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Fate of Pesticides in the Aquatic Environment: Determination and Identification of Dead End Degradation Products of Selected Pesticides and a Hydrological Tracer by Combination of Experimental and *In Silico* Methods.

Kumulative Dissertationsschrift zur Erlangung des akademischen Grades
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   DOI:10.1016/j.scitotenv.2014.11.025

2. **Lukasz Gutowski**, Ewelina Baginska, Oliver Olsson, Christoph Leder, Klaus Kümmerer (2015) Assessing the Environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water-sediment test and *in silico* methods. Chemosphere 2015;138:847-55
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3. **Lukasz Gutowski**, Oliver Olsson, Jens Lange, Klaus Kümmerer (2015) Photolytic transformation products and biological stability of the hydrological tracer Uranine. The Science of the Total Environment 2015;533:446-53
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Fate of Pesticides in the Aquatic Environment: Determination and Identification of Dead End Degradation Products of Selected Pesticides and a Hydrological Tracer by Combination of Experimental and *In Silico* Methods.

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

Carried out at the Institute of Sustainable and Environmental Chemistry
Leuphana University of Lüneburg

Dissertation submitted by
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The individual articles constituting this cumulative doctoral dissertation meet the formal requirements for a cumulative dissertation. The PhD thesis consists of the following publications:

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Zusammenfassung

Uranin (Fluorescein-Natrium, UR) wird seit Ende des 19. Jahrhunderts routinemäßig als hydrologischer Tracer verwendet, um Transport- und Mischprozesse in Oberflächengewässern und in Grundwasser zu beurteilen. Mit Hilfe erhaltener Daten zu Uranin können Schlussfolgerungen bezüglich Reichweite und Verhalten von Verunreinigungen, gezogen werden (teilweise auch mit Hilfe von Modellen). Die Verwendung von UR für qualitative (visuelle) Studien zur Beurteilung der Grundwasserkontamination ist üblich. Jedoch sind Daten über das Umweltverhalten von UR (z. B. Veränderung, Abbau oder Bildung und Schicksal von Transformationsprodukten, TPs) nur unvollständig oder nicht gut vergleichbar. Untersuchungen zu UR bezüglich biologischer Abbaubarkeit sind nur spekulativ. S-Metolachlor (SM) ist ein weltweit bekanntes Chloracetamid-Herbizid, das sehr viel zum globalen Pestizidverbrauch beiträgt. Auf dem französischen Markt wird es für vielerlei Anbaupflanzen gegen einjährige Gräser und bestimmtes Blattunkraut unter dem Namen Mercantor Gold® (MG) verwendet. Photoabbau beeinflusst das Schicksal von SM in der aquatischen Umwelt. Transformationsprodukte wurden in Oberflächengewässern und in Grundwasser bereits gefunden. Jedoch wurden die Transformationsprodukte nicht weiter bewertet und dessen Schicksal nicht weiter untersucht. Darüber hinaus beeinflussen Adjuvantien in MG die Löslichkeit, die Bioabbaubarkeit, die Photolyse und die Sorptionseigenschaften des wirksamen Inhaltsstoffs SM.

TPs können andere Eigenschaften haben (z. B. toxischer oder in höheren Konzentrationen vorliegend), die dafür sorgen, dass sie in anderen Umweltbereichen vorliegen, die nicht durch die Muttersubstanz (PC) beeinflusst werden. Um die ökologischen Auswirkungen von Pestiziden, Tracern und dessen jeweilige Transformationsprodukte auf Wasserorganismen zu beurteilen, kann das Verhalten dieser Substanzen mit Hilfe von Bioabbaubarkeitstests im Labor überprüft werden. Doch bisher gab es nur unvollständige Informationen über die Transformation von SM, MG und UR oder das Schicksal ihrer Photo-TP in Oberflächengewässern oder im Wassersedimenttest. Die Kombination aus Photolyse mit aerobem Bioabbau, um persistente Photo-TP zu identifizieren, gibt einen neuen Einblick in das Umweltverhalten der ausgewählten Verbindungen.

Deshalb war die Grundlage für diese Arbeit 1) die Einfluss von MGs Adjuvantien auf die Bioabbaubarkeit, die Photolyse (Xenonlampe) und die Sorption von SM zu bestimmen 2) die Photolyse und Bioabbaubarkeit von UR zu untersuchen 3) die Primärelimination (Photolyse)

von den PC mittels HPLC(-UV, -FLD) und das Ausmaß der Mineralisierung mittels non-purgeable organic carbon (NPOC) zu bestimmen 4) die Strukturen der Photo-TP von SM, MG und UR mittels LC-MS/MS zu bestimmen 5) die Bioabbaubarkeit der Photo-TP zu bestimmen, um das Schicksal und die Persistenz in der aquatischen Umwelt einschätzen zu können 6) *in silico*-Vorhersagen (Pestizide) in Bezug auf Humantoxizität (Karzinogenität, Genotoxizität und Mutagenität) und Ökotoxizität (Mikrotoxizität, Biokonzentrationsfaktor und Toxizität gegenüber der Regenbogenforelle) durchzuführen.

SM, MG und UR waren im Geschlossenen Flaschentest (CBT), im Manometrischen Respirationstest (MRT) und im Wassersedimenttest (WST) nicht leicht biologisch abbaubar. Die chemische Analytik der Photolyseproben zeigte, dass SM in MG, verglichen zu SM als Reinsubstanz, schneller eliminiert wurde. UR zeigte hingegen generell eine schnelle Primärelimination. Der geringe Grad an Mineralisierung der untersuchten Stoffe zeigt, dass viele Photo-TP gebildet wurden. Darüber hinaus wurde festgestellt, dass die Photo-TP in den durchgeführten Bioabbaubarkeitstests nicht biologisch abbaubar waren. Für UR konnte nur ein geringer Abbau in OECD 301D und WST festgestellt werden. Außerdem wurden in OECD 301D und WST ausgehend von den Photo-TPs von SM, SM in MG neue Bio-TPs gebildet. Die Ergebnisse lassen vermuten, dass die Formulierung in MG den biologischen Abbau nicht signifikant beeinflusst. Jedoch beeinflusst die Formulierung die Diffusion der Wirksubstanz SM in Sediment und beeinflusst damit auch die photolytische Effizenz. Dies könnte zu einer schnelleren Bildung von Photo-TPs in der Umwelt führen. *In silico*-Vorhersagen zeigten für viele Endpunkte, dass Bio-TPs im Vergleich zu SM zu einer erhöhten Humantoxizität und einer erhöhten Toxizität gegen Wasserorganismen führen könnte. Es gab keine Anhaltspunkte für eine Toxizität von UR. Trotzdem ist die Durchführung von zielführenden Untersuchungen in Bezug auf Langzeiteffekte von Photo-TPs von UR berechtigt.

Die vorliegende Arbeit zeigt, dass eine Kombination aus Labortests, analytischer Methoden und *in silico*-Tools, zu wertvollen Informationen über das Schicksal von TP ausgewählter Substanzen führt. Außerdem wurde gezeigt, dass nicht nur der Zerfall der Muttersubstanzen, sondern auch die Photo-TP, die in der aquatischen Umwelt gebildet werden, Aufmerksamkeit erhalten sollten.

Summary

Uranine (sodium fluorescein, UR) has been routinely used in hydrological research to monitor surface and subsurface water flow, transport and mixing processes since the end of nineteenth century. Based on such obtained data, further conclusions can be drawn on the spread and behavior of pollutants (partly on models). Use of UR for qualitative (visual) studies of underground contamination is common, however data available on its environmental behavior (e.g., conversion, degradation or formation and fate of the transformation products, TPs) are incomplete or not readily comparable. UR observations of biodegradation are still speculative. S-metolachlor (SM) is a popular worldwide chloroacetamide herbicide, which highly correspond to the global pesticide use. It is offered on the French market as an effective multi-crop herbicide against annual grasses and certain broadleaf weeds under the trade name Mercantor Gold® (MG). Photodegradation contributes to the fate of SM in the aquatic environment. TPs were already found in surface and groundwater. However, further fate and assessment of the TPs was not done. Moreover, adjuvants in MG's formula can affect the solubility, biodegradation, photolysis and sorption properties of the active compound SM.

TPs can have different properties (e.g. more mobile, toxic or present at higher concentrations) that enable them to reach the environmental compartments not affected by the parent compound (PC) itself. To assess the ecological impact of pesticides, tracers, and their respective TPs on water organisms, their behavior can be investigated in laboratory screening biodegradation tests. Yet, incomplete data was available on SM, MG and UR transformation or their photo-TPs' fate in surface and water-sediment systems. The combination of photolysis with aerobic biodegradation in order to identify persistent photo-TPs could provide new insight into the environmental behavior of the selected compounds.

Therefore, principle of this thesis was to 1) identify the impact of MG's adjuvants on the biodegradation, photolysis (Xe lamp) and sorption compared to the SM alone, 2) examine the photolysis and biodegradability of UR 3) monitor the primary elimination (photolysis) of the PCs by HPLC (-UV, –FLD) and measure the degree of mineralization by means of non-purgeable organic carbon (NPOC) 4) elucidate the photo-TPs of SM, MG and UR by using LC-MS/MS 5) analyze biodegradability of the photo-TPs in order to determine their fate and persistence in aquatic environment 6) conduct *in silico* toxicity predictions (pesticides) in human (carcinogenicity, genotoxicity and mutagenicity) and eco-toxicity (microtoxicity, bioconcentration factor and toxicity in rainbow trouts).

SM, MG and UR were found not readily biodegradable in Closed Bottle test (CBT), Manometric Respiratory test (MRT) and in water-sediment test (WST). Chemical analysis of photolysis samples showed higher elimination of SM in MG compared to SM alone whereas UR displayed high primary elimination rate in general. The overall low degree of mineralization indicated that abundant photo-TPs were formed. Furthermore, the photo-TPs were found not biodegradable in performed biodegradation tests. Only small degradation rates for UR could be observed in the CBT and WST. Additionally, in the MRT and WST new bio-TPs were generated from the photo-TPs of SM and SM in MG. Obtained results suggest that the MG formulation did not significantly affect the biodegradation, however it influenced the diffusion of the active substance (SM) to sediment and potentially affected the photolysis efficiency, which might result in faster formation of photo-TPs in the environment. *In silico* predictions showed that for many endpoints, biotransformation might lead to an increased toxicity in humans and to water organisms compared with the parent compound SM. No indications were found for UR toxicity. Still, target-oriented investigations on long term impacts of photo-TPs from UR are warranted.

The present work demonstrates that a combination of laboratory tests, analytical analysis and *in silico* tools result in valuable information regarding environmental fate of the TPs from selected compounds. Furthermore, it was shown that photo-TPs formed in the aquatic environment should be taken into account not only the parent compound and its decay.

List of Abbreviations

BCF	Bioconcentration factor
BOD	Biological oxygen demand
CBT	Closed Bottle Test
CFU	Colony forming units
COD	Chemical oxygen demand
EIC	Extracted ion chromatogram
FDA	Food and Drug Administration
FLD	Fluorescence detector
HO•	Hydroxyl radical
HPLC (LC)	High performance liquid chromatography
LC (HPLC)	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MG	Mercantor Gold®
MRT	Manometric Respiratory Test
MS	Mass spectrometry
m/z	Mass-to-charge-ratio
NPOC	Non purgeable organic carbon
OECD	Organization for Economic Co-operation and Development
QSAR	Quantitative structure activity relationship(s)
RSD	Relative standard deviation
RT	Retention time
SM	S-metolachlor
ThOD	Theoretical oxygen demand
TIC	Total ion chromatogram
TP	Transformation product
t_R	Retention time
UR	Uranine

UV	Ultraviolet
V. fischeri	*Vibrio fischeri*
WST	Water sediment test

Contents

1. Introduction and Motivation

1.1 Pesticides and hydrological tracers

The presence of pesticides in the natural environment is a known environmental issue (Fenner et al., 2013). Due to the rising human population, more food have to be provided to meet its growing needs. Thus, amount of pesticides used worldwide is increasing, as a result it causes new challenges to handle and reduce pesticide loads before they reach the natural water resources. The negative impact of pesticides in the environment might not only affect the various bodies of water, but in fact might have a negative impact on the environmental conditions, which are suitable for flora and fauna. Research on the transport of pesticides from their source (i.e. agricultural application) to surface water bodies and groundwater by processes such as spray drift, wash-off from plants, surface runoff, infiltration, lateral subsurface flow, leaching or via industrial waste water discharge has been conducted by numerous authors and the dominating processes are largely known (Brown and van Beinum, 2009; Gassmann et al., 2013; Reichenberger et al., 2007; Remucal, 2014; Shibata et al., 2011; Tang et al., 2012). Approximately 1 to 5% of load of field applied herbicides are removed by surface runoff and reach the aquatic compartment (Scott et al., 1999; Wauchope, 1978). Construction of artificial wetland is one of the well-known approaches to neutralize or minimize the negative effect on the environment by the pesticide contamination. In constructed wetlands, sorption, volatilization, hydrolytic and photolytic oxidation, biological degradation, bioaccumulation, and sedimentation may contribute to attenuation of organic contaminants (Gregoire at al., 2009; Imfeld et al., 2009). However, their role and influence on generation and fate of TPs is yet not fully examined.

Another environmental issue is that the application of pesticides introduces to the environment not only the active compound itself, but also other chemicals that make up the commercial product which is applied. In commercial formulations, adjuvants have been developed not only to maximize pesticide efficacy but also to minimize unfavorable environmental contamination from the active compound and its transformation products (TPs) (Katagi, 2008). Surfactants are some of the most important components among many other adjuvants such as stabilizers, thickeners or disperse and antifreeze agents (Katagi, 2008). Surfactants modify spray droplet size thus improving biological activity together with the retention, spreading on the leaf or even enhancing the uptake of pesticides by crops (Knowles, 2001).

Fluorescent dyes are routinely used as hydrological tracers to monitor surface and subsurface water movement. Hydrological tracing technique is an important tool for risk assessment of problematic sites (Käss, 1994; Reichert and Hoetzl, 1991). One important use of tracers is to verify pollutant dispersal and behavior in the environment, e.g. for pesticides. Hence, once in aquatic environments pesticides among many biotic and abiotic processes are subjected to sunlight photolysis. Therefore it is of relevance to gain knowledge on the photolytic degradation. In surface waters sunlight photolysis of UR is ubiquitous and its half-lifes have been quantified (Smart and Laidlaw, 1977; Käss, 1998, 2004). Hence, it was used as a reference substance to mimic photolytic decay of a herbicide (Isoproturon) in surface waters (Lange et al., 2011).

However, any organic substance released to the aquatic environment, may be transformed through the processes mentioned in the previous paragraphs. Thus, extensive use of even potentially safe UR for tracing experiments or commercial use might likely cause risk of long-term environmental contamination. Biotic degradation together with sun photolysis is the first line degradation step for the pesticides and hydrological tracers in aquatic environment. Biodegradation is based exclusively on the activity of microorganisms, hence, it is important to carry out simulation tests on the degradation of substances in the aquatic environment (Alexy et al., 2004). Abiotic elimination processes such as photolysis, hydrolysis, and sorption are also of great importance for the aquatic fate of chemicals. Knowledge on sorption processes can deliver significant information about mobility or distribution of a chemical in the environment. Photolysis is among the most important abiotic degradation mechanisms for many pollutants. Therefore, knowledge on the photolysis pathways and kinetics is essential to predict the environmental fate of these compounds in natural waters (Trovó et al., 2009).

1.2 Transformation products

Transformation is an important mechanism of dissipation for any chemical compound in the aquatic environment. It includes non-biotic and biotic processes such as photolysis, hydrolysis, oxidation and reduction (Fenner et al., 2013). Transformation products can be more toxic and present at higher concentrations than their parent compounds (PCs) (Mañas et al., 2009; Olsson et al., 2013). However, the description of TPs' fate in the environment and the assessment of their effects on aquatic ecosystems are impeded by a lack of data and by missing knowledge about their environmental fate and effects. Gómez et al., (2012) stated that the majority of the TPs have most likely not even been identified yet and much less is known about their environmental relevance. In regions with intensive agriculture, the detection of elevated

pesticide concentrations in water samples both surface- and groundwater becomes more and more frequent, and often times exceeding drinking water thresholds (Köck-Schulmeyer et al., 2014; Herrero-Hernandez et al., 2013). In recent years, there has been an increasing interest in gathering knowledge on sources, occurrence, fate, and possible effects on human health and aquatic organisms or bioaccumulation potential for these compounds (Mostafalou and Abdollahi, 2013).

It is important to better understand the rate and relevance of the TPs in the environment or at the catchment site. Therefore, it is relevant to identify the structures of TPs by analytical methods such as LC-MS/MS. Their further risk assessment can be done in first line by *in silico* approach, which would also contribute to pesticide regulation. The *in silico* approaches are gaining importance especially for analyzing environmental fate and impact of the TPs, because these compounds are usually formed in low concentrations within complex matrices so that isolation and purification is very difficult, tedious, expensive or not possible as TPs are often formed as new molecules. Further, many of these TPs are not available commercially, which makes the individual analysis of their environmental fate impossible. Therefore, it can be helpful to apply QSAR models to estimate the potential for biodegradation, photodegradation, and toxicity in the environment (European Commission, 2003a, 2003b; Rücker and Kümmerer, 2012; Trautwein and Kümmerer, 2012; Walker et al., 2004).

