5					0.98±0.01	0.97±0.00	1.10±0.11	0.70±0.10				
	40									116.55±19.82	0.90±0.72	0.15±0.06	1.59±0.14
128 min	1.35	2±1	3±2	5±2	5±2								
	1.5					0.92±0.07	0.99±0.02	1.13±0.02	0.78±0.04				
	40									71.35±13.47	2.50±1.61	0.26±0.07	2.92±0.38
	80									107.06±1.01	0.13±0.02	0.15±0.04	1.12±0.11
256 min	1.35	1±1	1±1	7±2	4±2								
	1.5					0.93±0.09	0.94±0.04	1.25±0.15*	0.91±0.16				
	40									*33.79±3.78**	*12.11±1.86**	0.39±0.19	5.30±2.40
	80									113.95±9.12	0.17±0.05	0.17±0.04	1.42±0.42

*p≤0.05, **Bold and italic** indicates statistical significant values that can be classified as positive based on test criteria defined in Material and Methods.

Table 3: Quantitative structure-activity relationship (QSAR) predictions of selected physico-chemical and toxicity properties for Atenolol (ATL) and its transformation products (ATPs).

TP ID	Physico-chemical Predictions[1]		Toxicity Predictions										
			CASE Ultra						Leadscope				Oasis
	Log kow	Log BCF	Genotoxicity			Mutagenicity		Bacterial Toxicity	Genotoxicity		Mutagenicity		Mutagenicity
			A		B	C		D	A	B	C	E	C
			A7U	A7V	A7S	GT1_A7B	SALM2013	AUA					
ATL	0.16	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 134	-0.88	0.5	-	-	-	-	-	OD	-	-	-	-	-
ATP 275	2.15	0.5	+	+	+	-	-	+	-	-	-	+	+
ATP 299	-1.24	0.5	-	-	+	-	-	-	+	-	-	-	-
ATP 223	-0.12	0.5	OD	OD	+	OD	OD	OD	+	-	-	-	-
ATP 152	0.07	0.5	-	+	+	-	-	+	+	-	-	+	-
ATP 318	-5.4	0.5	-	OD	OD	-	-	OD	-	-	-	-	-
ATP 254$_{1a}$	1.44	0.36	+	-	-	-	-	OD	-	-	-	-	-
ATP 254$_{1b}$	0.4	0.5	+	-	+	-	-	OD	-	-	-	-	-
ATP 238	1.14	0.16	+	-	-	-	-	OD	-	-	-	-	-
ATP 232	2.46	1.29	OD	OD	OD	OD	+	+	OD	OD	OD	OD	-
ATP 316$_{1a}$	-4.01	0.5	-	-	-	-	-	-	-	-	-	-	-
ATP 316$_{1b}$	-4.01	0.5	+	-	-	-	-	OD	-	-	-	-	-
ATP 316$_{1c}$	-3.76	0.5	+	+	+	-	-	OD	-	-	-	-	-
ATP 332	-4.52	0.5	-	-	-	-	-	OD	+	-	-	-	-
ATP 297$_{1}$	-0.32	0.5	+	-	+	-	-	+	-	-	-	-	-
ATP 297$_{2}$	-1.52	0.5	-	-	+	-	-	OD	+	-	-	-	-

ID	[1]												
ATP 210	-0.4	0.5	-	OD	OD	-	-	-	+	-	-	-	-
ATP 281_1	0.24	0.5	+	+	+	-	-	OD	-	-	-	-	-
ATP 281_2	-0.01	0.5	+	+	+	-	-	OD	-	-	-	+	-
ATP 281_3	-0.06	0.5	+	+	+	-	-	OD	-	-	-	-	-
ATP 281_4	-0.4	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 281_5	0.47	0.5	OD	+	+	-	-	OD	+	-	-	+	-
ATP 283_1	-0.51	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 283_2	-0.76	0.5	+	-	+	-	-	OD	-	-	-	-	-
ATP 283_3	-0.41	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 283_4	-1.16	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 283_5	-1.16	0.5	OD	OD	+	-	-	OD	-	-	-	-	-
ATP 283_6	-1.08	0.5	-	-	+	-	-	OD	-	-	-	-	-
ATP 283_7	-0.42	0.5	OD	OD	+	-	-	OD	-	-	-	-	-
ATP 301_1	-2.5	0.5	-	OD	+	+	+	+	-	-	-	-	-
ATP 301_2	-2.63	0.5	-	-	+	+	-	+	-	-	-	-	-
ATP 301_3	-2.63	0.5	-	-	+	+	-	+	-	-	-	-	-
ATP 301_4	-1.98	0.5	OD	OD	+	OD	-	+	-	-	-	-	-
ATP 301_5	-2.63	0.5	-	-	+	+	-	+	-	-	-	-	-
ATP 301_6	-2.5	0.5	-	OD	+	+	+	+	-	-	-	-	-

1= EpiSuite KOWWIN v1.68 estimate

A= In vitro Chromosome Aberration; B= In vivo Micronucleus Composite; C= Salmonella Mutagenicity; D= Environmental Bacteria Toxicity; E= Mammalian Mutagenicity

A7S: MN in vivo; A7U: Chromosome aberration composite in vitro; A7V: Chromosome aberration in CHO cells; SALM2013: Mutagenicity in Salmonella (improved); GT1_A7B: updated A7B; AUA:Toxicity to Environmental Bacteria

 - =Negative alert for activity; + = Positive alert for activity; OD= molecule fragments are out of domain

Table 4: Quantitative structure-activity relationship (QSAR) predictions of selected physico-chemical and toxicity properties for Metoprolol (MTL) and its transformation products (MTPs).

TP ID	Physico-chemical Predictions[1]		Toxicity Predictions										
			CASE Ultra						Leadscope				Oasis
	Log kow	Log BCF	Genotoxicity			Mutagenicity		Bacterial Toxicity	Genotoxicity		Mutagenicity		Mutagenicity
			A	B		C		D	A	B	C	E	C
			A7U	A7V	A7S	GT1_A7B	SALM2013	AUA					
MTL	1.88	0.65	-	-	-	-	-	OD	-	-	-	-	-
MTP 134	-0.88	0.5	-	-	-	-	-	OD	-	-	-	-	-
MTP 192	2.74	1.48	+	OD	OD	-	+	+	-	-	-	-	+
MTP 234$_1$	2.29	1.18	OD	OD	OD	-	+	+	OD	OD	-	OD	+
MTP 234$_2$	3.07	1.70	+	-	-	-	-	OD	+	-	-	+	-
MTP 226	0.32	0.5	-	OD	OD	-	-	-	+	-	-	-	-
MTP 240	0.91	0.5	-	-	-	-	-	OD	-	-	-	-	-
MTP 284$_1$	1.21	0.21	-	-	-	-	-	OD	-	-	-	-	-
MTP 284$_2$	0.96	0.5	+	-	+	-	-	OD	-	-	-	-	-
MTP 270	-0.14	0.5	-	-	-	-	-	OD	-	-	-	-	-
MTP 254	1.4	0.34	-	-	-	-	+	OD	-	-	-	-	-
MTP 238	2.46	1.03	-	-	-	-	-	+	-	-	-	-	-
MTP 252	0.97	0.5	-	-	-	-	-	OD	-	-	-	-	-
MTP 318	-2.09	0.5	-	OD	OD	+	+	+	-	-	-	-	-
MTP 210	-0.4	0.5	-	OD	OD	-	-	-	+	-	-	-	-

1= EpiSuite KOWWIN v1.68 estimate

A= In vitro Chromosome Aberration; B= In vivo Micronucleus Composite; C= Salmonella Mutagenicity; D= Environmental Bacteria Toxicity; E= Mammalian Mutagenicity

A7S: MN in vivo; A7U: Chromosome aberration composite in vitro; A7V: Chromosome aberration in CHO cells; SALM2013: Mutagenicity in Salmonella (improved); GT1_A7B: updated A7B; AUA:Toxicity to Environmental Bacteria

 - =Negative alert for activity; + = Positive alert for activity; OD= molecule fragments are out of domain

Figure 3: Growth inhibition (*GI14h*) short term luminescence inhibition (*LI30min*) and long term luminescence inhibition (*LI24h*) in *V. fischeri* caused by exposure to mixtures derived from photolysis of (A) Atenolol and (B) Metoprolol. Concentrations for treatment were 100 mg/L (Atenolol) and 400 mg/L (Metoprolol). Samples were twofold diluted in the test media.

Supplementary data

for

Genotoxicity and cytotoxicity characterization of mixtures generated by from photolysis of the ß-blockers Atenolol and Metoprolol using a combination of experimental and (Q)SAR approaches

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Text S1: Toxicity Testing

Photolytic samples were taken and left to stand for 24 h at 4°C to reduce the presence of short lived oxidative agents. Then, the samples were sterile filtered (0.2 μm) and frozen in aliquots at -150°C before the assay. All tests were performed at least twice with 3 replicates per bacterial test and 2 replicates for *in vitro* MN test. Sample pH was measured and adjusted to pH 7.0 ± 0.2, if needed, prior to performing bioassays.

Modified luminescent bacteria test (modified LBT)

Materials: Supplemented seawater complete (SSWC) media containing 5% (w/v) peptone from casein, 0.5% (w/v) yeast extract, 0.3% (w/v) glycerol, 3% (w/v) NaCl, 44.2 mM NaH_2PO_4, 12.1 mM K_2HPO_4, $MgSO_4$, 0.8 mM 7 H_2O, 3.8 mM $(NH_4)_2HPO_4$ adjusted to pH 7. Positive controls were 3,5-dichlorophenol (3,5-DCP, 97%, 591-35-5, Sigma-Aldrich Chemie GmbH) and chloramphenicol (CAM, 98%, 56-75-7, Sigma-Aldrich Chemie GmbH).

Test organism: The freeze-dried luminescent bacteria (*V. fischeri* NRRL-B-11177) for the LBT were purchased from Hach-Lange GmbH, Düsseldorf.

Method: On the day before testing, a pure culture of the luminescent bacteria strain *Vibrio fischeri* NRRL-B-11177 was prepared in SSWC media. After overnight incubation at 20 °C for 22-24 h, the culture was diluted with fresh SSWC media to an initial cell density of 20 formazine turbidity units (FTU) and subsequently transferred to into a 96-well plate. After 30 min of pre-incubation at 15 °C, an initial measurement of luminescence emission and optical density (578 nm) was conducted, using a multimode micro-plate reader (Varioskan Flash, Thermo). Subsequently, the photolytic samples were added to the test cultures in triplicates and a kinetic measurement of luminescence, as well as optical density, was carried out for 24 h at 15 °C. In each experiment, 3,5-dichlorophenol

and chloramphenicol were used as positive controls at final assay concentrations of 4.5 mg/L and 0.05 mg/L, respectively. Prior to testing, all investigated samples were supplemented with NaCl to a final salinity of 2% (w/v).

Analysis: The raw data were normalized to percent inhibition in relation to the negative controls. This was conducted for three different endpoints which were: short-term luminescence inhibition after 30 min (LI_{30min}), long-term luminescence inhibition after 24 h (LI_{24h}) and growth inhibition after 14 h (GI_{14h}). Calculations and data analysis were performed using the recommendation of Menz et al. (2013). Regardless of the measured endpoint, 20% was considered as the threshold value for inhibition. Analysis of concentration-response relationships of parent compounds was performed by plotting the normalized inhibition values against the respective concentrations followed by a logistic regression analysis, applying the function "Four Parameter Logistic Curve" (1).

$$y=\min+(\max-\min)/(1+(x/EC_{50})\text{-Hillslope}) \tag{1}$$

where,

y   is the inhibition in %

min   is the bottom of the curve (0%)

max   is the top of the curve (100%)

Hillslope   is the slope of the curve at its midpoint

Bacterial mutagenicity: Ames MPF 98/100 Aqua

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

Test organisms: Salmonella typhimurium TA 98 and TA 100 were supplied by Xenometrix AG.

Method: An overnight culture was grown until the optical density at 600 nm (OD_{600nm}) reached ≥ 2.0. In a 24-well plate, bacteria were added to the exposure medium and the samples in the presence or absence of metabolic activation (+/- S9). The plates were then exposed for 90 min at 37°C (MaxQ600, Thermo Scientific) while shaking (250 rpm). After which, the exposed mixture was diluted with reversion indicator medium and transferred into 384-well plates for 48 h incubation at 37°C. During this time, the pH dependent reversion indicator dye would change from purple to yellow in the presence of bacterial growth. The yellow coloured wells were scored to give the number of revertant wells out of the 48 wells for each irradiation time. Positive controls used for the MPF assay without metabolic activation were a mixture of 4-NQO and 2-NF at a final concentration of 0.1 µg/ml and 2 µg/ml respectively. 2-AA at a final concentration of 5 µg/ml was used for the test performed with S9 mix. Millipore water was used as the negative control.

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

Analysis: Classification as positive for mutagenicity occurred when the response was ≥ 2 fold increase in the number of revertants over that of the baseline number of revertants (the mean revertants of the negative control plus standard deviation (SD)). Variations between observed effects at different sampling times were evaluated by one way analysis of variance (ANOVA), following post hoc multiple comparisons (Dunnett's method), in which the untreated samples (0 min) were defined as the control group.