1.3 Research gap

It should be noted that information and data regarding the SM, MG, UR, and their photolysis products' behavior in the aquatic environment is still plainly lacking in the international scientific literature. It has been reported by several studies that biodegradation plays the most important role in the fate of chloroacetanilide pesticides in the environment while other factors like photo-oxidation and chemical hydrolysis are of minor importance under typical (physio-chemical) conditions of soil and water (Humburg et al., 1989; Liu et al., 1991; Stamper et al., 1998). Contradictory findings have been reported concerning photolysis as the main abiotic degradation pathway for chloroacetanilides (R-metolachlor and SM) in the environment (Dimou et al., 2005; Ruth et al., 2000). However, more data is available for the R-enantiomeric form than for the SM. Both processes (biodegradation and photolysis) may occur simultaneously in surface water bodies, where SM, MG and UR were applied. Additionally, knowledge regarding a possible transformation of photo-TPs and the products' fate and effects in surface waters and water-sediment system is especially limited. Studies focused on the active ingredient of the pesticide itself neglecting possible effects of additional chemicals (adjuvants)

that are found in commercial products. Hence, the influence and effects of the adjuvants on the SM and its TP's behavior in the surface and water-sediment system is largely unknown.

This thesis will address this knowledge gap by an in depth analysis of the fate of parent compounds and their photo-TPs in surface waters and water-sediment system. Prediction of the potential toxicity in humans and eco-toxicity against aquatic organisms of bio-TPs found in the study presented here.

2. Aims and objectives

The main objectives for this PhD thesis were:

- Development of analytical methods used for the identification of TPs of SM, MG UR and elucidation of reaction pathways.
- Identification of stable TPs of selected pesticides and a hydrological tracer.
- Investigation of photolysis and biodegradation for the selected pesticides and hydrological tracers in order to gather deeper knowledge on their fate.
- Investigation of MG' adjuvants influence on the biodegradation, photolysis and sorption processes compared with the SM alone.
- Investigation of the TPs' fate in the aquatic environment.
- Assessment of not biodegradable TPs: Preliminary toxicity assessment (e.g. ecotoxicity, mutagenicity, carcinogenicity and genotoxicity) for the identified TPs.

3. Research approach and methods

In order to fulfill the main objectives, the following tasks were addressed in the three research papers. Selection criteria for the investigated compounds were: a) insufficient or outdated knowledge available up to now (SM, UR), b) high sales volume and usage worldwide (SM, UR), c) missing data about MG adjuvants influence on the behavior of the active substance SM d) possible toxic effects (SM TP's and bio-TPs). Criteria for compound and methods selection is shown in Fig. 1.

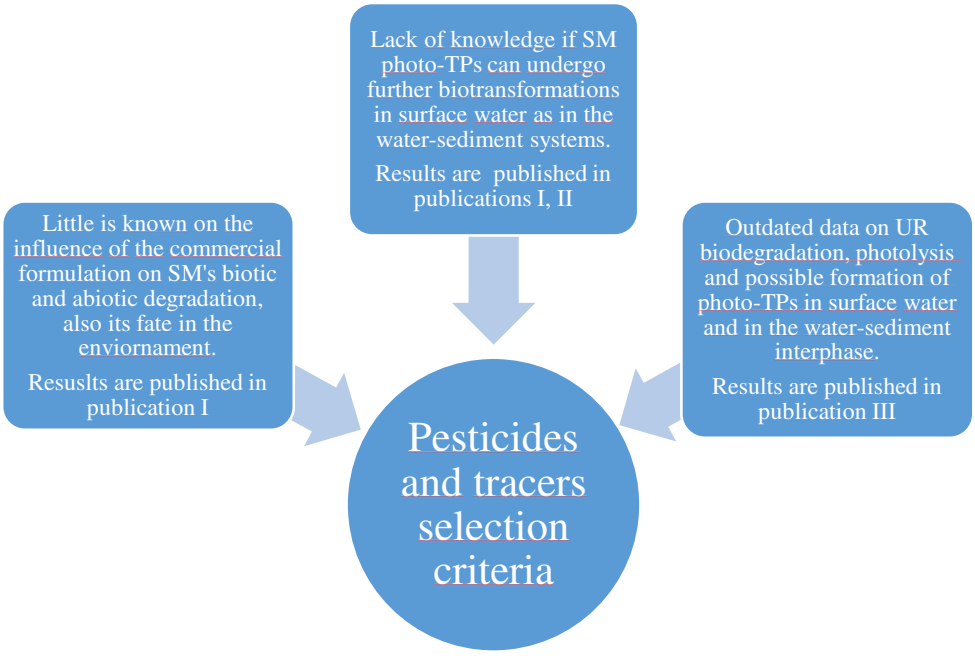

Fig. 1. Selection criteria for investigated pesticides and hydrological tracers in the articles of the thesis.

The first publication focuses on the comparative assessment of the environmental fate and connected risk of SM and its commercial product MG. Two tests from the OECD series were used for biodegradation testing: Closed Bottle test (CBT; OECD 301 D) and Manometric Respiratory test (MRT; OECD 301 F). Photolysis in water of two formulations of SM with simulated sunlight (Xe-lamp) was studied. Both compounds (SM and MG) were subjected to photolysis at two initial concentrations of 20 mg L^{-1} and 40 mg L^{-1}, respectively. The primary elimination of SM and SM in MG was monitored by high-performance liquid chromatography-ultraviolet (HPLC-UV) at 220 nm and structures of photoproducts were identified by LC-MS/MS (ion trap). Furthermore, CBT and MRT was performed for samples after 8 hours of photolysis. Additionally, a set of *in silico* prediction programs was applied for supporting analytical results and human toxicity (endpoints: carcinogenicity, genotoxicity and mutagenicity) assessment of SM and TPs.

Yet, little was known about the influence of MG adjuvants on sorption and biodegradability in water-sediment system. Therefore, as a follow up study, **the second publication** concentrates on the environment fate of SM and MG in the simulated water-sediment test, and the *in silico* eco-toxicity assessments of SM's bio-transformation products (bio-TPs). To achieve this goal, a newly developed screening water sediment biodegradation test (WST) was applied to investigate the biodegradation and sorption processes of MG compared with SM. Additionally, based on photolysis experiments (Xe lamp) described in the first paper the biodegradability of the photolysis mixtures was examined in this WST. The primary elimination of SM and SM in MG from water phase was monitored and structures of its bio-TPs were elucidated by the same LC-MS/MS method as in the first article. Extraction of SM from sediment was conducted in order to estimate the role of sorption processes.

The third publication focuses on assessing the photolytic transformation products and environmental fate of the hydrological tracer UR. Photolysis in water using Xe lamp (8 h irradiation time) was studied with three initial concentrations of 10 mg L^{-1}, 20 mg L^{-1} and 60 mg L^{-1}. The primary elimination of UR was monitored and structures of its TPs were elucidated by high-performance liquid chromatography-fluorescence tandem mass spectrometry HPLC-FLD-MS/MS. The excitation and detection wavelengths were 476 and 515 nm, respectively. By means of AutoMS^n mode, each m/z of TPs identified in the TIC was used as precursor ion and further fragmented up to MS^3. To assess the biodegradability and ecological impact of UR, two OECD (301 D and 301 F) tests and WST were applied. Subsequently, the biodegradability of the photolysis mixture was examined.

4. Results and Discussion

In the first publication the xenon photolysis and further the biodegradability of the obtained photolysis of SM and its commercial product MG is reported. To monitor primary elimination of the parent compound alone and in the formulation, an HPLC method was developed. The sufficient resolution, good sensitivity and acceptable analysis time for the developed liquid chromatographic separation of all studied pesticide compounds were obtained by adjusting different chromatographic factors mainly stationary-phase composition (column type and size), column oven temperature, flow rate, and optimum mobile phase compositions.

Regardless used concentrations both compounds were efficiently degraded during photolysis. The HPLC analysis showed elimination of 74.2% (± 0.9%) for the commercial product in comparison to 68.9% (± 0.7%) for the SM alone at two initial concentrations (20 mg L^{-1} and 40 mg L^{-1}). Slight difference in photo elimination could be seen in favour of the commercial formulation over the SM alone. This was probably due to the surface-active components in the MG formula, which can influence the physico-chemical properties of the parent compound. The NPOC measurement was conducted in parallel with each experiment to monitor any possible mineralization during photolysis. After irradiation for 8 hours the mineralisation rate did not exceed 1% of NPOC measured for all tested compounds. This indicated that these pesticides were transformed into TPs that were resistant to further Xe photolysis.

The formation of new peaks in the chromatogram from photo-treated samples was observed by means of LC-MS/MS. Peaks were gradually increasing with the irradiation time reaching the maximum intensity at 8.0 h. This demonstrated the formation of photo-TPs. Fig. 2a shows the total ion chromatogram (TIC) of SM in ultrapure water obtained at the time point 0.0 h. Fig. 2b shows the new peaks ($TP1_{a-g}$, TP2, TP3 and TP4) resulting from photolysis of SM after 8.0 h. Moreover, aforementioned TPs tend to be of higher polarity than parent compound itself. A total of 10 identical compounds were identified as SM and SM in MG photo-TPs (Fig. 4). The most abundant photo-product was of *m/z* 266.2 and had been labelled as $TP1_a$, $TP1_b$, $TP1_c$, $TP1_d$, $TP1_e$, $TP1_f$ and $TP1_g$ related to its different retention times. This product was considered as an example of a mono-hydroxylation consisting of mostly isomers eluting at different retention times.

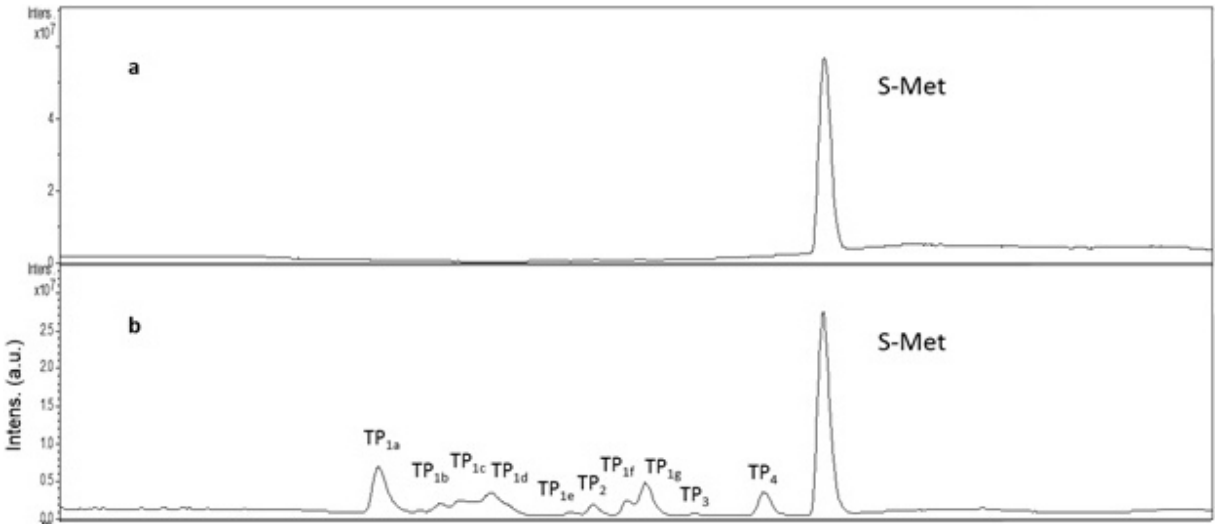

Fig. 2. Total ion chromatogram (TICs) of SM during the photolysis experiment: a) sample at time point 0.0 min, b) sample after photolysis for 8.0 h submitted to the biodegradability test.

The compound with *m/z* of 248 (TP4) only differs by 18 Da from the isomers described in previous paragraph. It can be assumed that its formation resulted by a loss of water from the mono-hydroxylated TPs mentioned above. The product with *m/z* 234.1 (TP2) (Fig. 4) differs only 14 Da from TP4 suggesting that TP2 has kept the $-(CH_3)CH\text{-}CH_2$ chain. It is interesting to mention formation of a photo-TP with *m/z* 222.1 (TP3) which previously was described as minor photoproduct of Alachlor (Hogenboom et al. 2000; EPA 2006).

Both substances displayed similar behaviour in all biodegradation tests indicating that MG adjuvants had no impact on the SM biodegradation. No biodegradation has been observed for MG, SM and their photolysis samples (8.0 h) in the CBT classifying them as being *not readily biodegradable.* Similarly to the CBT the MG, SM and their photolysis mixtures were *not readily biodegradable* in the MRT. The validity criteria were met since 60% of the quality control substance was biodegraded within 10 days. No toxic effects on bacteria were observed in the toxicity control as well as no degradation was observed in the sterile control. The measurements with HPLC-UV confirmed that no elimination of SM in MG and SM along with their photo-products occurred during the tests. However, LC-MS/MS analyses of the MRT samples revealed the formation of bio-TPs from 8.0 h photolysis samples. Some photoproducts of m/z 266.2 were degraded after 28 days of MRT duration. As a result, two bio-TPs of m/z 264.2 were formed in the solution due to the microbial transformation of the photo-TPs.

In the second publication further fate of the SM, MG and their transformation products is described. For this purpose newly developed screening water sediment test was applied. It was of special interest to examine the behaviour of the photolysis mixture in the presence of water-sediment interphase. Biodegradation in WST was examined and formation of bio-TPs from photo-TPs was reported here too. Due to the sediment phase it was possible to investigate the

sorption processes and to monitor the dissipation of the parent compound from the water phase. What is more, the influence of MG adjuvants on the diffusion of the parent compound to the sediment was measured and compared to the active substance alone.

SM was found not biodegradable in the WST as well as its photolysis mixture. Likewise the parent compound alone, the commercial formulation and its photo-TPs were not biodegraded in the WST. However, SM in MG reached slightly higher degradation rates compared with the active substance alone. That could be due to the adjuvants in the commercial formulation or different inoculum used in this test. These adjuvants, which were in fact a mixture of hydrocarbons, might have served as a source of carbon for the bacteria in the inoculum or directly influence the biodegradation (Katagi, 2006 and 2008). Surfactant Dodecylbenzenesulfonic acid is known to be biodegradable (Khleifat, 2006; Scott and Jones, 2000), and it could contribute to biodegradation results in low extent.

HPLC-UV measurements revealed that SM alone reached 33.6% (±2.8%) removal from water phase on 28th day whereas the SM in MG to reached higher removal of 52.8% (±5.1%) on 28th in all three test series. This result could be once more explained by presence of surfactants in the MG formula, which could directly interact with the sediment. Generally, this finding could be confirmed by a study of Bayer (1967), who reported increased mobility of four urea herbicides in soil when applied in a mixture with anionic and nonionic surfactants. The extraction of SM was conducted in order to investigate whether SM was reversibly or irreversibly adsorbed to sediment taken from WST. Extraction with highly polar organic solvent (ACN/water) turned out to be more effective with recovery rates of 96.9 % (SM) and 93.1 % (MG), compared to 55.4% (SM) and 50.0 (MG) for the solution of 0.01 M $CaCl_2$, as suggested by the OECD Test Guideline 106 (OECD 1997). This might suggest, that part of SM in both formulations could be immobilized in the sediment under natural conditions.

The results of the HPLC-UV analysis for WST samples after 28 days confirmed that neither the SM, SM in MG nor of their photolysis mixtures were biodegraded during the test. However, the formation of the bio-TPs in the test samples on the 28th day for the photolysis mixtures of SM and MG occurred. Moreover, the identical bio-TPs were found at the end of the MRT (article I). To investigate bio-transformation in the WST the same analytical method was applied, as described in the first article. The LC-MS/MS results, shown in Fig. 3 indicate for the photolysis mixture that the photo-TP1$_a$ was eliminated, whereas intensity of photo-TP1$_b$ increased by co-elution of the second bio-TP. In other words, new bio-TPs (a-c and d-f are isomers, respectively, see below) were formed as found in samples on the 28th day for SM alone as for the MG formulation. SM and SM in MG showed similar behavior.