Bacterial genotoxicity: umu-Test

Materials: TGA- culture medium, B-Buffer, phosphate buffer and stop reagent were prepared according to ISO 13829. Ortho-nitrophenol-ß-d-galactopyranoside (ONPG) was obtained from Carl Roth GmbH. Positive controls included 4-nitroquinoline-1-oxide (4-NQO) and 2-aminoanthracene (2-AA) (Sigma-Aldrich Chemie GmbH) dissolved in dimethyl sulfoxide (DMSO). Aroclor 1254-induced rat liver homogenate (S9) was bought from Xenometrix AG while the co-factor salt NADP sodium salt and D-glucose-6-phsophate di sodium salt were obtained from Carl Roth GmbH and Applichem, respectively.

Test organism: Salmonella typhimurium TA1535 psk 1002 was bought from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig).

Method: The umu-test for genotoxicity testing was performed according to ISO 13829 protocol. An overnight culture of *S. typhimurium* TA1535 psk 1002 was grown for 15 h at 37°C shaking at 250 rpm (MaxQ600, Thermo Scientific). After that the OD_{600nm} was measured and a 1:10 dilution of the overnight culture was re-incubated until the bacteria were in log phase (OD_{600nm} = 0.4-0.6). Then, plate A was prepared containing the samples, 10x concentrated TGA medium, and bacteria with or without S9 mix. Plate A was incubated for 2 h at 37 °C while shaking at 250 rpm. Then, plate B was prepared by a 1:10 dilution of the contents of plate A in TGA media. Plate B was incubated further for 2 h at 37 °C and 250 rpm. Thereafter, the OD_{600nm} of the contents of plate B was measured using BioTek synergy HT. Then the β-galactosidase activity was determined by placing 30 μl of the contents of plate B to a new plate (plate C) containing the B-Buffer and following with the addition of the ONPG solution. Plate C was incubated for 30 min at 28°C, 250 rpm, after which the reaction was stopped using the stop reagent. Then the absorbance at 420 nm of plate C was measured to calculate the induction ratio of the *umuC* gene.

Calculation and Analysis: The calculation of growth (G) and induction ratio (IR) was performed according to ISO 13829. Classification as positive for *umuC* induction was assessed when the IR > 1.5 and G ≥ 0.5. Significant changes of *umuC* induction after photolysis were determined by ANOVA (Dunnett method, overall significance level p≤0.05) using the samples collected at 0 min irradiation as the control group.

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

Materials: Reagents for the staining and lysis of cells for flow cytometry analysis were purchased from Litron Laboratories, Rochester, USA (*In Vitro* MicroFlow kit). The content of the *In Vitro* MicroFlow kit included Buffer Solution, Nuclei Acid Dye A Solution (EMA dye), RNase Solution, Nucleic Acid Dye B Solution (SYTOX Green dye) and Incomplete Lysis Solutions 1 and 2. Invitrogen 6 μm PeakFlow™ Green flow cytometry reference beads were bought from Life Technologies. Positive controls used were mitomycin C (MMC) and vinblastin sulphate (VB) dissolved in dimethyl sulphoxide (DMSO) all obtained from Sigma Aldrich Chemie GmbH.

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

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

Method: CHO-K1 Cells were maintained for at least two weeks prior to the test in the combined HAM's F12 medium at 37°C, 5% CO_2 in a humid atmosphere (Thermo Scientific MIDI 40 CO_2 incubator). Then the cells were trypsinized and plated at 12,000 cells/ml/well into a 24 well pate. The cells were allowed to attach for 44 h at 37°C, 5% CO_2 in a humid atmosphere. After that the media was aspirated and replaced with 1 ml

solutions of the samples in media. The positive controls were MMC at 0.1 µg/ml and VB at 0.01 µg/ml. The 24 well plate was then placed for 30 h at 37°C, 5% CO_2 in a humid atmosphere. Then the cell staining and lysis protocol of the *InVitro* MicroFlow Kit was followed.

Briefly, the cell staining and lysis protocol included placing the 24 well plate on ice for 20 minutes after the 48 h exposure time. After that, the solution was aspirated and 300 µl of EMA solution was added to each well. The plate was exposed to fluorescence light for 30 min to undergo photo activation of the dye. Then the EMA dye was aspirated and the cells were washed with 1ml of cold buffer solution. 500 µl of complete lysis solution 1 was added to each well and incubated for 1 h at 37°C, 5% CO_2 in a humid atmosphere. Cytometry reference beads were added to complete lysis solution 2 which was later added to each well after the incubation period. The plate was then kept at room temperature in dark for at least 30 min prior to flow cytometry analysis.

Analysis: Flow cytometry analysis was performed using BD Biosciences FACSCalibur with data acquired according to the gatings and settings recommended by the *InVitro* MicroFlow Kit protocol. 20,000 nucleated cells per samples were analysed for MN formation, cytotoxicity (EMA+ and relative survival), and cell cycle perturbation. The validity criteria for the test were defined as suggested by Bryce et al (2010). Samples were classified as positive when MN frequency ≥3 SD of the mean negative control value. Samples were determined to be cytotoxic if there was 50% reduction in relative survival to negative control. The statistical significance determined by ANOVA (Dunnett method, overall significance level $p \leq 0.05$) using 0 min irradiation as the control group was also used to assist in the determination of positive results.
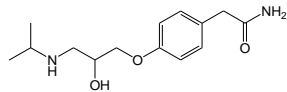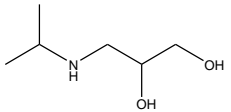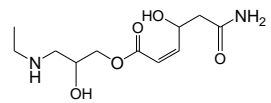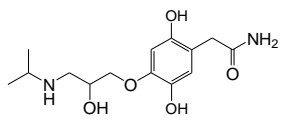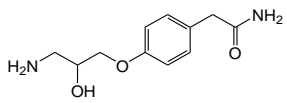
Table S1: Description of the applied QSAR models