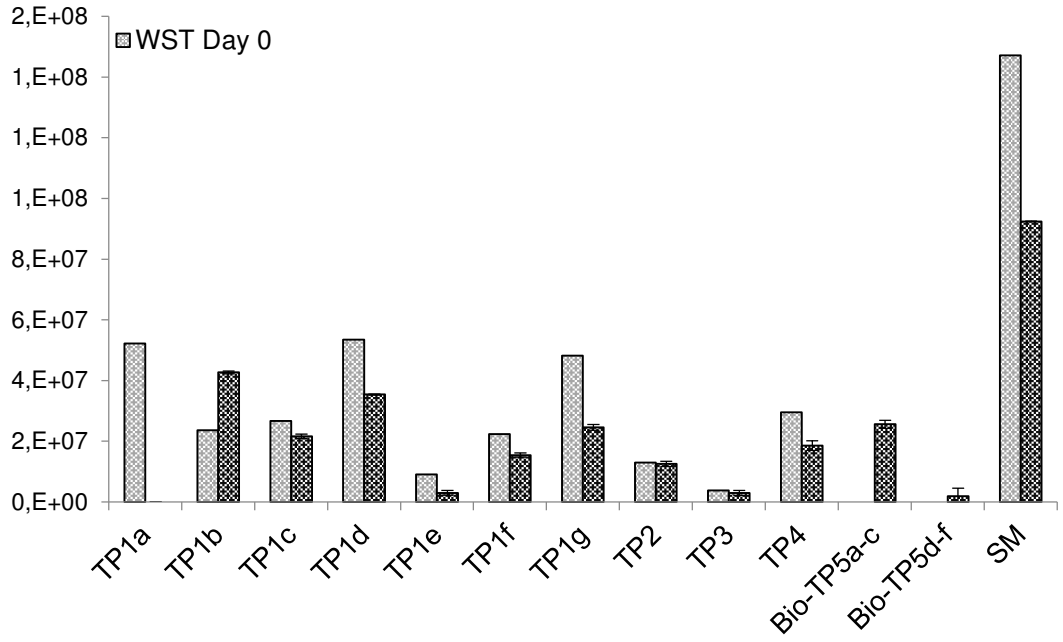

Fig. 3. Relative peak area of the photo-TPs in photolysis mixture (8h irradiation time) at the start and end of biodegradation test assays. WST of SM in MG formulation. Day 0 (n=1), day 28 (n=2), respectively.

Based on these outcomes, it can be assumed that the bio-TPs' formation occurred through a formation of double bond within the structures of dechlorinated and mono-hydroxylationed *m/z* 266.2 photo-TPs of SM, as reported in literature (Coffinet et al., 2012; Gutowski et al., 2015). Neither a specific mass of *m/z* 264.2 was found nor were the 6 TPs of *m/z* 266.2 degraded in the sterile control, respectively. The latter ones were still detected by LC-MS at the end of the test at the same intensity. Therefore it can be concluded that found bio-TPs of *m/z* 264.2 resulted from microbial transformation of the photo-TPs *m/z* 266.2. Therefore abiotic processes like hydrolysis or other non-biotic chemical transformations could be excluded to play a significant role in the parent compound and its photo-TPs fate in water. Suggested abiotic and biotic degradation pathway for SM and SM in MG can be seen in the Fig. 4. Thus, it appears that found bio-TPs could be formed in the environment from the direct transformation of the parent compound, or by further transformation of the photo-TPs, not leading to the direct mineralization as yet.

Moreover, QSAR models were applied to assess the human and eco toxicity potential of formed bio-TPs. In the first article the QSAR estimations showed that the carcinogenicity, genotoxicity and mutagenicity might be altered after biotransformation. Particularly, it is of interest that several alerts for bacterial mutagenicity and micronucleus formation were predicted in a set of biotransformation products (bioTP1$_a$ and bioTP1$_a$, bioTP1$_c$, bioTP1$_e$, respectively) but not in the parent compound. In the second article the *in silico* predictions provided initial indications that on the one hand toxicity should be alerted towards environmental bacteria (bio-TP1$_e$) and

towards rainbow trouts (bio-TP1$_d$ and bio-TP1$_f$), respectively. On the other hand, no indication was found that the bioconcentration factor (BCF) of the transformation products was altered compared to the parent compound. This gives a first indication that the biotransformation might increase the ecotoxicological potential of a chemical compound, which would deserve further experimental attention.

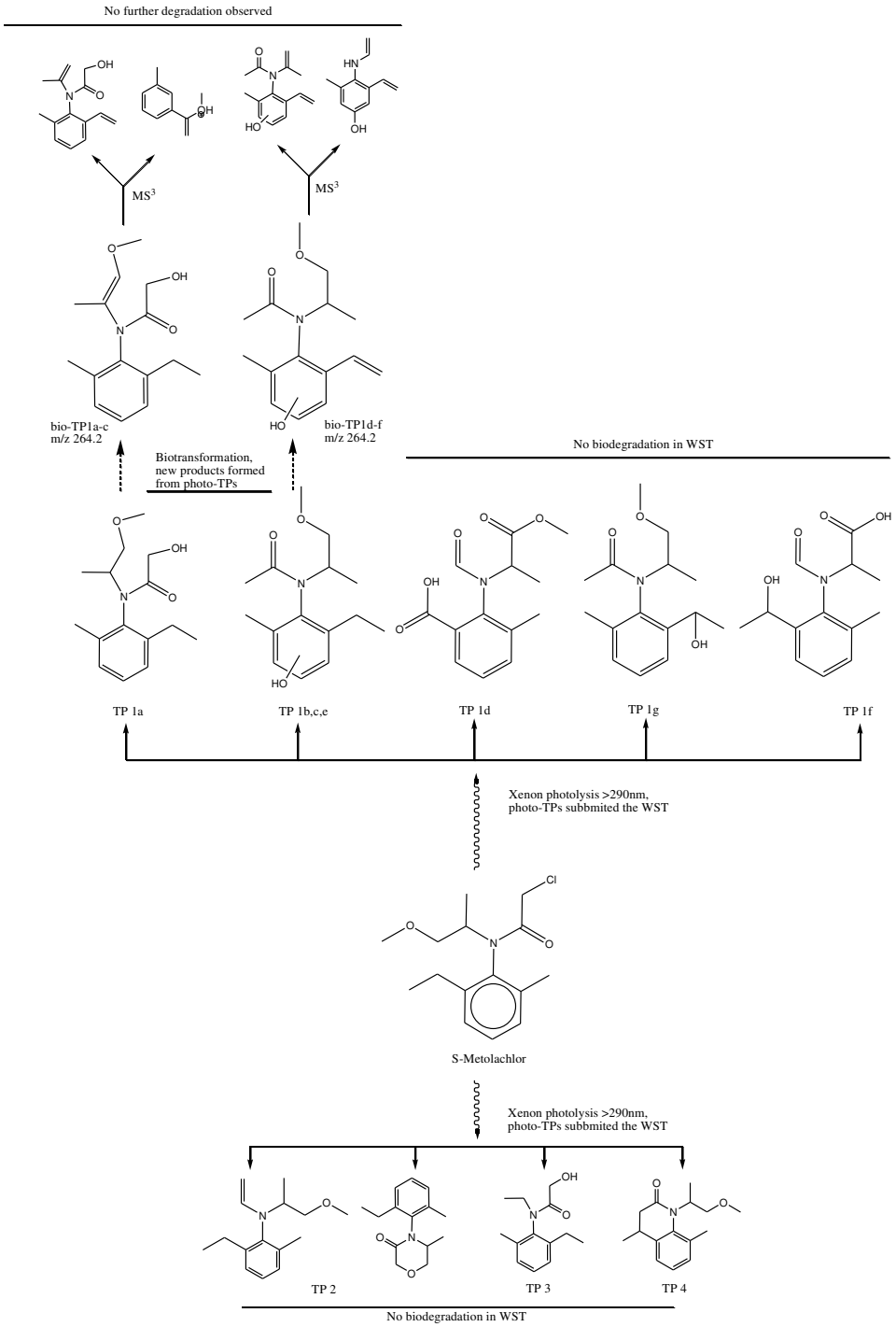

Fig. 4. SM and MG abiotic and biotic degradation pathway.

The third publication deals with biodegradability and photo-transformation of fluorescence tracer UR. In this study the biodegradability of UR was examined in CBT, MRT and WST where biodegradation of UR have not been observed. It is worth to mention that in CBT and WST only a small extent of biodegradation occurred, however not significant. Photolysis in water was conducted by Xe lamp irradiation for 8 hours. The primary elimination of UR was monitored and structures of its TPs were elucidated by HPLC-FLD-MS/MS. Thereby, elimination of the photolysis mixture was examined. Although the HPLC analysis showed high elimination of parent compound from 75.4% to 83.0%, varying on the initial concentration, the mineralization rate (NPOC) of UR was relatively low. The variation of NPOC removal ranged from 8.2% to 17%, depending on the UR concentration. The low degree of mineralization indicated that the tested substance was not fully degraded, instead transformed to photo-TPs, more resistant than their PC to photolysis.

Formation of new peaks in the chromatographic analysis of the samples collected during irradiation was observed by means of LC-MS/MS. The retention time for UR was 6.3 min and molecule ion was found at 333 *m/z*. A total of 5 compounds were identified as UR photo-TPs (Fig. 5). For structural elucidation each peak was isolated and further fragmented by means of AutoMS$^{(n)}$. Detected photo-TPs were investigated as possible mono- and di-hydroxylated derivatives of UR. They were labeled as $TP_{1a,b}$ (*m/z* 264.9) and $TP_{3a,b}$ (*m/z* 377).

It is interesting to mention that only one identified photo-TP ($TP_{1a,b}$) had a lower mass compared with the PC. This product eluted at two different retention times probably because of OH group, which could be added to 10 sites of UR aromatic rings. The TP_2 differs only 16 Da (higher) from the parent compound suggesting that generation of this compound could occur due to addition of a hydroxyl group to the one of UR' aromatic rings. Addition of the hydroxyl group might occur at ten possible sites of the UR molecule. The fragmentation patterns confirmed that TP_1 and TP_2 were hydroxylated products whereas TP_3 belong to the carboxylated compounds.

Similarly to the parent compound, only small extent of biodegradation occurred for the photolysis mixture. These results classify UR and UR-TPs as *not readily biodegradable.* The measurements with HPLC-FLD indicated that no elimination of UR and the photoproducts occurred during the CBT and MRT. However, at the end of WST the HPLC-FLD analysis showed elimination of 2.4 mg L^{-1} (11.7%) of initial UR concentration from the water phase. This might have been a result of a partial sorption to the sediment particles or that a small part of the parent compound as well as its photo-TPs were degraded by the microorganisms.

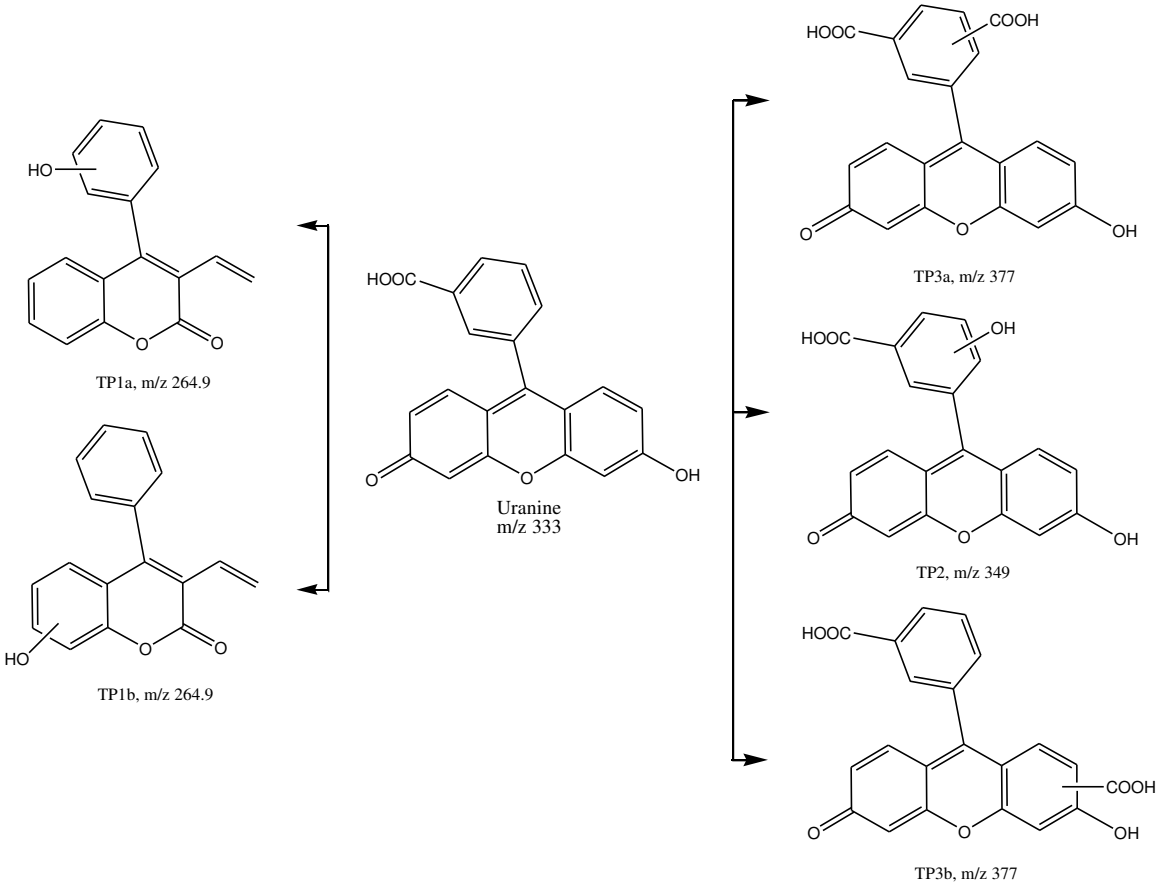

Fig. 5. Proposed photo transformation products of UR identified by means of LC-MS/MS.

5. Synopsis

The principle aim of this thesis was the comprehensive characterization and identification of the stable transformation products resulting from photolysis and biodegradation processes for all investigated compounds. Additionally, the adjuvants influence on above mentioned processes was examined and *in silico* predictions for toxicity in humans and eco-toxicity of the bio-TPs. For this purpose, a chemical-analytical method based on a combination of innovative approach, convenient sample generation and collection with sensitive LC-MS/MS analysis was successfully developed. Benefitted from this approach, the objectives addressed in the thesis have been achieved and the following conclusions can be drawn:

- This study demonstrates that for all investigated compounds, the photolysis is an important abiotic elimination process, however without complete mineralization, leading to formation of many TPs. Additionally, similar elimination rates for UR and SM might suggest that UR could be used as proxy to mimic SM photolytic decay in the field experiments.
- It was shown that neither of the tested compounds nor their photo-TPs were readily biodegradable in the performed tests, although small, but not significant elimination occurred for the hydrological tracer. Therefore, no biodegradation can be expected for the investigated chemicals when they directly reach into surface water. There, they can undergo photolysis by sunlight.
- This study found that the adjuvants do not significantly affect the biodegradation process but can result in enhancing photolysis yield and increase the diffusion of the active substance to the sediment.
- It was found for the first time that some of the SM's and MG's photo-TPs can be biotransformed to new products (bio-TPs) whereas other remaining photo-TPs of UR and pesticides were persistent to biodegradation in the aquatic environment.
- The applied techniques within this study emphasize the importance of *in silico* approaches such as QSAR models as a tool for getting additional information on environmental fate and effects of the bio-TPs. The *in silico* predictions provided evidence that these compounds might have an increased genotoxic, carcinogenic, mutagenic and eco-toxic potential compared to the parent pesticide.

Because of no, or at least incomplete mineralization of SM, SM in MG, UR or their TPs, the presence of these compounds in the environment may therefore pose a risk to the aquatic environment due to their persistence and the unknown properties of the photo-TPs. In other

words, the results found in this thesis highlight that not only there is an environmental risk by the PC, but also even higher by the persistent TPs, which can undergo further transformations in the surface waters. The combination of LC–UV-FLD-MS/MS analysis, NPOC monitoring, and QSAR gave valuable insights into the transformation processes of the PCs and the resulting bio-TPs of pesticides.

6. Conclusions

The work presented here shows how important is the assessment of environmental fate and risk for understanding the pesticides and hydrological tracer behavior in the aquatic environment. Furthermore, the results contribute to better understanding of the rate and relevance of the TPs, which provide more urgently required information within pesticide regulations. Outcomes also indicate that there is a case by case approach and investigation necessary. This holds on the one hand with respect to the individual compounds – even the structurally related (especially SM photo-TPs) showed similar behavior and fate within different simulation tests. On the other hand, these results show that different conditions within applied tests have an impact on the outcomes, especially formation of the bio-TPs from photo-TPs. This confirmed some of the knowledge already reported in literature and also extended it in new data. However, the biotransformation of photo-TPs and role of adjuvants and even the influence of the water-sediment interphase is new knowledge as reported here. The identification of TPs is one of the most difficult and challenging aspects in environmental chemicals analysis of micro-pollutants. It is important to underline the fact that knowledge on the TPs is still very limited, especially in terms of predicting their formation and the assessment of their physico-chemical and (eco) toxicological properties. Therefore, it was of high importance to identify the TPs by analytical methods and to characterize their fate by use different experiments (simulation tests) with *in silico* prediction approaches. New knowledge was gained due to combination of the Xe photolysis with the biotic degradation, especially to identify behavior of the photo-TPs. The toxicity predictions of identified bio-TPs were investigated with the goal of prioritizing their relevance in contribution to environmental risk assessment.

This research has led to many new findings that call for further investigation. In order to fill this gap, future studies should therefore concentrate on the development of the bio-TPs from identified photo-TPs of selected pesticides, and formation of stable photo-TPs of UR in the aquatic environment. Additionally, investigation of UR biotic elimination should be further examined as this study showed that it might contribute to its removal is some extent. This research also suggest that UR could be used as a reference substance to mimic photolytic decay of SM in surface waters as their photolysis rates were similar. Moreover, this thesis could provide information for the assessment and application of the artificial wetlands to reduce pesticide transport into surface waters. It could contribute to better understanding the role of biotic and abiotic process in the PC and its TPs fate in constructed wetlands. As this study identified relevant bio-TPs, the application of artificial wetlands has to be further discussed.

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Declaration 1

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

Lüneburg, 1st March 2015

Appendixes

Curriculum Vitae

Lukasz Gutowski, Born on 11.06.1986 in Elblag, Poland.

Education:

03/2011-12/2014
Ph.D. candidate (Dr. rer. nat.) in Institute for Sustainable and Environmental Chemistry, Faculty of Sustainability Sciences at Leuphana University in Lüneburg, Germany.

03/2009-11/2009
Master in Process Engineering and Biotechnology in Environmental Protection, University of Applied Sciences Offenburg and University of Warmia and Masuria, Poland.

2005-2009
Engineer in Environment Protection, *University of Warmia and Masuria, Poland.*

2002-2005
High school certificate, high school, Elblag, Poland

Employment and Professional Experience

2011-2014
Research fellow, doctorate student**,** Member of European project: PhytoRet Interreg IV, Supervision of B.Sc. Students + Theses, Supervision of B.Sc. Students + Theses, Supervision of experimental lab practical and tutoring. Leuphana University Lüneburg, Germany

4/2010- 10/2010
Research assistant, master student. The Universitätsklinikum Freiburg, Germany

List of Publications

1. **Lukasz Gutowski**, Oliver Olsson, Christoph Leder, Klaus Kümmerer (2015) A comparative assessment of the transformation products of S-metolachlor and its commercial product Mercantor Gold® and their fate in the aquatic environment by employing a combination of experimental and *in silico* methods. The Science of the total environment 2015;506-7:369–79)
   DOI:10.1016/j.scitotenv.2014.11.025

2. **Lukasz Gutowski**, Ewelina Baginska, Oliver Olsson, Christoph Leder, Klaus Kümmerer (2015) Assessing the Environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water-sediment test and *in silico* methods. Chemosphere 2015;138:847-55
   DOI:10.1016/j.chemosphere.2015.08.013

3. **Lukasz Gutowski**, Oliver Olsson, Jens Lange, Klaus Kümmerer (2015) Photolytic transformation products and biological stability of the hydrological tracer Uranine. The Science of the Total Environment 2015;533:446-53
   DOI:10.1016/j.scitotenv.2015.07.002

Article I

A comparative assessment of the transformation products of S-metolachlor and its commercial product Mercantor Gold® and their fate in the aquatic environment by employing a combination of experimental and *in silico* methods

Lukasz Gutowski, Oliver Olsson, Christoph Leder, Klaus Kümmerer

(**2015**)

The Science of the Total Environment 506-7:369–79

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A comparative assessment of the transformation products of S-metolachlor and its commercial product Mercantor Gold® and their fate in the aquatic environment by employing a combination of experimental and *in silico* methods

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HIGHLIGHTS

- S-metolachlor (SM) and SM in Mercantor Gold® were not biodegraded in water.
- Photolysis of SM was increased in the commercial formulation compared to pure SM.
- A total of 10 photo TPs were found for SM and MG, structures were elucidated.
- New bio-TPs were generated by aquatic micro-organisms from photo-TPs.
- New bio-TPs might be of higher toxicity compared with the parent compound.

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ABSTRACT

Even appropriately used, pesticides can enter the surface and groundwater by several routes where photochemical degradation along with biotic processes contributes to their fate, resulting sometimes in the formation of stable transformation products (TPs). Yet, little is known about S-metolachlor (SM) transformation in the aquatic environment. Furthermore, commercial formulation of a pesticide might have different physical and biological properties compared to its pure grade. The present study assessed the biodegradability of the pure SM and its commercial product Mercantor Gold® (MG) by employing two OECD biodegradation (301D, F) tests. Photolysis in water was investigated by using a Xe lamp. Subsequently the biodegradability of the photolysis mixtures was examined. The primary elimination of SM was monitored and structures of its TPs were elucidated by HPLC–UV–MS/MS. Additionally, a set of *in silico* prediction programs was applied for supporting analytical results and toxicity assessment of SM and TPs. S-metolachlor and Mercantor Gold® were not biodegraded. HPLC–UV analysis showed higher elimination of SM in MG compared to pure SM during photolysis. A total of 10 photo-TPs of SM and MG were identified. According to MS data and *in silico* predictions, chemical structures were proposed for all found photo-TPs. Likewise for the parent compounds, no biodegradation has been observed for their photo-TPs. However, in the 301F test new bio-TPs have been generated from photo-TPs which were observed for the first time according to authors' best knowledge. The results suggest that the MG formulation does not affect the biodegradation process, but it influences the photolysis efficiency and potentially might result in faster formation of TPs in the environment. This study also demonstrates that photo-TPs can be further transformed into new products due to bacterial activity in the water phase. Moreover biotransformation might lead to an increased toxicity compared with the parent compound.

1. Introduction

In regions with intensive agriculture, the detection of elevated pesticide concentrations in water samples both surface- and groundwater becomes more and more frequent, and often times exceeding drinking water thresholds (Köck-Schulmeyer et al., 2014; Herrero-Hernandez et al., 2013). Pesticide and TP environmental studies showed that TP

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concentrations can be more abundant than the parent compound (PC) concentrations in rivers (Olsson et al., 2013).

S-metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl]acet-amide) is a selective chloroacetanilide herbicide. From the chloroacetamide class, S-metolachlor (SM) is one of the three most commonly used worldwide (Martins et al., 2007) equating to 4.2% of the global pesticides used (Fenner et al., 2013). It is largely applied to annual grassy weeds, corn, soybeans, peanuts, and other crops. It is offered on the French market as an effective multi-crop herbicide against annual grasses and certain broadleaf weeds under the trade name Mercantor Gold® (MG). A reason for the global success of SM is that the S-enantiomer has replaced the existing racemic mixture of different metolachlor enantiomers (RM), which was introduced to the global market in 1977. The replacement of RM by SM allowed for the application of 35% less pesticide while providing the same effectiveness in weed control (O'Connel et al., 1998). RM has been intensively studied over the past years, thus providing a large amount of data in scientific literature (Liu et al., 2012; Xu et al., 2010; Ye et al., 2010; Shaner et al., 2006), however information about the S-enantiomer is scarce (Bedmar et al., 2011).

According to the EU regulation concerning plant protection products a degradation product or metabolite is either formed in the environment or by an organism respectively. Such a metabolite is only of relevance when there is a reason to assume it has a negative affect (i.e. toxicity) higher than the parent substance on organisms (Regulation (EC) No, 1107/2009). However, a clear description of metabolite fate in water phase is not well addressed. Stable TPs of chloroacetanilides herbicides were reported in surface and groundwater thanks to large body of monitoring data (Hladik et al., 2005). Moreover they have been detected more frequently and often in similar or even higher concentrations than their PC (Battaglin et al., 2003; Huntscha et al., 2008). Formed TPs are often smaller and more polar than their respective PC, therefore they have a higher potential to reach drinking water resources where they can be detected at fairly concentration throughout the year (Huntscha et al., 2008). Presence of pesticides TPs in drinking water might cause unexpected new problems like increase of carcinogenic potential during the water treatment with ozone (Schmidt and Brauch, 2008).

Once in an aqueous environment, pesticides have different pathways for their elimination from water such as hydrolysis, oxidation, biodegradation or photolysis (López et al., 2014; Martins et al., 2007). It has been reported by several studies that biodegradation plays the most important role in the fate of chloroacetanilide pesticides in the environment while other factors like photo-oxidation and chemical hydrolysis are of minor importance under typical (physio-chemical) conditions of soil and water (Humburg et al., 1989; Liu et al., 1991; Stamper and Tuovinen, 1998). Biodegradation tests were mainly carried out *in vitro*, focusing on isolation of soil microorganisms (*Bacillus* sp., *Fusarium* sp., *Mucor* sp., *Paracoccus* sp.), which are capable of metabolizing metolachlor (Saxena et al., 1987). Martins et al. (2007) reported degradation of S-metolachlor using the four possible isolated bacteria strains of about 35% on average after 10 days. However, Liu et al. (1995) showed that metolachlor is very stable in water when incubated for a period of 170 days in three different types of lake water resulting in no observed biodegradation. Other existing methods are focused on micro or mesocosm scale (Fenner et al., 2013).

Natural photodegradation of chloroacetanilide pesticides (data mostly available for metolachlor and several other widely used herbicides as alachlor, butachlor etc.) was studied in a lab scale as well as in the natural waters (Dimou et al., 2005). Contradictory findings have been reported concerning photolysis as the main abiotic degradation pathway for chloroacetanilides (metolachlor and SM) in the environment (Dimou et al., 2005; Wilson and Mabury, 2000). However, more data is available for the R-enantiomeric form than for SM. Kochany and Maguire (1994) found four compounds in lake waters resulting from dechlorination after 40 days of sunlight irradiation. Dimou et al. (2005) reported formation of up to nine photoproducts from simulated sun photolysis of metolachlor in aqueous media of different compositions and six out of nine to be formed in distilled water due to the direct photolysis with the Xe lamp. However there are still several knowledge gaps:

(i) Especially in photolysis, many TPs are often formed because of the radical reactions involved. However, knowledge regarding their fate and properties is very limited. Furthermore, if these TPs are persistent they may be of special interest for risk assessment. The combination of photolysis with aerobic biodegradation in order to identify persistent photo-TPs has already been applied to investigate the behavior of pharmaceuticals like antipsychotic or anti-hypertension drugs (Trautwein and Kümmerer, 2012a, 2012b; Mahmoud and Kümmerer, 2012). However, such studies have not yet been performed for chloroacetanilide pesticides.

(ii) Studies focused on the active ingredient of the pesticide itself neglecting possible effects of additional chemicals (adjuvants) that are found in commercial products. Adjuvants can affect the solubility, biodegradation and sorption properties (Katagi, 2008) and may act as a photo-sensitizer. A comparison of bio- and photodegradation of a commercial product with its pure active ingredient alone in terms for S-metolachlor has not yet been reported.

(iii) The possible formation of TPs and their environmental fate is largely unknown. Generation of data based on quantitative structure activity relationship (QSAR) are gaining importance especially for analysis and assessment of environmental TPs (Mahmoud et al., 2014; Rastogi et al., 2014a, 2014b) because these compounds are usually formed only in low concentrations within complex matrices so that isolation and purification is very difficult. Further, many of these TPs are not available commercially, which makes the individual experimental analysis of their toxicity impossible.

Therefore, this paper addresses specifically (i) the impact of MG's adjuvants on the biodegradation compared to the pure SM. (ii) The fate of the photo-TPs in simulated water environment was investigated. Thus, a combination of photolysis and two biodegradation tests was carried out to evaluate the primary elimination of the parent compound monitored by using HPLC–UV. The degree of mineralization was evaluated with the non-purgeable organic carbon (NPOC) analysis. This approach allowed for a comparison of the degradation and transformation potential of SM with the commercial product Mercantor Gold®. Furthermore, generated TPs were analyzed in terms of ready biodegradability and the observed biotransformation products (iii) *in silico* (QSAR) prediction tools were applied a) to support structure elucidation of the generated photoproducts as identified with LC–UV–MS/MS and b) for the assessment of toxicity of TPs.

2. Materials and methods

2.1. Chemicals

The analytical standard of S-metolachlor (98.4% chemical purity, CAS number 87392-12-9) was obtained from Fluka (Sigma-Aldrich, Steinheim, Germany) and the commercial product Mercantor Gold® from Syngenta Crop Protection, France. This product consists of S-metolachlor (86.5% w/w), a mixture of aromatic hydrocarbons (2–12% w/w), dodecylbenzenesulfonic acid, calcium salt (1–5% w/w), poly(oxy-1,2-ethanediyl),alpha-2,4,6-tris(1-phenylethyl)phenyl-omega-hydroxy(1–5% w/w, CAS number 70559-25-0), and 2-methyl-1-propanol (1–2% w/w). HPLC grade acetonitrile was purchased from VWR (VWR International, GmbH, Darmstadt, Germany). All aqueous solutions were prepared using ultrapure water 18.2 MΩ·cm (Ultra Clear UV TM, Barsbüttel, Germany).

2.2. Simulated solar photolysis experiments in aqueous solution

S-metolachlor and Mercantor Gold® solutions were dissolved in ultrapure water the day prior to the experiment and stored in the dark at room temperature. Both compounds (SM and MG) were subjected to the photolysis at two initial concentrations of 20 mg L^{-1} and 40 mg L^{-1}, respectively. 800 mL of the test solution was transferred to the photo-reactor under constant mixing using a magnetic stirrer. Temperature was set to 20–22 °C controlled by a circulating cooler (WKL230, LAUDA, Berlin). Photodegradation in water was performed in an ilmasil quartz immersion tube using a Xe lamp (TXE 150, UV Consulting Peschl, Mainz, Germany) as the source of radiation. The lamp emits spectra similar to natural sun light (200–800 nm) with the highest intensity in the visible range (200–280 nm: 1.61 e^{-2} W/m^2, 280–315 nm: 1.16 e^{-2} W/m^2, 315–380 nm: 3.75 e^{-2} W/m^2, 380–780 nm: 5.58 e^{-1} W/m^2) (data provided by the manufacturer). Before every experiment the lamp was warmed up for 3 min to reach maximum intensity. Photolysis experiments were performed for 8.0 h. Samples were collected every hour for HPLC and LC–MS/MS analysis. Samples for NPOC determination were taken at the time increments of 0.0 h, 4.0 h and 8.0 h.

Samples before (0.0 h) and after (8.0) h of photolysis were collected and subsequently submitted to the ready biodegradability tests: Closed Bottle test (CBT) and Manometric Respiratory test (MRT). The final concentration of SM and MG was adjusted by measuring NPOC of the tested substance (i.e. before photolysis) and photolysis treated samples, to provide required carbon content, and to reach adequate theoretical oxygen demand (ThOD), for each CBT and MRT, respectively (described further in 2.3 and 2.4). In parallel to every experiment, an HPLC analysis was run to support the NPOC results and determine primary elimination of the parent compound.

2.3. Closed Bottle test (OECD 301D)

CBT was performed according to the guidelines of the Organisation for Economic Co-operation and Development (OECD) (1992a). This test is characterized by low bacteria density (10^2–10^5 colony forming units (CFUs) mL^{-1}), low nutrients content, temperature at 20 ± 1 °C and kept in the dark as described elsewhere in detail (Kümmerer et al., 1996). Inoculum for the test was derived from the secondary effluent of a municipal sewage water treatment plant (SWT) (Lüneburg, Germany; population 73,500 equivalents). Two drops of inoculum were added to 1 L of mineral medium, which corresponded approximately to the 500 CFU mL^{-1}. The concentrations of standard solutions for S-metolachlor and Mercantor Gold® were 2.2 mg L^{-1} and 2.4 mg L^{-1}, respectively, corresponding to the theoretical oxygen demand ThOD of 5 mg L^{-1}.

The test consisted of four different series: (i) blank series (containing only the mineral medium and inoculum), (ii) quality control (contains readily biodegradable sodium acetate as the only relevant carbon source apart from the inoculum), (iii) test series (containing the target compound), and (iv) toxicity control (contains the target compound and sodium acetate as source of carbon). Toxicity was assessed by comparing oxygen consumption as measured in the toxicity controls with the predicted level computed from the oxygen consumption in the quality control and the test vessel, respectively. The amount of sodium acetate for each series corresponds to ThOD of 5 mg L^{-1}. All tests were run in duplicates.

The whole process was monitored by measuring dissolved oxygen concentration in the test vessels with Fibox 3 (Fiber-optic oxygen meter connected with Temperature sensor PT 1000) (PreSens, Precision Sensing GmbH, D-93053 Regensburg, Germany) in accordance with the international standard (ISO, 1990) for the 28th day period (OECD, 1992a). A compound is qualified as "ready biodegradable" when 60% of ThOD expressed as percentage of oxygen consumption is consumed within the period of 10 days after the oxygen uptake reached 10% of ThOD. Samples from the beginning (day 0) and the end of the test (day 28) were collected and stored at −20 °C until analysis with HPLC–UV and LC–MS/MS.