QSAR software	Model	Description	Endpoint	References
CASE Ultra V.1.5.0.1 (MultiCASE Inc.)	A7U Chromosome Aberration *In vitro* composite	model develop from FDA databases	*In vitro* Chromosome aberration	Chakravarti et al., 2012; Saiakhov et al., 2013
	A7V Chromosome Aberration *In vitro* CHO cells	model develop from FDA databases		
	A7S Micronucleus *In vivo* composite	model develop for rat and mice data from FDA databases	*In vitro* MN formation	
	GT1_A7B Mutagenicity for 7 major strains of *S. typhimurium*	model developed *S. typhimurium* strains (+/- S9) based on FDA database	Bacterial mutagenicity	
	SALM 2013 Salmonella mutagenicity	model developed *S. typhimurium* strains (+/- S9) as a composite from Aggregated Salmonella mutagenicity from NTP, GENETOX, RTECS, regulatory agencies, and other sources database		
	AUA MICROTOX toxicity to environmental bacteria	model developed for toxicity to environmental bacteria using MICROTOX EPA	Bacterial toxicity	
Leadscope V. 3.2.4-1	*In vitro* Chromosome Aberration average model	model develop with CHO, CHL, HPBL and other mammalian cell culture from 2012 SAR Genetox Database from Leadscope	*In vitro* Chromosome abberation	Roberts et al., 2000

	Micronucleus *in vivo* composite model	model develop with rat and mice data from 2012 SAR Genetox Database from Leadscope	*In vitro* MN formation	
	Salmonella composite	model develop with data from *S. typhimurium* TA 97, TA 98, TA 100, TA1535, TA 1536, TA 1537, TA 1538 from 2012 SAR Genetox Database from Leadscope	Bacterial mutagenicity	
	Mammalian mutation *in vitro*	model develop for mammalian mutation including mouse lymphoma mutation gene mutation assays at the thymidine kinase (tk) locus using L5178Y cells in culture from 2012 SAR Genetox Database from Leadscope	Mammalian mutagenicity	
Oasis	Mutagenicity v .04	model developed with data from *S. typhimurium*+/-S9 using NTP database	Bacterial mutagenicity	Laboratory of Mathematical Chemistry, 2014

Table S2: List of detected transformation products of Atenolol

Peak ID	Retention time (min)	m/z	Structure ID	SMILES code	Proposed structure
ATL	10.9	267.1	ATL	NC(CC1=CC=C(OCC(O)CNC(C)C)C=C1)=O	
TP134	2	134.1	ATP134	OCC(CNC(C)C)O	
TP275	2	275.2	ATP275	O=C(OCC(O)CNC(C)C)/C=C\C(CC(N)=O)O	
TP299	3.3	299.1	ATP299	NC(CC1=C(O)C=C(OCC(O)CNC(C)C)C(O)=C1)=O	
TP225	5.5	225.1	ATP225	NC(CC1=CC=C(OCC(O)CN)C=C1)=O	

TP151[1-2]	9.1, 9.5	151.1	ATP151[1]	CC1=CC=C(OCCC)C=C1	
			ATP151[2]	CC(COC1=CC=CC=C1)=O	
TP207	9.2	207.1	ATP207	N=CCC1=CC=C(OCC(O)C=N)C=C1	
TP265	9.4	265.1	ATP265	NC(CC1=CC=C(OCC(CNC(C)C)=O)C=C1)=O	
TP193	9.5	193.1	ATP193	NCCC1=CC=C(O/C=C/CN)C=C1	
TP223	10.7	223.1	ATP223	NC(CC1=CC=C(OCC(O)C=N)C=C1)=O	
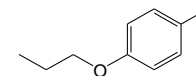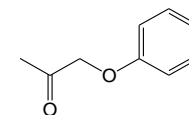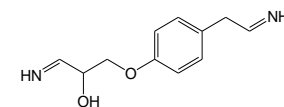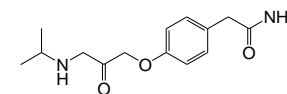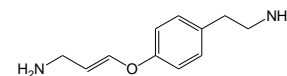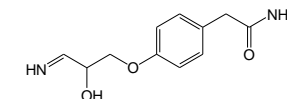
TP309[1-2]	11.2, 14.2	309.2	ATP309[1]	NC(C(C(C1=CC=C(OCC(O)CNC(C=O)C=O)C=C1)=O)=O	
			ATP309[2]	NC(C(C1=CC=C(OC(C(O)C(NC(C)C)=O)=O)C=C1)=O)=O	
TP152	11.7	152.1	ATP152	NC(CC1=CC=C(O)C=C1)=O	
TP176	12	176	ATP176	C=CC1=CC=C(O/C=C/CN)C=C1	
TP194	12	194.1	ATP194	OCCC1=CC=C(O/C=C/CN)C=C1	
TP387	12	387.1	ATP387	OCCC1=CC=C(O/C=C/CNNC/C=C/OC2=CC=C(CCO)C=C2)C=C1	
TP318	12.2	318.2	ATP318	OC(CC(C=O)\C=C(O)/C(OCC(O)CNC(C)C)C=O)=O	
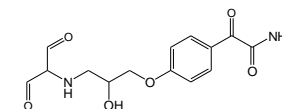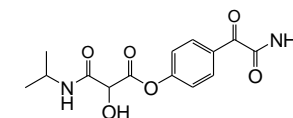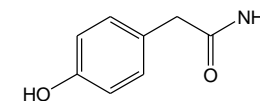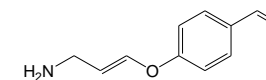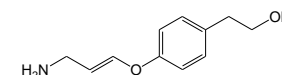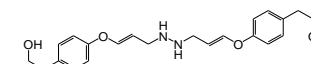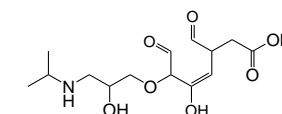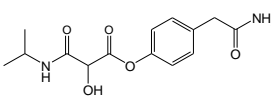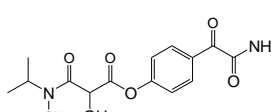
TP254	12.3	254.1	ATP254$_{1a}$	OC(CNC(C)C)COC1=CC(O)=C(C=O)C=C1	
			ATP254$_{1b}$	OC(CNC(C)C)COC1=CC=C(C=O)C=C1O	
TP295$_{1-2}$	12.6, 17.6	295.2	ATP295$_1$	NC(CC1=CC=C(OC(C(O)C(NC(C)C)=O)=O)C=C1)=O	
			ATP295$_2$	NC(C(C1=CC=C(OCC(O)C(NC(C)C)=O)C=C1)=O)=O	
TP238	12.9	238.1	ATP238	OC(CNC(C)C)COC1=CC=C(C=O)C=C1	
TP325	12.9	325.2	ATP325	NC(C(C1=CC=C(OC(C(O)C(N(O)C(C)C)=O)=O)C=C1)=O)=O	