2.4. Manometric Respiratory test (OECD 301F)

The MRT works with higher bacterial density (5–10 × 10^6 CFUs mL^{-1}) and diversity as the CBT thus increasing the probability for biodegradation. This test was also performed according to the OECD guidelines (OECD, 1992b) in the dark at room temperature (20 ± 1 °C) under gentle stirring. The test series were as described for CBT in 2.3 with addition of abiotic control vessel (containing added sodium azide, to obtain sterile conditions). CO_2 production as the parameter of the endpoint biodegradation is measured indirectly by the OxiTop OC110-system (WTW, Weilheim, Germany). The so called pressure heads which are sealing the test vessel are used for this purpose. By biodegradation process, oxygen is consumed and carbon dioxide formed. Carbon dioxide is removed by reaction with sodium hydroxide under formation of sodium carbonate. The results are a drop of the pressure inside the test vessel which is proportional to the degree of mineralization of the test compound. The concentrations of standard solutions for S-metolachlor and Mercantor Gold® were 13.1 mg L^{-1} and 13.5 mg L^{-1}, respectively, corresponding to ThOD of 30 mg L^{-1}. Inoculum was derived from the municipal sewage treatment plant (Lüneburg, Germany; population 73,500 inhabitants). Aliquots (measuring) of 80 mL of inoculum were added to 1 L of mineral medium. The validity criteria are the same as for the CBT.

2.5. Analysis of SM and TPs by HPLC–UV and LC–MS/MS

The primary elimination was monitored by means of HPLC–UV (Prominence series Shimadzu, Duisburg, Germany). The chromatographic separation was achieved with RP-18 column (EC 125/4 mm NUCLEODUR 100–5 μm C18 ec, Macherey and Nagel, Düren, Germany) protected by a EC 4/3 mm NUCLEODUR 100–5 μm C18 ec guard column. Mobile phase consisted of ultrapure water (solution A) and 100% acetonitrile (solution B). For elution, the following gradient was used: 0.01 min 20% B, 3.0 min 20% B, 13.0 min 80% B, 20 min 80% B, 24 min 20% B. Sample injection volume was 20 μL and the oven temperature was set at 40 °C, flow rate was set at 0.7 mL min^{-1}. Retention times for SM and SM in MG were 14.20 min, the total run time was 30 min and the wavelength was set at 220 nm.

SM and MG standards (1.25, 2.5, 5, 10, 20, 40 and 80 mg L^{-1}) were used to obtain calibration curves and linear relationships were obtained. Regression coefficients for SM and MG were $r^2 = 0.999$ and $r^2 = 0.999$; $n = 2$, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for SM were 0.02 mg L^{-1} and 0.06 mg L^{-1}, respectively, and for SM in MG 0.07 mg L^{-1} and 0.2 mg L^{-1}, respectively.

The identification and elucidation of the TPs were performed with the LC–MS/MS Bruker Daltonic Esquire 6000 plus ion-trap mass spectrometer (IT-MS) equipped with the Bruker data analysis system (Bruker Daltonic GmbH, Bremen, Germany). The mass spectrometer was connected to an Agilent Technologies HPLC system (Agilent Technologies, Böblingen, Germany, HPLC 1100 series). The analytical separation was carried out using the same C18 column previously described. For elution, the same gradient method was applied as for HPLC analysis. Flow rate was 0.7 mL min^{-1} in LC part, before MS a T cap was applied reducing the flow to the half (0.35 mL min^{-1}). Injection volume was 20 μL and oven temperature was set to 40 °C. The retention time for SM was 14.25 min and molecule ion was found at 284.4 *m/z*. The MS was operated in a positive mode polarity. More information about the MS settings can be found in Text S1 in Supplementary information (SI). Analysis of total ion chromatogram and corresponding mass spectrum was used for structural identification of TPs. By means of AutoMS(n) mode, each *m/z* of TPs identified in the TIC was used as precursor ion and further fragmented up to MS^3.

2.6. In silico prediction of TPs

The photodegradation pathway of SM in MG and SM were predicted with the software MetaPC (version 1.8.1, MultiCASE Inc., Beachwood, USA) in order to get information on the chemical structure of TPs in addition to mass spectrometry that in turn allows improving the reliability of the proposed structural formula of TPs. This software predicts the chemical transformation under different conditions such as mammalian metabolism, aerobic and anaerobic degradation and photo-degradation (Sedykh et al., 2001). Meta software consists of a library of known pairs of transforming ("transforms") and target sequences. The followed test molecules are scanned for these target sequences. The thermo stability of the investigated chemical as well as its spontaneous reaction module for unstable structural moieties are also monitored and taken under account. The output is a list of generated TPs. The obtained and identified TPs from the mass spectrometry were compared to those predicted by the software.

2.7. Calculation of photolysis half-life

In order to check whether the photodegradation was a first order rate, the linear regression was made based on the logarithmic concentration values ($\ln[C_t]/[C_o]$) determined as a function of time. The photodegradation constant k_{obs} was obtained by subtracting the exponents of different degradation curves represented by the apparent degradation (k_{app}) and degradation factors such as volatilization, hydrolysis and biodegradation (as dark experiment, k_{dark}). In that matter the k_{obs} constants can be expressed as $k_{obs} = k_{app} - k_{dark}$ where the estimated half-lifes can refer to the actual experiments, without the contribution of other factors. The half-life of both substances was determined by using the equation $t_{1/2} = \ln 2/k$.

2.8. In silico prediction of ready biodegradability

To assess whether or not the identified TPs can be classified as readily or not readily biodegradable Biowin models, which origin from the Environmental Protection Agency (U.S. EPA, 2004) as well as Syracuse Research Corporation are included in the EPA's EPI suite software (EPIWEB 4.1) package were used. They gained popularity due to their easy usability and for producing acceptable data collected by US regulatory authorities. To have a rough overview on the biodegradability potency with the correlation to the OECD test guidelines the Biowin models 5 and 6 were taken under consideration. Those are linear and non-linear regressions models that can predict the biodegradability potential of the substance in the MITI-I test (OECD 301C) based on the MITI data (Ministry of International Trade and Industry (Japan)). As an outcome the prediction of readily/none readily biodegradable in MITI-I test is coded from 0 to 1, where a result higher than 0.5 is indicative for readily biodegradable. Due to higher bacterial density and diversity, respectively, predicted values from MITI test are not directly comparable to the Closed Bottle test (Trautwein and Kümmerer, 2012a, 2012b). Therefore these results were used in this study only as a rough first orientation. As a prediction input the simplified molecular input line entry specification (SMILES) codes from the molecular structure of the TPs were taken as derived from the molecular structures as established with LC–MS/MS (ion trap).

2.9. In silico assessment of TPs by QSAR models

In silico toxicity prediction and physicochemical parameters of SM and its TPs were assessed using a set of different QSAR software each with their own strength because of different algorithms and training sets. The set of software used were the CASE Ultra V.1.5.0.1 (MultiCASE Inc.) (Saiakhov et al., 2013) and Leadscope software V.3.2.3-1 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.

CASE Ultra and Leadscope software provide a positive, negative and out of domain (OD) estimations for the selected models. OD means that the test chemical is not included in the applicability domain of the model used. Often CASE Ultra software provides alerts for all its selected models like 'Inconclusive' and Inconclusive with asterisk symbol (*). 'Inconclusive' alert means that a significant portion of the test chemical is covered by unknown structural fragments and Inconclusive with asterisk symbol (*) means both positive and deactivating alerts were found in the same molecule and therefore a clear result cannot be given. The above mentioned models and software are described in detail elsewhere (Mahmoud et al., 2014; Rastogi et al., 2014a, 2014b). Table S1 in SI enlisted all the *in silico* software and their respective models used in the present study.

3. Results and discussion

3.1. Biodegradation of the parent compound in CBT and MRT

The validity criteria for CBT according to the OECD guideline (>60% ThOD of the quality control — sodium acetate is required to be degraded within 14 days) were met (Organisation for Economic Co-operation and Development, 1992c). No toxic effects on bacteria (biodegradation in toxicity control >25%, Fig. 1) were observed by any tested substance in the toxicity control bottles. Both substances acted much alike in all biodegradation tests indicating that MG adjuvants had no impact on the SM biodegradation, therefore only SM in MG degradation is presented here (Figs. 1 and 6). No biodegradation has been observed for MG and SM in CBT classifying them as being *not readily biodegradable* (Fig. 1). The average biodegradation values after 28 days for MG and SM (0.0 h photolysis time, respectively) monitored by measurement of the oxygen concentration were 4.1% and 4.3%, respectively and in fact presented no difference on the background of the nominal variation of the test results.

Similarly to the CBT the MG and SM were *not readily biodegradable* in the MRT. The validity criteria were met since 60% of the quality control substance was biodegraded within 10 days. No toxic effects on bacteria were observed in the toxicity control as well as no degradation was observed in the sterile control. The average biodegradation values after 28 days for MG and SM (0.0 h photolysis time) were 3.8% and −3.1% respectively. The reason for the negative values in MRT might be interpreted as high degradation in the blank controls and should be considered as 0% degradation of the test substance.

3.2. Photodegradation

The preliminary elimination of both SM and SM in MG compounds was monitored with HPLC–UV analysis. As a result all photodegradation experiments first order rate constants for SM and SM in MG were obtained as a very good fit of the data by a linear regression of logarithmic concentration values ($\ln[C_t]/[C_o]$) determined as a function of time (Fig. 2). The first-order linear relationship of $\ln C/C_0$ versus t (from 0.0 h to 8.0 h) was found based on the obtained results for SM and SM in MG (Fig. 2). Obtained results demonstrate that SM and SM in MG photolysis obeyed first-order kinetics. The rate constants and half-times for SM and SM in MG (20 mg L^{-1} and 40 mg L^{-1}) were $k_{obs} = 0.1867$, 0.147 h^{-1} and $t_{1/2} = 222.6$ min, 283.2 min (SM) and $k_{obs} = 0.1992$, 0.169 h^{-1} and $t_{1/2} = 208.8$ min, 246 min (MG) respectively. Generally, obtained first-order rate results fit to the outcomes of studies of Dimou et al. (2005) and Kochany and Maguire (1994) for photodegradation of metolachlor in purified water, however differ in obtained half-time values. In both studies calculated half-time were much higher (87 h and 192 h, respectively) than presented here. One of the reasons

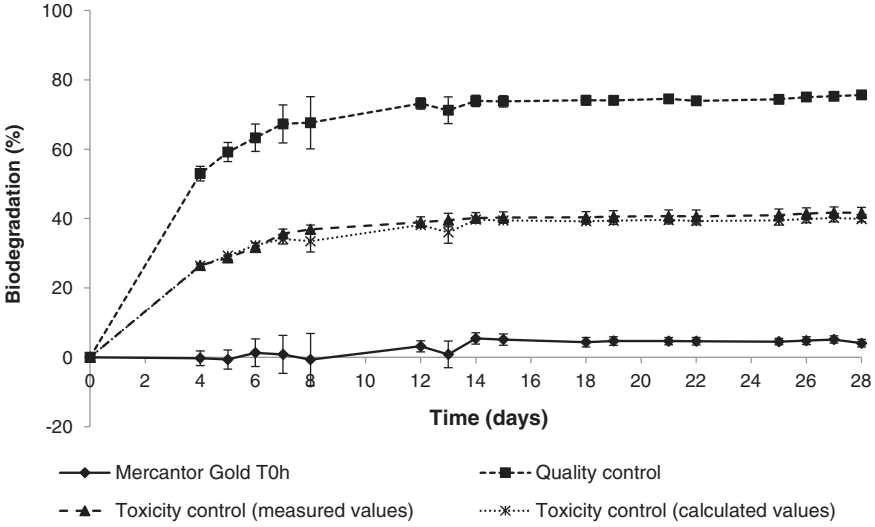


Fig. 1. Biodegradation in Closed Bottle test of MG at the time point 0.0 h (without phototreatment). No biodegradation (solid lane) can be observed during 28 days of the test duration. All values represent the means ± SD (n = 4).

might be a different experimental setup or concentration of the active substance used.

The comparison of SM and SM in MG degradation dynamics and their rate constants are shown in Fig. 3. The HPLC analysis showed a degradation of about 74.2% (±0.9%) of the initial concentrations of the commercial product (20 mg L^{-1} and 40 mg L^{-1}) in comparison to 68.9% (±0.7%) of the pure pesticide at the same concentrations. The SM in MG was slightly better degraded than SM alone. The reason might be the complex mixture of this commercial product. Water solubility of pure SM is 488 mg L^{-1} at 20 °C (PPDB, 2014). One of the MG adjuvants (dodecylbenzenesulfonic acid calcium salt) is a surface-active substance as its mode of action is to solubilize, suspend or disperse the active ingredient of SM. Therefore the solution of MG might be easier accessible to the Xe lamp radiation than its pure active substance as oily droplets dissolved in water. However, Katagi (2008) indicated that it is unlikely for surfactants to act as photosensitizer because they are transparent to UV–visible region, except when they contain an aromatic moiety (i.e. alkylphenoxy ethoxylates) in their structures. Dodecylbenzenesulfonic acid has an aromatic element (benzenesulfonic) within its structure, therefore it might act as photosensitizer, and thus result in higher efficiency of MG photolysis. The measurement of NPOC removal was conducted in parallel with each experiment to monitor the possible mineralization of tested compounds during the photodegradation (Fig. 3). In all cases the results indicated that after the 8.0 h irradiation variation of NPOC no higher than 1% was observed. This indicated that the tested substances were not mineralized, instead transformed into TPs that were resistant to further photolysis under Xe irradiation. The monitoring of the pH showed that at the beginning of the experiment the SM solution had pH of 6.2 (0.0 h) and at the end (8.0 h) the pH was 4.1. Likewise, the initial pH of MG-solution was 6.1 and after 8.0 h of irradiation was 4.1. To some

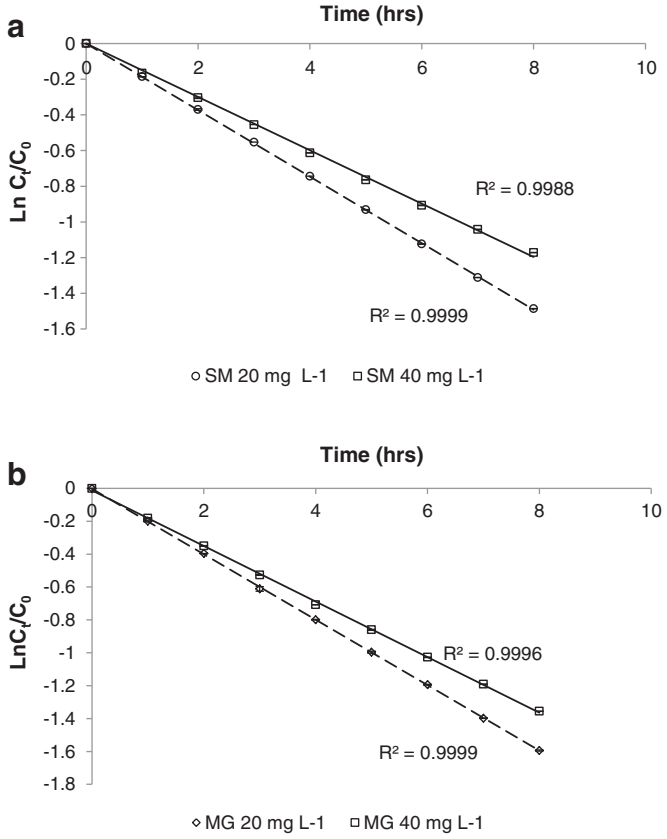


Fig. 2. First order photodegradation kinetics of a) S-metolachlor and b) Mercantor Gold® at 20 mg L^{-1} and 40 mg L^{-1}, photolysis with Xe lamp for 8.0 h. All values represent the means ± SD (n = 2).

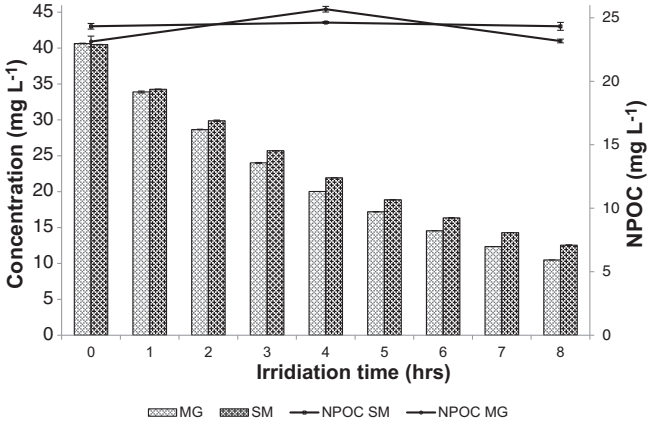


Fig. 3. Elimination of S-metolachlor and Mercantor Gold® during the irradiation with Xe lamp for 8.0 h. Secondary y-axis represents evaluation of non-purgeable organic carbon. All values represent the means ± SD (n = 2).

extent this might indicate acidic nature of newly formed transformation products. Furthermore, obtained UV–vis spectra (SI, Fig. S1) showed that in acidic pH there was no effect of hypochromic or bathochromic shift for better absorbance of radiation emitted from the lamp. Therefore, it clearly indicates that the pH change could not be a reason for faster photo degradation of MG.

By means of LC–MS/MS the formation of new peaks in the chromatogram from samples of the photodegradation test were observed. The peaks were gradually increasing with the irradiation time reaching maximum intensity after 8.0 h. This demonstrated the formation of photo-TPs. The primary investigation was based on suspected-target approach by comparing the chromatograms from the beginning of the experiment (0.0 h) with samples taken at each time point (every 60 min) until 8.0 h.