TP253	13.1	253.1	ATP253	NC(CC1=CC=C(OC(C(C(N)=O)O)=O)C=C1)=O	
TP232	13.2	232.1	ATP232	O=CCC1=CC=C(O/C=C/C=N/C(C)C)C=C1	
TP316	13.2	316.2	ATP316$_{1a}$	OC(C(O)C1=C(O)C=C(OCC(O)CNC(C)C)C(O)=C1)=O	
			ATP316$_{1b}$	OC(C(O)C1=C(O)C(O)=C(OCC(O)CNC(C)C)C=C1)=O	
			ATP316$_{1c}$	OC(C(O)C1=C(O)C=C(OCC(O)CNC(C)C)C=C1O)=O	
TP332	13.5	332.3	ATP332	OC(CC1=C(O)C(O)=C(OCC(O)CNC(C)C)C(O)=C1O)=O	
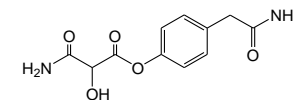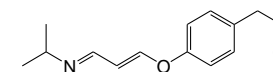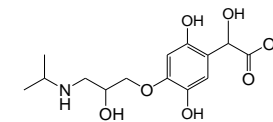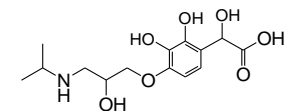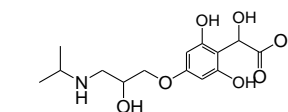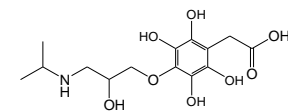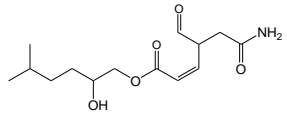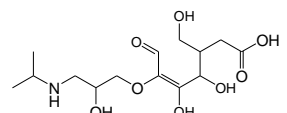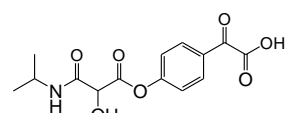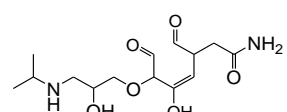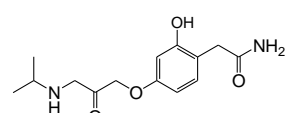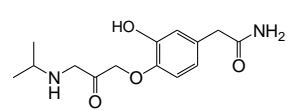
TP285	13.8	285.3	ATP285	NC(C/C(CO)=C/C=C(O/C=C/CNC(C)C)\CO)=O
TP249	14	249.1	ATP249	NC(CC1=CC=C(O/C=C/CNC(C)C)C=C1)=O
TP297₁₋₂	14.1, 17.6	297.1	ATP297₁	NC(C(C1=C(O)C=C(OCC(O)CNC(C)C)C=C1)=O)=O
			ATP297₂	NC(CC1=C(O)C=C(OCC(O)C(NC(C)C)=O)C=C1)=O
TP210	14.6	210	ATP210	O=CCC1=CC=C(OCC(O)CN)C=C1
TP419	14.6	419.1	ATP419	O=CCC1=CC=C(OCC(O)CNNCC(O)COC2=CC=C(CC=O)C=C2)C=C1

TP287	14.8	287.3	ATP287	O=C(OCC(O)CNC(C)C)/C=C\C(CC(N)=O)C=O
TP336	14.8	336.3	ATP336	OC(CC(CO)C(O)\C(O)=C(OCC(O)CNC(C)C)/C=O)=O
TP310	17.3	310.2	ATP310	OC(C(C1=CC=C(OC(C(O)C(NC(C)C)=O)=O)C=C1)=O)=O
TP317	17.6	317.2	ATP317	NC(CC(C=O)\C=C(O)/C(OCC(O)CNC(C)C)C=O)=O
TP281$_{1-5}$	Many	281.2	ATP281$_1$	NC(CC1=C(O)C=C(OCC(CNC(C)C)=O)C=C1)=O
			ATP281$_2$	NC(CC1=CC(O)=C(OCC(CNC(C)C)=O)C=C1)=O