Fig. 4a shows the total ion chromatogram (TIC) of SM in ultrapure water obtained at the time point 0.0 h. Fig. 4b shows the new peaks ($TP1_{a–g}$, TP2, TP3 and TP4) resulting from photolysis of SM after 8.0 h. Moreover, aforementioned TPs tend to be of higher polarity than parent compound itself. SM in MG showed the same chromatographic behavior as SM, presenting adjuvants with no impact on analytical separation. Retention times and TPs were identical to those presented from analytical grade of SM. The MS/MS fragmentation pattern generated based on their peak intensity to achieve structural elucidation are shown in Table 1. The kinetics of appearance of photo-TPs which were formed during the photolysis are provided in detail is SI (Figs S2, S3, S4 and S5).

It has been mentioned by many authors that chlorine removal, hydroxylation and cyclizations (ring formation) are the most often occurring reactions during photolysis (Coffinet et al., 2012; Khaleel et al., 2013; Souissi et al., 2013). This was found here too. A total of 10 identical compounds were identified as SM and SM in MG photo-TPs (Fig. 5). In both cases the most abundant peak was *m/z* 266.2 (R_t = 8.7 and 12.5 min). Taking the above into consideration the mass of 266.2 *m/z* compared to 284.4 *m/z* of SM observed at seven different retention times (R_t = 8.6, 9.5, 9.8, 10.3, 11.4, 12.3 and 12.6 min) could be identified mostly as isomers and an example of monohydroxylation. Photo-TPs were labeled as $TP1_a$, $TP1_b$, $TP1_c$, $TP1_d$, $TP1_e$, $TP1_f$ and $TP1_g$ related to their retention times.

For structural elucidation each peak was isolated and further fragmented (Table 1). The fragmentation pattern of *m/z* 266.2 delivered almost identical mass spectra making it difficult to distinguish the different isomers and compounds formed. However, the proposed structures were supported by *in silico* (MetaPC) predictions (Fig. 5, $TP1_d$ and $TP1_f$). All of the identified products of *m/z* 266.2 are assumed to result from dechlorination process and addition of a hydroxyl group to the structure, which might occur at eight sites of the molecule. Fragmentation pattern for these compounds is similar since all lose 32 Da (CH_3OH), when the functionality of the ether remains and 18 Da (H_2O) in accordance with hydroxyl moiety addition.

$TP1_b$, $TP1_c$ and $TP1_e$ were identified as phenol containing isomers because they display similarity in their mass spectra and retention times. However, the correct position of either *ortho*, *para* or *meta* of hydroxyl group could not be elucidated. Coffinet et al. (2012) has also identified seven photolysis products of metolachlor with *m/z* of 266 and many others similar to those found in the present study. Above mentioned photo-TPs were also found in water using high-pressure UV lamp. It should be noted that one of identified isomers of the *m/z* 266.2 was widely detected before as important photoproduct hydroxyl-metolachlor (Dimou et al., 2005; EPA, 2006; Kochany and Maguire, 1994).

The compound with *m/z* of 248 (TP4) only differs by 18 Da from the isomers described in previous paragraph. It can be assumed that it resulted by a loss of water from the monohydroxylated TPs mentioned

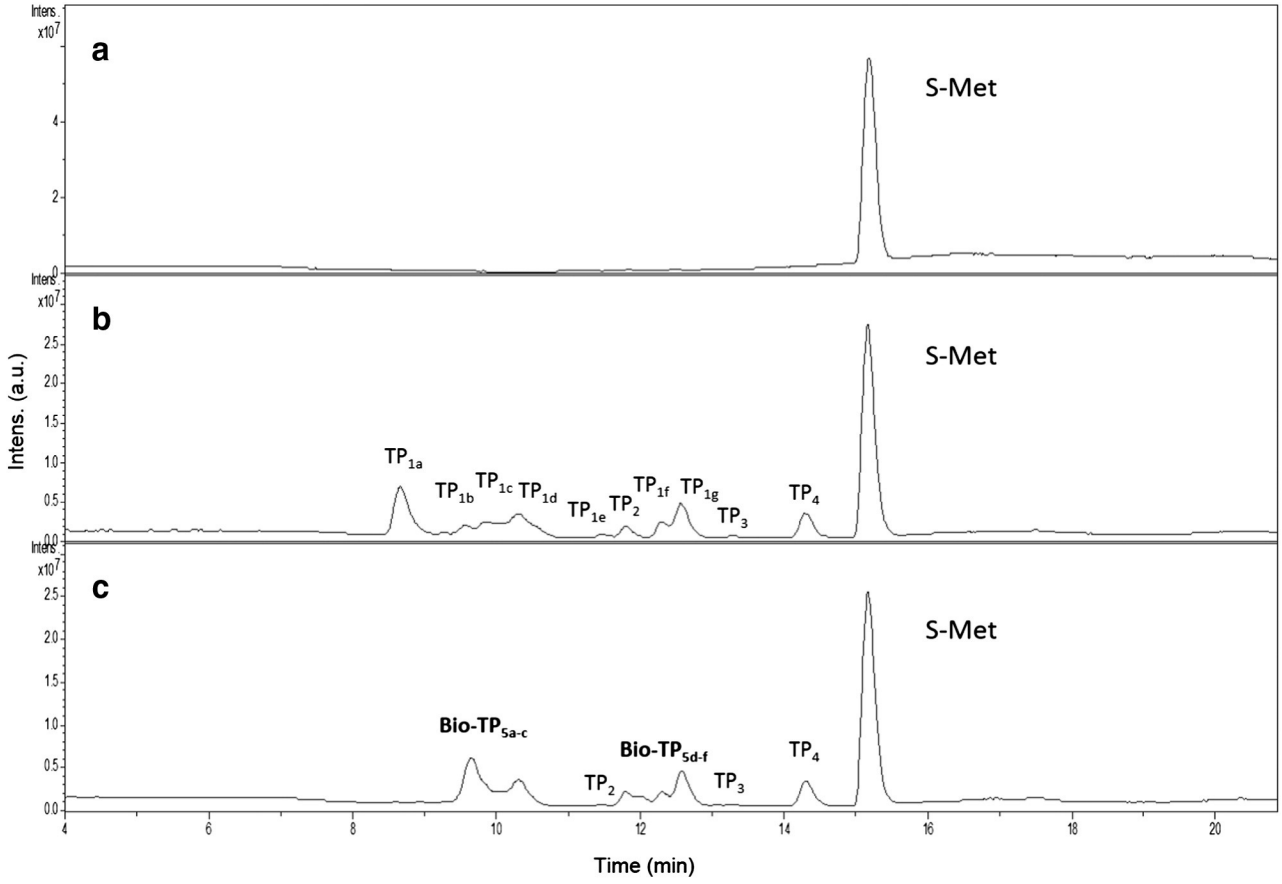


Fig. 4. Total ion chromatogram (TIC) of SM in the MRT test: a) sample at time point 0.0 min, b) sample after photolysis for 8.0 h and subjected to the MRT test, at day 0, and c) sample containing TPs after incubation for 28 days. Note that the scale varies.

Table 1
Chromatographic parameters of SM, MG and their transformation products analysis by LC/MS–MS (Rt-retention time, *m/z*-mass to charge ratio, relative abundance in bracets).

Compound	Rt (min)	Main precursor ion (*m/z*)	Product ions (*m/z*), % of relative abundance in brackets
$TP1_a$	8.6	266.2	234 (100), 149.1 (28.45), 135.1 (89.56), 147.1 (100)
$TP1_b$	9.5	266.2	234 (100), 216.2 (28.85), 134.9 (100), 159.9 (17.61), 174.1 (23.57)
$TP1_c$	9.8	266.2	234.2 (100), 248.2 (94.77) 177.1 (67.82), 216.0 (100), 149.0 (100)
$TP1_d$	10.3	266.2	177.1 (100), 149.1 (44.56), 149.0 (100), 120.9 (100)
$TP1_e$	11.4	266.2	176.98 (100), 234 (57.90), 149 (100)
TP2	11.8	234.4	147.6 (98), 174.0 (20.86), 188.0 (19.17)
$TP1_f$	12.3	266.2	177.1 (100), 149.1 (50.04), 234.2 (32.21), 122.0 (37.28)
$TP1_g$	12.6	266.2	234.2 (100), 145.9 (100)
TP3	13.3	222.1	172.0 (100), 204.1 (40.36)
TP4	14.3	248.4	216.2 (100), 176.1 (9.55)
S-metolachlor	15.2	284.4	252.4 (100), 176 (100)
MRT product day 28, bio-$TP5_{a–c}$	9.6	264.2	232.2 (100), 149.2 (17.72), 175.0 (18.54), 204.0 (100)
MRT product day 28, bio-$TP5_{d–f}$	12.0	264.2	232.2 (100), 175.0 (100), 204.0 (18.97)

above, as suggested by Coffinet et al. (2012) and Souissi et al. (2013). The product ions of this compound lose 32 and 72 Da to provide *m/z* ions of 216 and 176 respectively. Due to the fragmentation pattern it can be postulated that this molecule corresponded to the cyclic compounds, which was previously suggested by Wu et al. (2007). However, taking into account the evolution of all the irradiation products as a function of time of photolysis, the most visible trend was that their concentration increased during the photodegradation experiments to reach its maximum after 8.0 h. Therefore, it could be suggested that generation of this product occurs directly from the parent compound SM by loss of chlorine followed by cyclization.

The product with *m/z* 234.1 (TP2) (Fig. 5) differs only 14 Da from TP4 suggesting that TP2 has kept the $–(CH_3)CH–CH_2$ chain. Thus, it could be inferred the loss of one CH_2 group, as found by Coffinet et al. (2012). It is worth mentioning that this compound has been detected in previous studies as major photo-TP (Dimou et al., 2005; EPA, 2006; Mathew and Kahn, 1996). Due to unclear fragmentation of this product, the obtained mass spectrum was difficult to interpret. As a result two possible

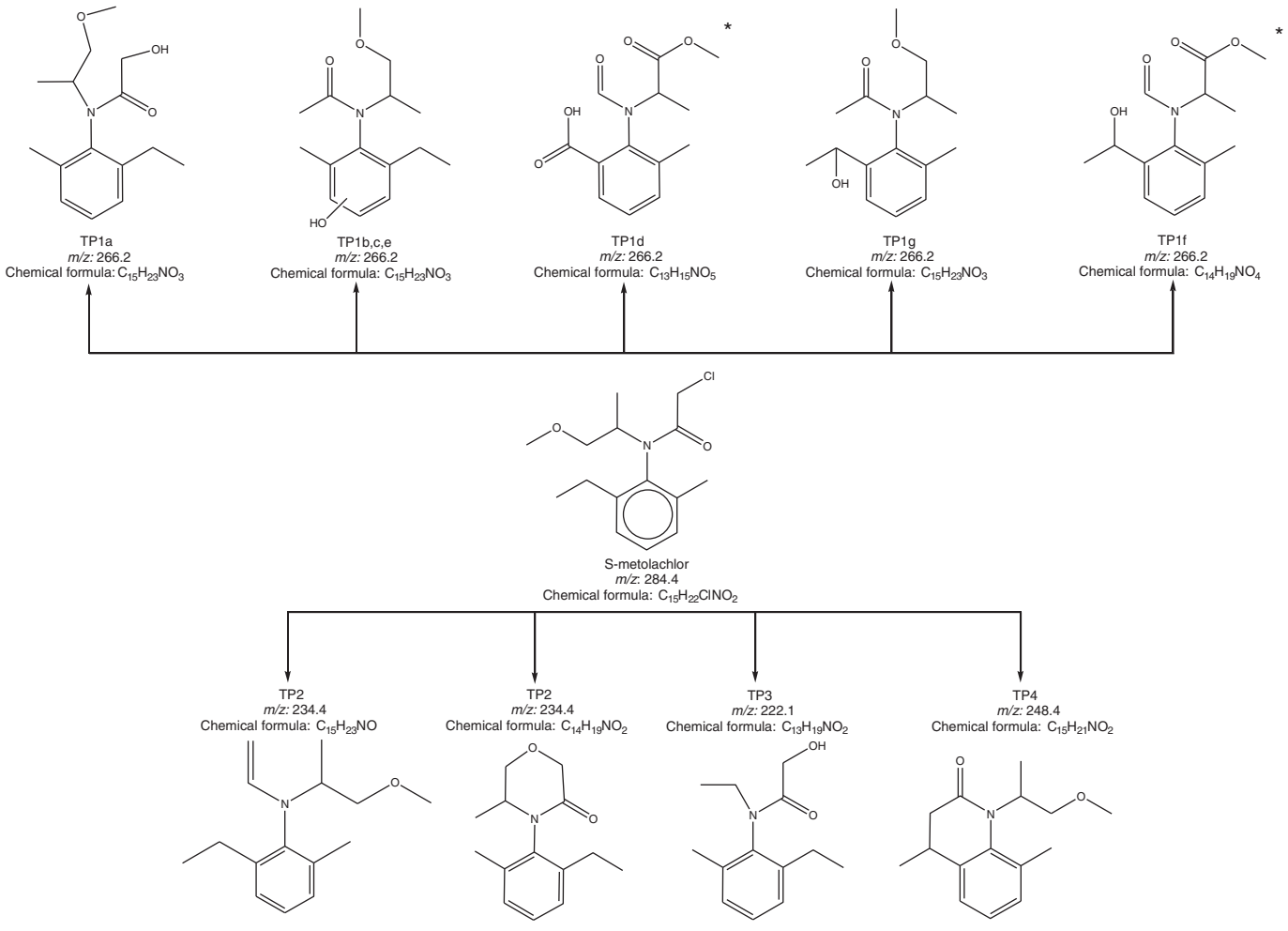


Fig. 5. Scheme of the proposed photodegradation pathway for SM. * — Structures predicted by the MetaPC software.

structures of the same mass have been suggested in this study. Likewise, Coffinet et al. (2012) have not provided mechanism of formation for this compound.

It is interesting to mention formation of a photo-TP with *m/z* 222.1 (TP3) which previously was described as minor photoproduct of Alachlor (Hogenboom et al., 2000; EPA, 2006). The fragment ion of this compound loses 18 Da (water) and 50 Da to provide ions of *m/z* 204 and 172, respectively. It could be suggested that this compound resulted from the photoproduct of the *m/z* 266.2 due to the loss of 44 Da (CH_2CH_2O).

3.3. Biodegradability of the photo-TP's in CBT and MRT

In the CBT, for samples after 8.0 h photolysis the average biodegradation values for MG (Fig. 6) and SM were both 5.5% on the 28th day. Those values classified MG (i.e. SM and the adjuvants), and SM-TPs as *not readily biodegradable*. The measurements with HPLC–UV confirmed that no elimination of SM in MG and SM along with their photoproducts occurred during the test. In other words the photo-TPs were resistant against biodegradation in CBT. No toxic effects on bacteria (biodegradation in toxicity control >25%, Fig. 6) were observed by the photo-TPs.

Likewise in the CBT, no biodegradation has been observed for the photo-TPs in the MRT. In the samples after 8.0 h of photolysis, the average biodegradation values for MG and SM were 6.3% and −2.4% on the 28th day, respectively. Also here, no toxic effects on bacteria were reported in the toxicity control. Generally, due to greater microbial density it can be expected to obtain higher degradation rates in MRT compared to CBT. However, lower on negative values might be explained as a result of a higher concentration of test substance subjected to the MRT which might inhibit bacteria from biodegradation as suggested by Rastogi et al. (2014a, 2014b).

The measurements with HPLC–UV confirmed that no elimination of SM in MG, SM alone and their photoproducts occurred during the tests. However, LC–MS analyses of MRT test samples and the generation of bio-TPs in the samples after 8.0 h of photolysis for MG and SM indicated that these can be attributed to SM or photo-TPs of SM (Fig. 4c). Some of the TPs with the *m/z* 266.2 were degraded after 28 days. Instead, two products with the *m/z* 264.2 (R_t 9.7 and 12.0 min) were formed during the MRT and were present in the solution on the 28th day (Table 1). To get further information about these two possible bio-TPs ($TP5_{a-c}$, $TP5_{d-f}$; Fig. 4c), the ions with *m/z* 264.2 were investigated using the MS^n mode, where they were isolated, used as precursor ions and further fragmented up to MS^3. The product ions and the percentage of relative abundance are given in Table 1. Neither a specific mass of *m/z* 264.2 was found in the abiotic control nor were the 7 TPs of *m/z* 266.2 degraded in the sterile control. The LC–MS analysis on the 28th day showed that the photo-TPs (*m/z* 266.2) were not abiotically degraded. Therefore it can be concluded that the formed bio-TPs (*m/z* 264.2) are resulting from bacterial transformation of photo-TPs (*m/z* 266.2) and not from any abiotic elimination like hydrolysis or sorption, which did not occur in the sterile control.