			ATP281₃	NC(C(O)C1=CC=C(OCC(CNC(C)C)=O)C=C1)=O	
			ATP281₄	NC(CC1=CC=C(OC(O)C(CNC(C)C)=O)C=C1)=O	
			ATP281₅	NC(CC1=CC=C(OCC(C(O)NC(C)C)=O)C=C1)=O	
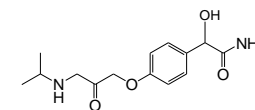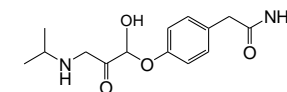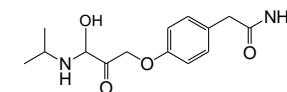
TP283₁₋₇	Many	283.2	ATP283₁	NC(CC1=C(O)C=C(OCC(O)CNC(C)C)C=C1)=O	
			ATP283₂	NC(CC1=CC(O)=C(OCC(O)CNC(C)C)C=C1)=O	
			ATP283₃	NC(C(O)C1=CC=C(OCC(O)CNC(C)C)C=C1)=O	
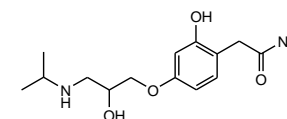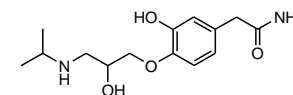
| | | | ATP283₄ | NC(CC1=CC=C(OC(O)C(O)CNC(C)C)C=C1)=O | |
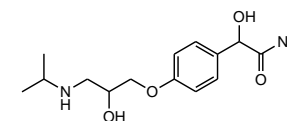
ATP283₄   NC(CC1=CC=C(OC(O)C(O)CNC(C)C)C=C1)=O
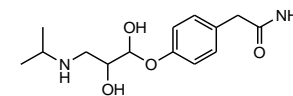
ATP283₅   NC(CC1=CC=C(OCC(O)C(O)NC(C)C)C=C1)=O
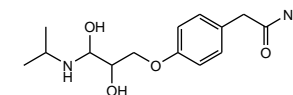
ATP283₆   NC(CC1=CC=C(OCC(O)CNC(C)CO)C=C1)=O
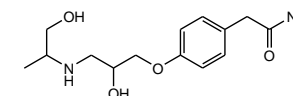
ATP283₇   NC(CC1=CC=C(OCC(O)CNC(C)(O)C)C=C1)=O
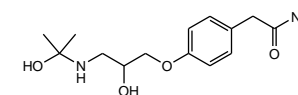
TP301₁₋₆   Many   301.2   ATP301₁   NC(C/C(CO)=C/C=C(OCC(O)CNC(C)C)\C=O)=O
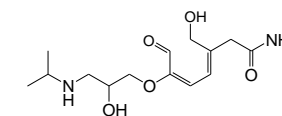
ATP301₂   NC(CC(O)\C=C/C(OCC(O)CNC(C)C)=C\C=O)=O
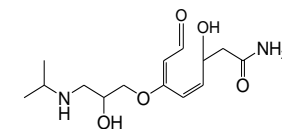
ATP301$_3$ NC(C/C(\C=C/C(O)OCC(O)CNC(C)C)=C/C=O)=O
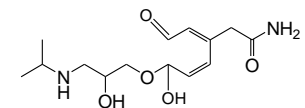
ATP301$_4$ NC(C/C(CC=O)=C/C=C(O)/OCC(O)CNC(C)C)=O
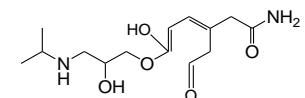
ATP301$_5$ NC(CC(O)/C=C\C(OCC(O)CNC(C)C)=C/C=O)=O
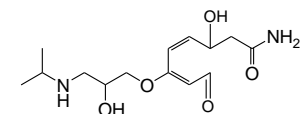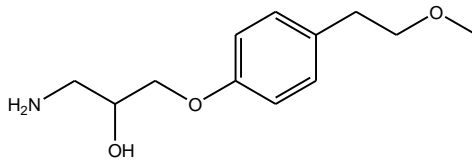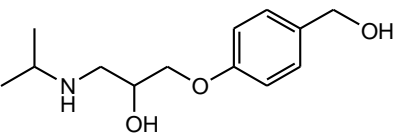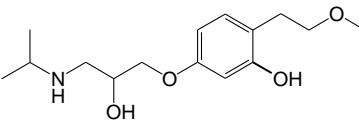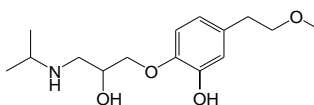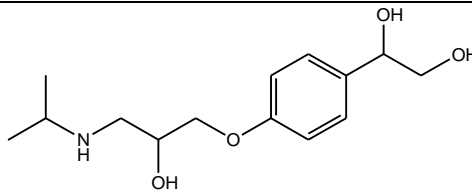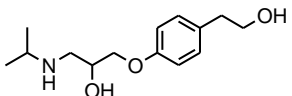
ATP301$_6$ NC(C/C(C=O)=C/C=C(OCC(O)CNC(C)C)\CO)=O
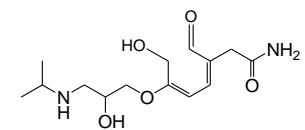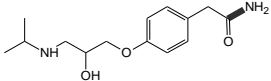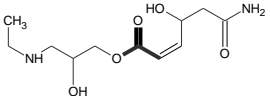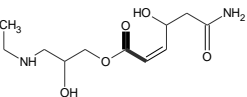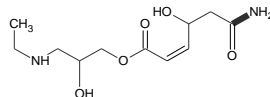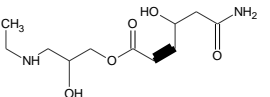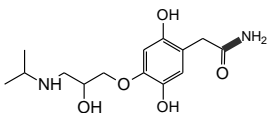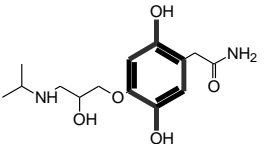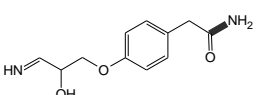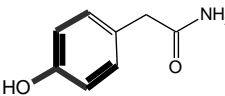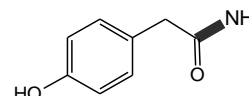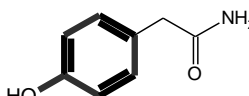
Table S3: List of detected transformation products of Metoprolol

Peak ID	Retention time (min)	m/z	Structure ID	SMILES code	Proposed structure
MTL	16.1	268.1	MTL	COCCC1=CC=C(OCC(O)CNC(C)C)C=C1	
TP134	2.5	134.2	MTP134	OCC(O)CNC(C)C	
TP192	3.5	192.1	MTP192	CC(C)NC/C=C/OC1=CC=CC=C1	
TP234$_{1-2}$	4.4, 7.1	234.1	MTP234$_1$	c1(OC=CCNC(C)C)ccc(CC=O)cc1	
			MTP234$_2$	c1(C=C)ccc(OCC(=O)CNC(C)C)cc1	
TP302	10.7	302.2	MTP302	COCC/C(\C=C/C(OCC(O)CNC(C)C)=C/O)=C\O	

TP226	11.2	226.1	MTP226	COCCC1=CC=C(OCC(O)CN)C=C1	
TP240	11.9	240.1	MTP240	CC(C)NCC(O)COC1=CC=C(CO)C=C1	
TP284[1-2]	12.3, 15.1	284.1	MTP284[1]	COCCC1=CC=C(OCC(O)CNC(C)C)C=C1O	
			MTP284[2]	COCCC1=CC=C(OCC(O)CNC(C)C)C(O)=C1	
TP270	12.4	270.1	MTP270	OCC(O)C1=CC=C(OCC(O)CNC(C)C)C=C1	
TP254	12.7	254.1	MTP254	OCCC1=CC=C(OCC(O)CNC(C)C)C=C1	

TP238		13.4	238.1	MTP238	CCC1=CC=C(OCC(O)CNC(C)C)C=C1

TP252		14.4	252.1	MTP252	O=CCC1=CC=C(OCC(O)CNC(C)C)C=C1

TP210		14.5	210	MTP210	c1(OCC(O)CN)ccc(CC=O)cc1

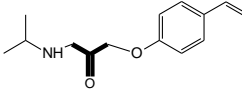|					
TP318	several		318.1	MTP318	OCC/C(CO)=C/C=C(OC(O)C(O)CNC(C)C)\C=O

Table S4: Positive alerts for Genotoxicity and bacterial toxicity according to CASE Ultra models prediction for Atenolol and selected transformation products

	Genotoxicity			Mutagenicity		Bacterial Toxicity
	A		B	C		D
	A7U	A7V	A7S	GT1_A7B	SALM2013	AUA
ATL						
ATP275						
ATP299						
ATP223						
ATP152						
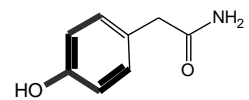
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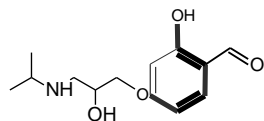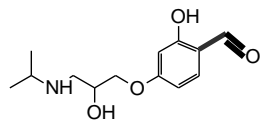
ATP254₁ᵦ
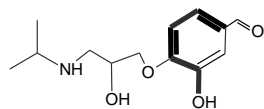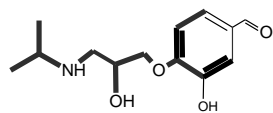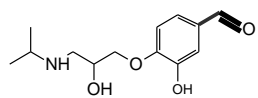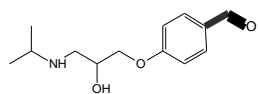
ATP238