The results of biodegradation tests are in accordance with predictions from EPI Suite software. The predictions of Biowin 5 and 6 confirmed in principle the results obtained from CBT and MRT tests. Predicted biodegradation probability was well below 0.1 for S-metolachlor in both analytical standard and in the commercial product. Predicted values for the TPs of SM and SM in MG fitted well to the experimental data: degradation probability of TPs with the *m/z* 233, 221 and 248 were 0.2, 0.45 and 0.18, respectively. Thus they have to be classified as not readily biodegradable which was found by the CBT and MRT tests. From proposed structures of 6 TPs with the *m/z* 266 two ($TP1_d$ and $TP1_f$) achieved higher than 0.5 score and thus can be assumed to be readily biodegradable. Their proposed structures can be found in Fig. 5. The remaining 4 TPs of the same *m/z* 266 were not predicted as readily biodegradable by achieving in average score of 0.2 and 0.3, respectively. Although obtained results are higher than of the parent compound, nevertheless still below 0.5. It might indicate their possible biotransformation due to the inoculum activity or spontaneous hydrolysis in aquatic environment which is not taken under consideration in the model (Mahmoud et al., 2013).

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

S-metolachlor and the observed biotransformation products were assessed by a set of *in silico* predictions for toxicity, since it cannot be excluded that these biotransformation products will appear and possibly will accumulate in the environment (Baran and Gourcy, 2013). A set of programs for predicting carcinogenicity, genotoxicity and mutagenicity was applied in order to take into account that the available programs

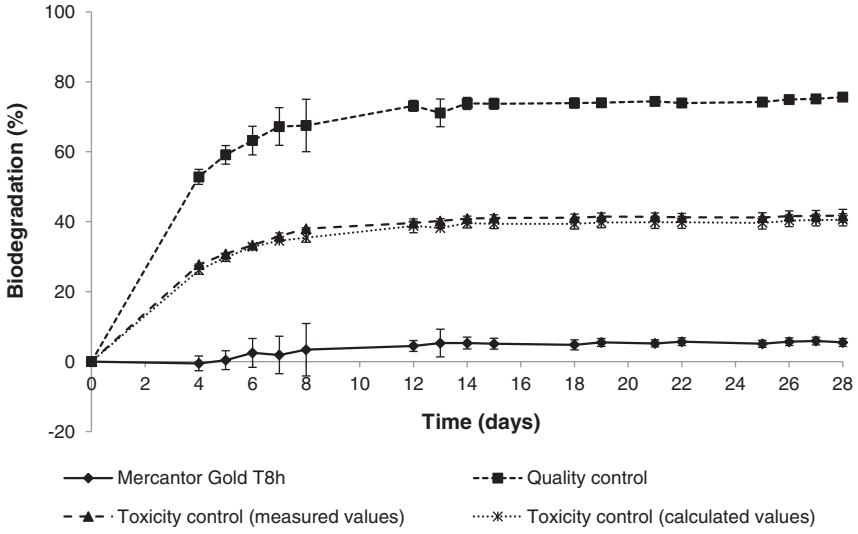
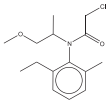
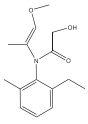
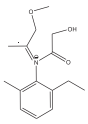
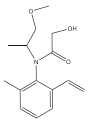
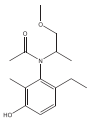
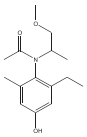
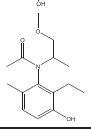


Fig. 6. Biodegradation of MG and its photo-TPs ("T8h") in the Closed Bottle test. Samples subjected the CBT were taken from photodegradation experiments, after irradiation for 8.0 h with Xe lamp. No biodegradation (solid lane) can be observed during 28 days of the test duration. All values represent the means ± SD (n = 4).

Table 2
In silico toxicity prediction by different models of CASE Ultra and Leadscope for S-metolachlor and the observed newly formed bio-TPs in MRT test.

Name, MS (m/z), R_t (min)	Structures	QSAR model								
		CASE Ultra					Leadscope			
		A	B	C	D	E	C	D	E	B
S-metolachlor 284.4; 15.2		+	+	+	+	−	+	OD	−	−
Bio-TP5$_a$ 264.2; 9.6		+	OD	+	+	+	+	OD	−	+
Bio-TP5$_b$ 264.2; 9.6		+	OD	+	+	OD	−	+	−	OD
Bio-TP5$_c$ 264.2; 9.6		+	−	−	+	−	+	OD	−	+
Bio-TP5$_d$ 264.2; 12.0		+	OD	−	+	−	+	OD	−	−
Bio-TP5$_e$ 264.2; 12.0		−	+	+	OD	−	+	OD	−	+
Bio-TP5$_f$ 264.2; 12.0		+	OD	+	+	−	+	OD	−	−

A — Human carcinogenicity; B — Micronucleus in vivo composite; C — Chromosome aberration; D — Chromosome aberration in vitro CHO; E — Bacterial mutagenicity; OD: Out of Domain means that the test chemical is not included in the applicability domain of the applied model; +: a positive alert for corresponding activity; −: a negative alert for corresponding activity.

might have individual strengths because of different algorithms and training sets. For evaluating bacterial mutagenicity results from statistical models and a rule-based model were compared. Structural identification of the biotransformation products was first based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum. Furthermore, to obtain structural elucidation of the bio-TPs, the MS^3 spectra were generated using the Auto MS^n mode. Due to possible position of the double bond within the structure, a total of 6 possible bio-TPs are presented in the Table 2.

Table 2 shows that the carcinogenicity, genotoxicity and mutagenicity might be altered after biotransformation. Since the main question is, whether novel toxicological activities are generated in the molecule after transformation or metabolization, it is particularly of interest to search the QSAR results for differences in predictions, where the parent compound is predicted to be negative but not the transformation products. Hence, it was intriguing that several alerts for bacterial mutagenicity (CASE Ultra, E) and micronucleus formation (Leadscope, B) are predicted in a set of biotransformation products (bio-TP1$_a$ (CASE Ultra, E) and bio-TP5$_a$, bio-TP5$_c$, bio-TP5$_e$ (Leadscope, B), respectively) but not in the parent compound. This provides a first indication that not only artificial or abiotic transformations but also biotransformations might lead to an increased toxicity compared with the parent compound. This strongly suggests that it is recommended to confirm the predicted toxicities of these bio-TPs by experimental analysis once they are available in sufficient amounts.

4. Conclusion

The approach demonstrated that by a well selected combination of suitable experimental and *in silico*-tools a deeper insight on the role and nature of TPs deriving from chemicals in the aquatic environment can be gained. Two parameters (direct photolysis and biodegradation) were the focus of the present study for two formulations of S-metolachlor. No significant difference has been observed in the biodegradation tests (CBT and MRT) where both pesticide forms were not readily biodegraded. This suggests that the MG formulation does not affect the biodegradation process. A difference was observed for the photodegradation, showing that commercial MG was better photo degraded than the pure SM. This suggests that MG adjuvants might have an impact on higher photolysis yield. In fact could potentially result in faster formation of TPs in the environment. The outcomes presented highlight that photodegradation should be considered as an important degradation pathway for SM and MG in the aquatic environment, however do not lead to the mineralization of the parent compound. Instead photolysis leads to formation of many stable transformation products. As a new insight a biodegradation/biotransformation occurred in MRT for some

main photo-transformation products ($TP1_{a–f}$), thus resulted in the formation of two new compounds (bio-TPs). Additionally the biotransformation might lead to an increased toxicity compared with the parent compound. Other remaining photo-TPs were completely stable to microbial degradation. This study is therefore another demonstration that chloroacetanilides and their TPs should be considered as persistent in the aquatic environment. It shows the importance of the fact that longer persistence time of investigated TPs might appear in long term contamination of surface and ground waters. Further study should be developed to investigate the behavior and toxicity of TPs under environmental conditions with special focus on commercial formulations of the active substance.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.11.025.

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Supplementary information to the article:

"A comparative assessment of the transformation products of S-metolachlor and its commercial product Mercantor Gold® and their fate in the aquatic environment by employing a combination of experimental and in-silico methods"

1. *The mass spectrometer settings (Text S1)*

The mass spectrometer was operated in positive polarity. The operating conditions of the source were: -500 V end plate, - 4833 V capillary voltage, 30 psi nebulizer pressure, and 12 L min^{-1} dry gas flow at a dry temperature of 350 °C. The selected lens and block voltages were: + 95.8 V capillary exit, 245.8 Vpp octopole reference amplitude and -61.0 V lens two. The scan range was determined from m/*z* 100 to 900 and the scan time was 200 ms.

2. *In silico software and their respective models used in the present study*

Table S1: List of *in silico* software and their respective models used for the prediction of biotransformation pro products and toxicity of

Activity	QSAR Software	Models	End points	References
Photodegradation products	METAPC v 1.8.1	Photodegradation	Photoproducts of chemicals under natural-like conditions	(Sedykh et al., 2001)
Toxicity	CASE Ultra v.1.5.0.1 (MultiCASE Inc.)	Human carcinogenicity (A0J)	Carcinogenicity	(Chakravarti et al., 2012; Saiakhov et al., 2013)
		Micronucleus formation in vivo composite (A7S)	Genotoxicity	
		Chromosome aberration in vitro composite (A7U)	Mutagenicity	
		Mutagenicity Ames (SALM2013)	Mutagenicity against *Salmonella Typhimurium*	
		Microtox toxicity environmental bacteria (AUA).	Bacterial toxicity	
	Leadscope V. 3.2.3-1	Bacterial mutagenesis (BM) model	Mutagenicity as a result of interaction with DNA of *Salmonella Typhimurium* or *Escherichia coli*	Training sets from 2012 SAR Genetox Database provided by Leadscope (Roberts et al., 2000)
		Mammalian mutagenesis (MM)	Mutagenicity	
		In vitro chromosome aberration (IVCA)	Mutagenicity	
		In vivo micronucleus (IVMN)	Genotoxicity	

3. UV/Vis spectrum of S-metolachlor

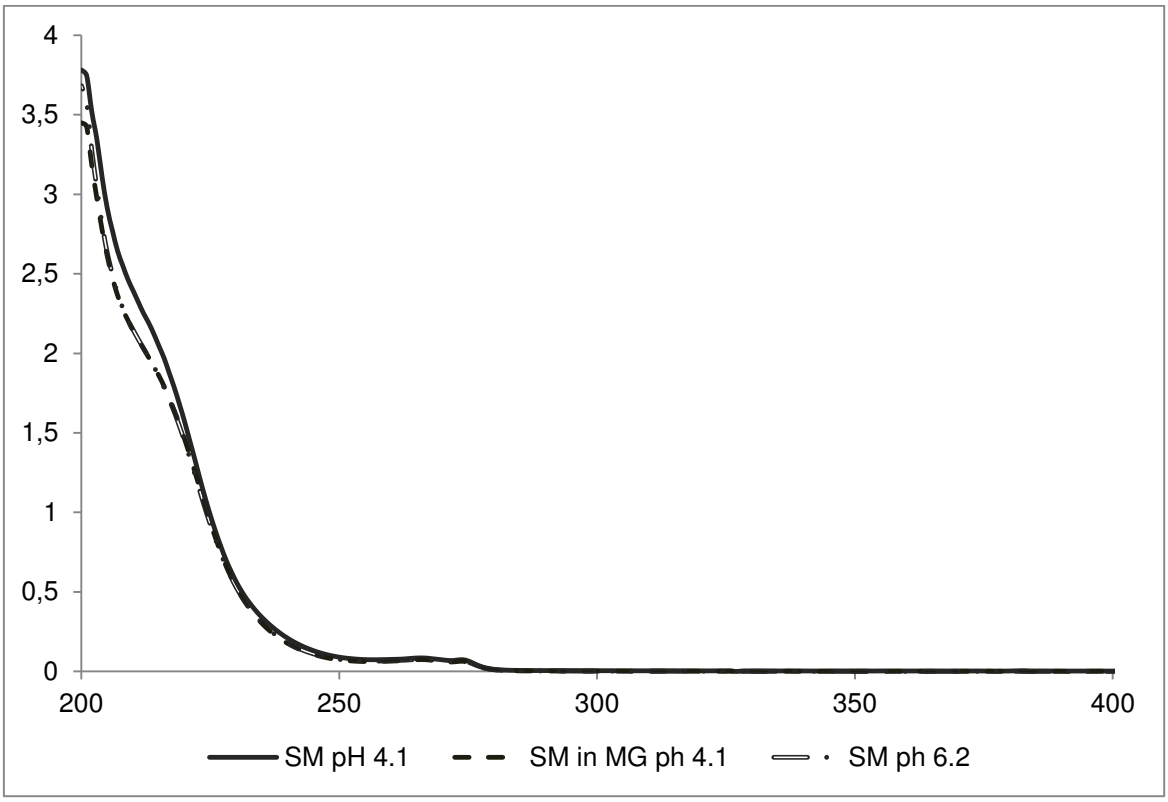

Figure S1. UV/vis spectra of SM and SM in MG at different pH.

4. Kinetic profiles of the photo-TPs

Fig.S2, S3, S4 and S5 shows the course appearance of peak area of the photo-TPs (relative abundance above 1%) measured by LC-EC-MS in positive mode (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM = 40 mg L^{-1}).

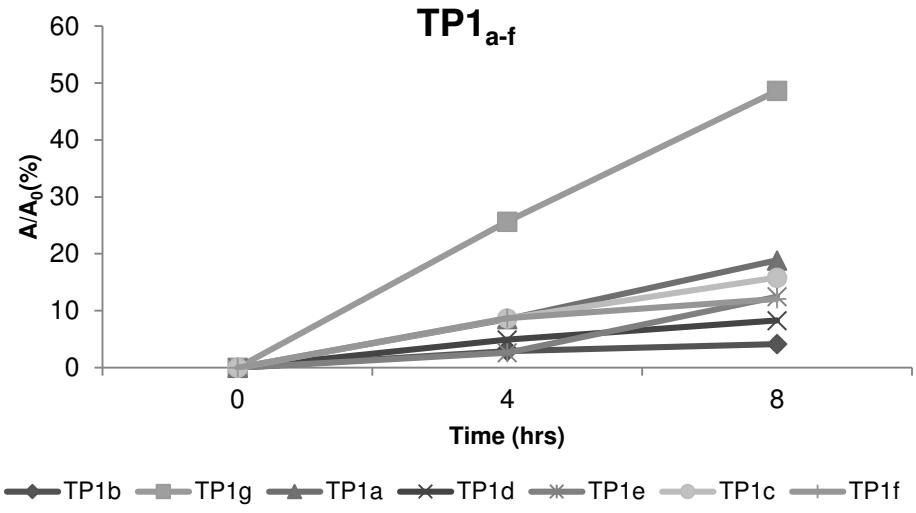

Fig.S2. The course appearance of peak area of the photo-TPs$1_{a\text{-}f.}$ (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM= 40 mg L^{-1}).

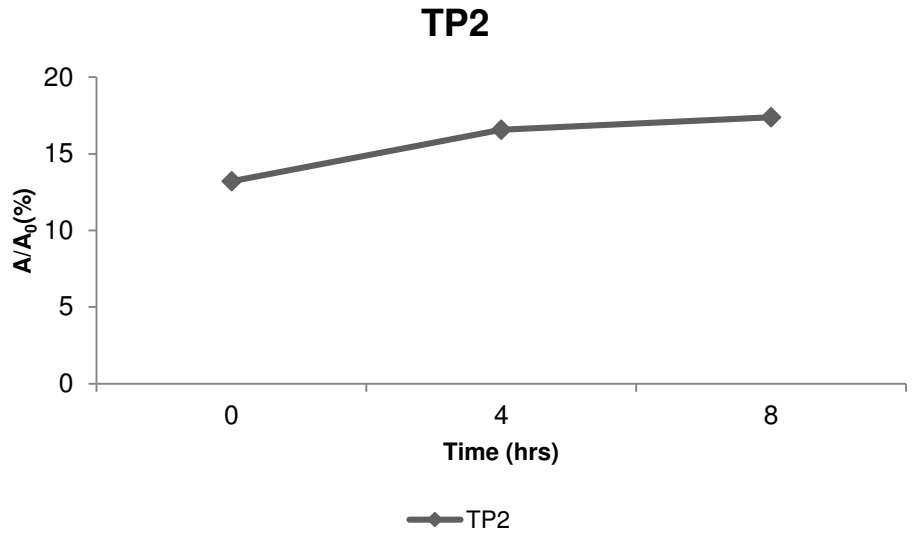

Fig.S3. The course appearance of peak area of the photo-TP2. (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM= 40 mg L^{-1}).