ATP232
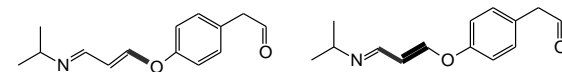
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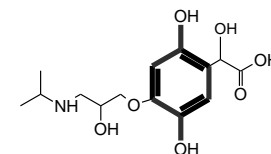
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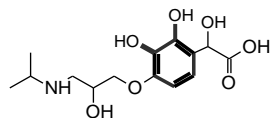
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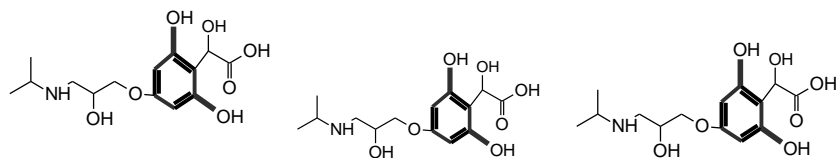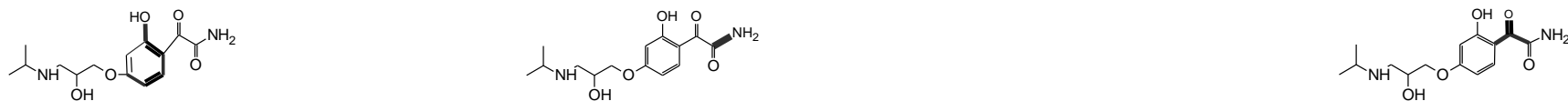
ATP297₁

ATP297₂

ATP281₁
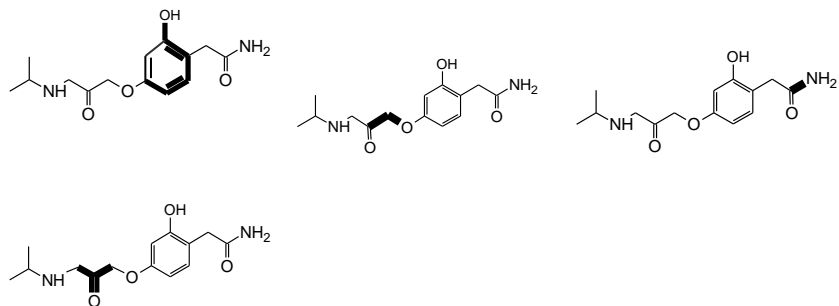
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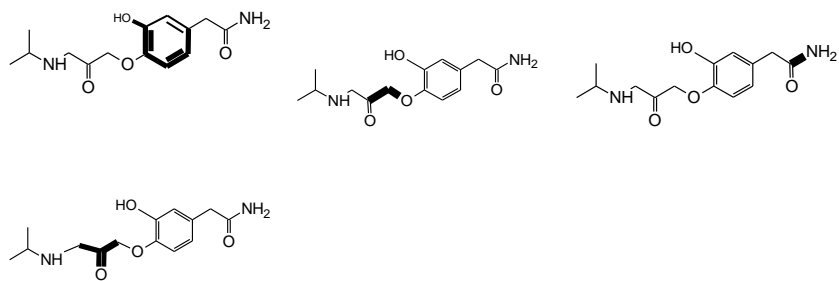
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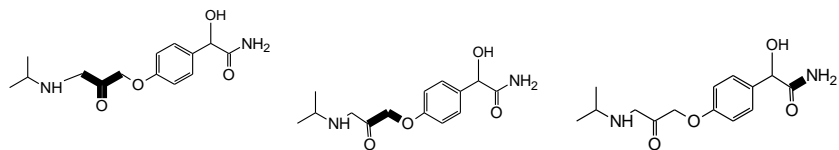
ATP281₄
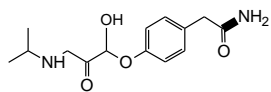
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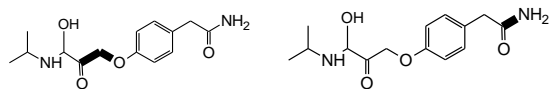
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ATP283₂

ATP283₃
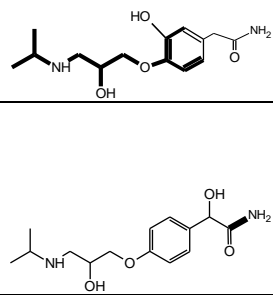
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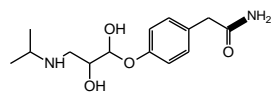
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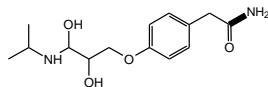
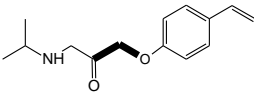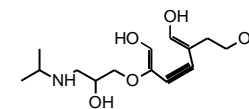ATP283₆
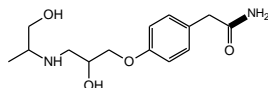
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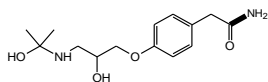
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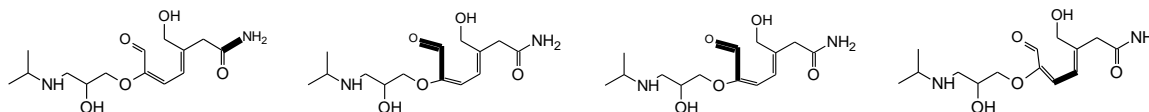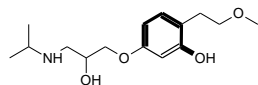
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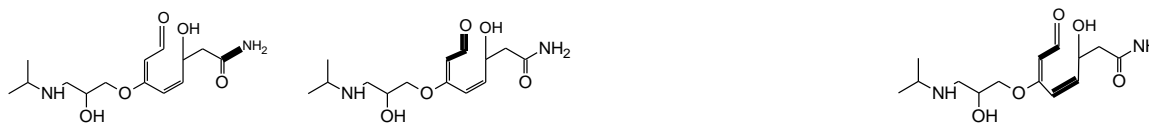
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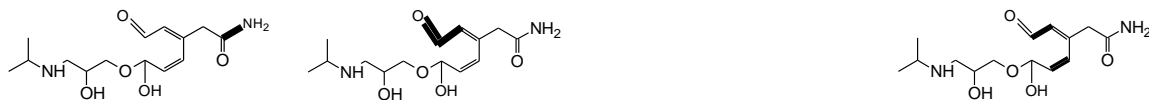
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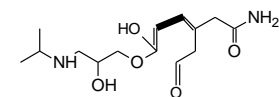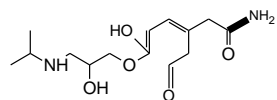
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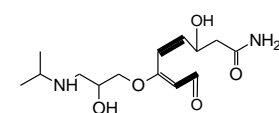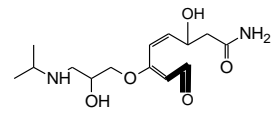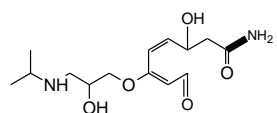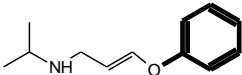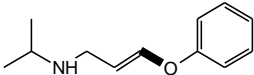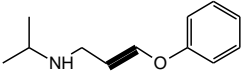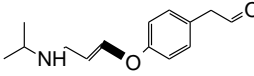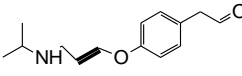
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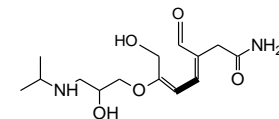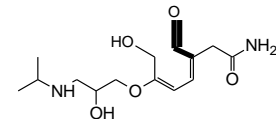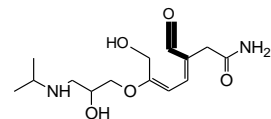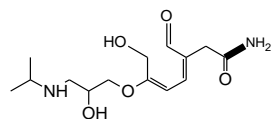
Table S5: Positive alerts for Genotoxicity and bacterial toxicity according to CASE Ultra models prediction for Metoprolol and selected transformation products
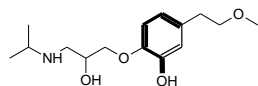
TP ID	Genotoxicity				Mutagenicity		Bacterial Toxicity
	A		B		C		D
	A7U	A7V	A7S	GT1_A7B	SALM2013		AUA
MTP192							
MTP234$_1$							
MTP234$_2$							

MTP302

MTP284₁

MTP284₂
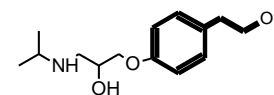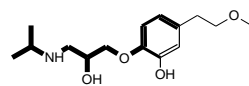
MTP254
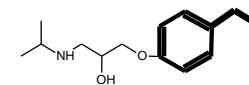
MTP238

MTP318

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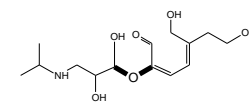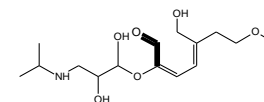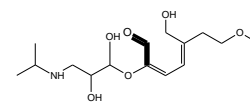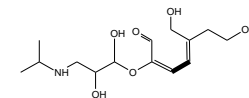
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Appendix 5

Supplementary study

<u>Genotoxicity and cytotoxicity characterization of Propranolol</u>

Supplementary data to Beta Blocker

Table S1 Genotoxicity of Propranolol and its photolytic mixtures

Treatment	Dilution Factor [concen-tration (mg/L)]	Ames Number of Revertants TA 98 -S9	+S9	TA 100 -S9	+S9	Umu Growth -S9	+S9	Induction Ratio -S9	+S9	In vitro Micronucleus Relative survival (%)	EMA+ (%)	Hypo-diploid (%)	MN (%)
Millipore water	1.35	2±1	2±2	4±3	5±1								
	1.5					1.00 ±0.01	0.99 ±0.08	1.03 ±0.06	1.01 ±0.19				
	10									100 ±6.68	0.44 ±0.09	0.17 ±0.01	1.43 ±10.19
PPL	1.35 [74]	1±1	4±2	3±2	5±4								
	1.5 [67]					1.02 ±0.08	0.84 ±0.14	1.07 ±0.17	1.08 ±0.18				
	10 [10]									78.50 ±27.52	2.61 ±3.34	0.28 ±0.04	1.84 ±0.25
	20 [5]									110.73 ±6.61	0.35 ±0.17	0.22 ±0.03	1.82 ±0.34
8 min	1.35												
	1.5					1.02 ±0.07	0.90 ±0.05	1.24* ±0.13	1.00 ±0.11				
	10									*29.54* *±6.09*	*17.24* *±6.87*	0.29 ±0.11	2.32 ±0.70
	20									107.90 ±9.76	0.38 ±0.12	0.31 ±0.13	1.53 ±0.01
16 min	1.35	1±0	2±2	6±6	7±1								
	1.5					0.93	0.86	1.34*	1.19				

						±0.07	±0.06	±0.16	±0.39			
	10									*50.17**	6.74	0.21	2.22
										±14.74	±4.73	±0.01	±0.52
	20									55.08	5.45	0.19	2.28
										±0.41	±0.11	±0.03	±0.49
32 min	1.35	4±0	5±2	8±2	7±4								
	1.5					0.98	0.94	1.43*	1.01				
						±0.04	±0.10	±0.08	±0.17				
	10									*47.72*	6.99	0.25	2.35
										*±5.86**	±1.44	±0.05	±0.37
	20									108.74	0.32	0.26	1.98
										±5.04	±0.01	±0.07	±0.26
64 min	1.35	4±1	7±2*	10±2	6±1								
	1.5					0.91	0.92	*1.51**	1.24				
						±0.08	±0.08	*±0.13*	±0.19				
	10									*32.84**	11.39	0.36	2.28
										±17.00	±1.91	±0.07	±0.25
	20									79.92	1.05	0.21	1.97
										±8.26	±0.26	±0.01	±0.00
128 min	1.35	2±3	5±3	*14±1**	8±3								
	1.5					0.97	0.96	*1.58**	1.15				
						±0.05	±0.06	*±0.03*	±0.20				
	10									*38.54**	11.95	0.54	3.68
										±1.74	±2.94	±0.08	±0.69
	20									70.14	2.05	0.51	2.27
										±12.33	±1.35	±0.08	±0.16
256 min	1.35	3±2	2±2	*15±3**	9±0								
	1.5					0.94	0.94	1.35*	1.27				
						±0.09	±10.12	±0.23	±0.14				
	10									*48.84*	6.71	0.27	2.69
										*±16.13**	±3.71	±0.04	±0.70

| | 77.52 | 4.01 | 0.32 | 1.96 |
| 20 | ±3.71 | ±0.22 | ±0.04 | ±0.16 |

*p≤0.05 when compared to PPL; **Bold and italic** are samples that have met the test criteria to be classified as mutagenic, *umuC* inducing and cytotoxic

Figure S1: Formation kinetic for transformation products of Propranolol during photolysis (Source: raw data provided by Tushar Rastogi).