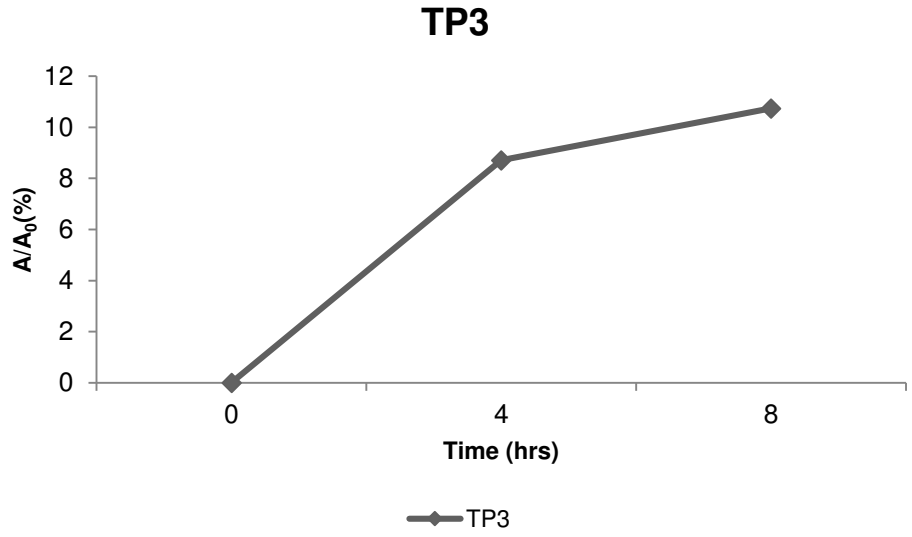

Fig.S4. The course appearance of peak area of the photo-TP3. (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM= 40 mg L^{-1}).

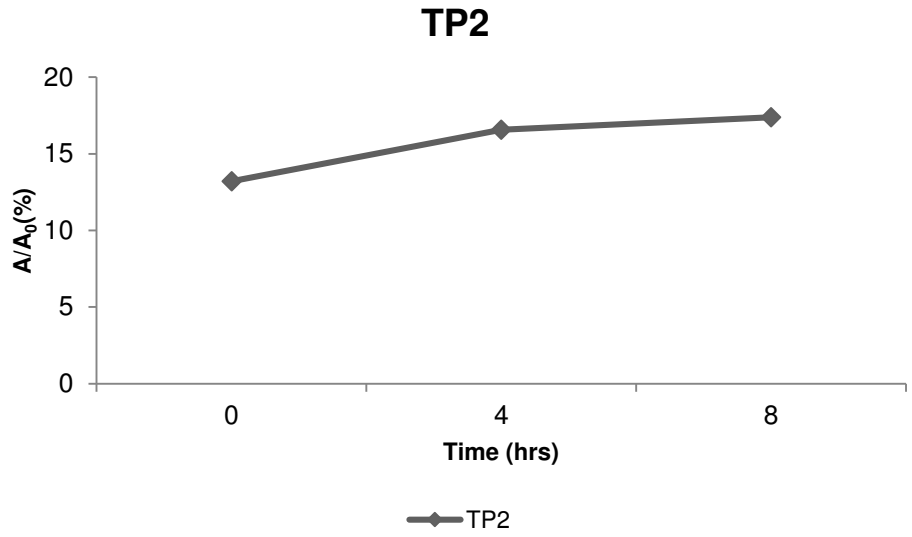

Fig.S3. The course appearance of peak area of the photo-TP2. (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM= 40 mg L^{-1}).

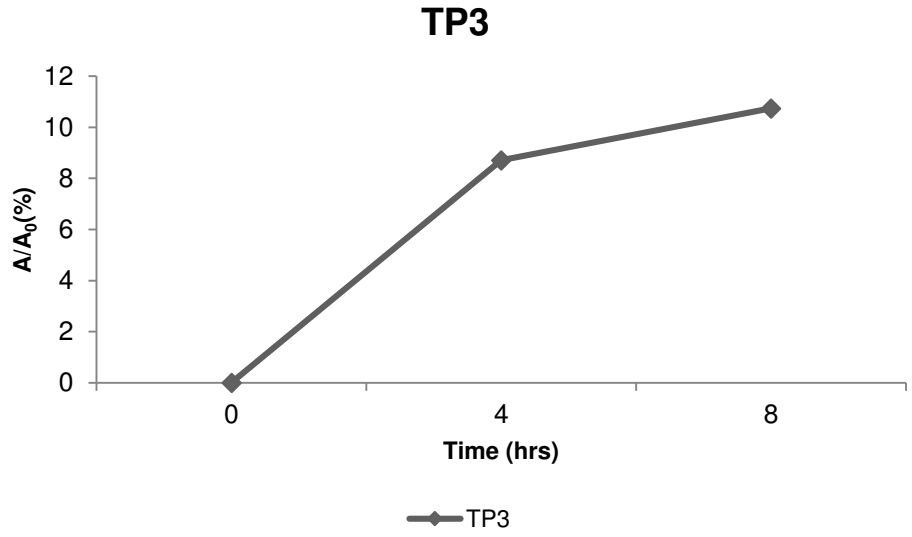

Fig.S4. The course appearance of peak area of the photo-TP3. (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of SM at 0 min.) (Initial concentration of SM= 40 mg L^{-1}).

Article II

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

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

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HIGHLIGHTS

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

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

2.2. Simulated solar photolysis experiments in aqueous solution

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

2.3. Water sediment test (WST)

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

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

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

2.4. Extraction of S-metolachlor from WST sediment

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

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

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

2.6. In silico QSAR models for ecotoxicity

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

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

3. Results and discussion

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

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

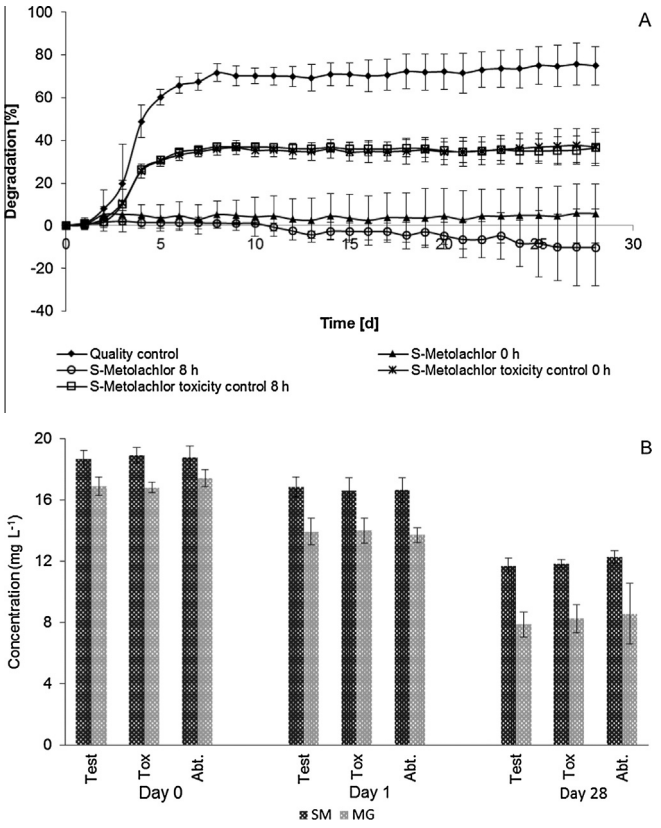


Fig. 1. Biodegradation and dissipation from water phase in water–sediment test. A – Degradation of SM and photodegradation mixture of SM (Xe-lamp irradiation time 8.0 h), in screening water sediment test ($n = 2$, each bottle measured three times). B – Primary elimination of SM and SM in MG in WST water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

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

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

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

3.2. Dissipation of the SM from water phase

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

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

3.3. Determination of transformation products in WST

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

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

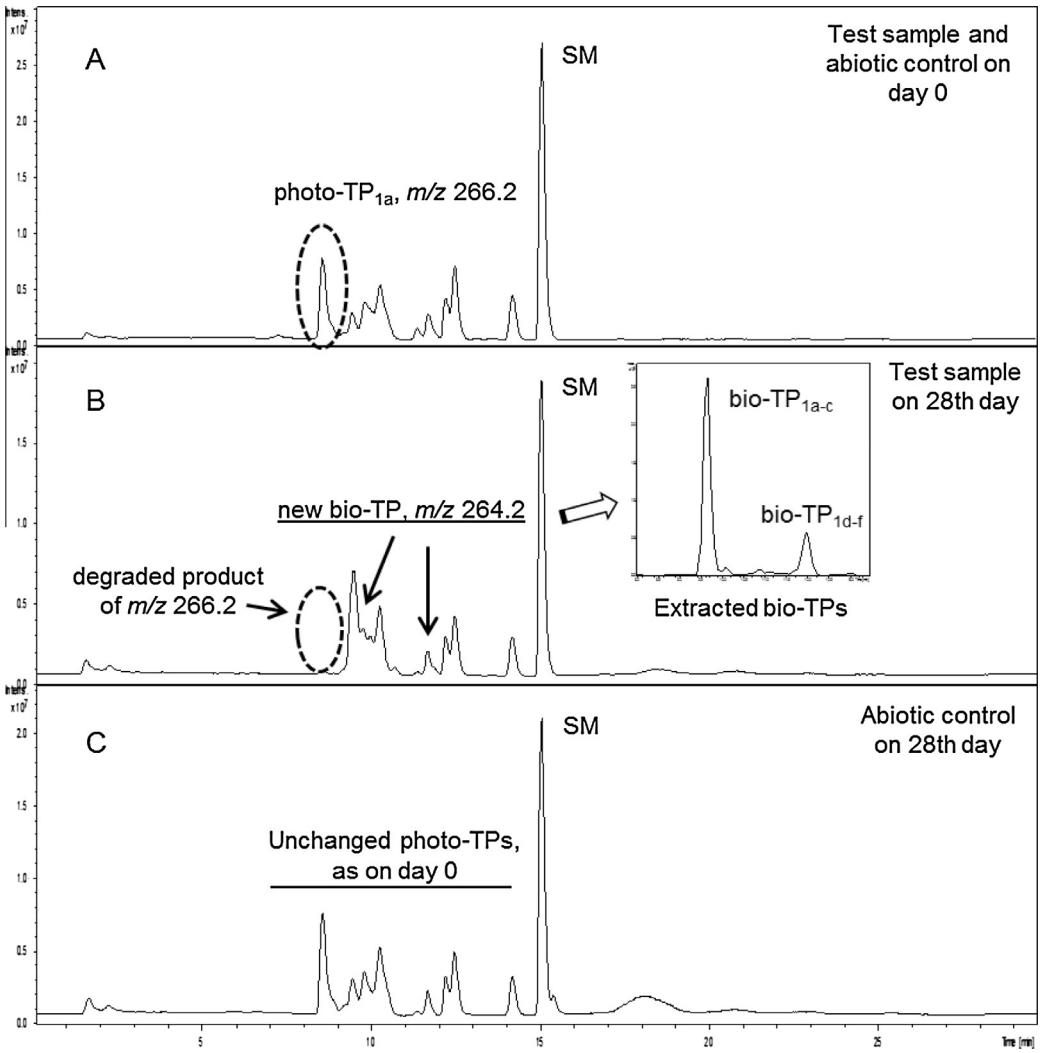


Fig. 2. Total on chromatogram of the photolysis mixture of SM obtained from the WST at day 0 (A) and 28th (B), and the abiotic control at day 28th (C). Dashed ellipse indicates the biodegraded TP (TP1a), arrows indicate newly formed bio-TPs (Bio-TP1a and Bio-TP1b, which co-elutes with present photo-TPs).

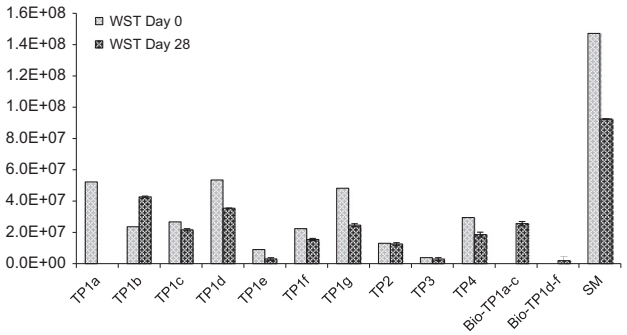


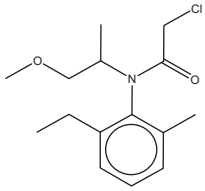
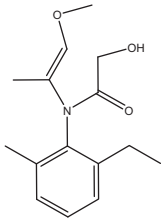
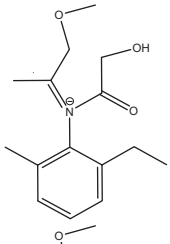
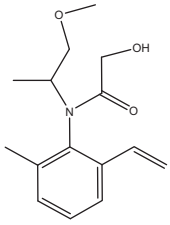
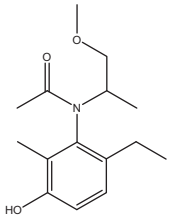
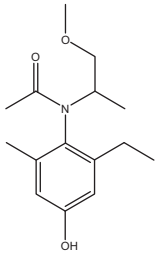
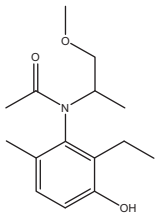
Fig. 3. Relative peak area of the photo-TPs in photolysis mixture (8 h irradiation time) at the start and end of biodegradation test assays. WST of SM in MG formulation. Day 0 ($n = 1$), day 28 ($n = 2$), respectively (lower case letters indicate isomers of the respective TPs, see also Fig. 4).

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

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

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

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

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

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

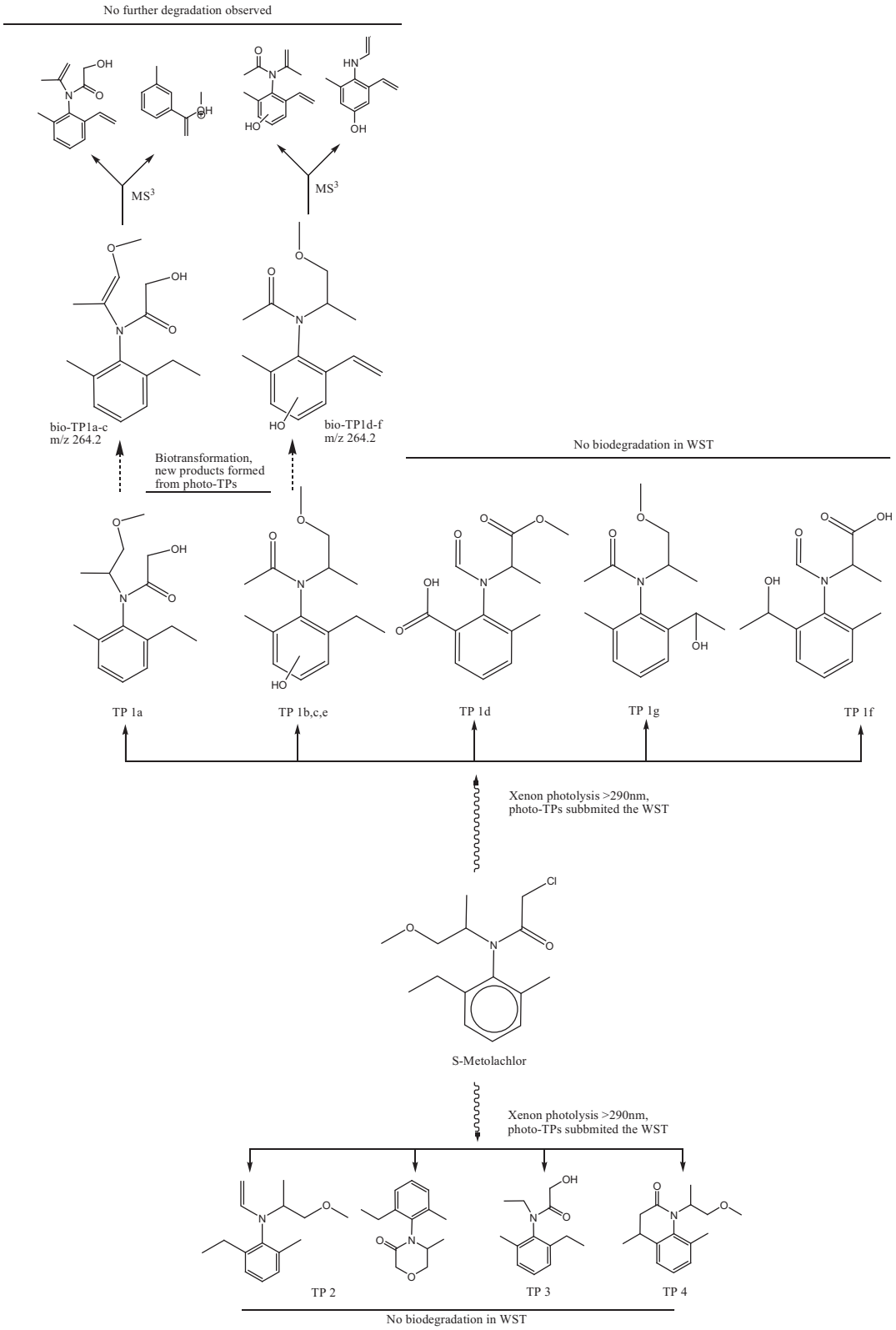


Fig. 4. Proposed abiotic (photo-) and biotic (WST) pathway for most intensive photo-TPs (m/z 266.2) of SM and SM in MG. Bio-TPs were further fragmented up to MS^3 spectra. All photoproducts were submitted to the WST. Only some the most intensive TPs of m/z 266.2 were further transformed into two new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

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

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

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

The BCF is predicted to be positive for both parent compound and transformation products (Table 1), but an additional refined analysis with several available models revealed indications, that the BCF might be mitigated after biotransformation (SI, Table S7). Moreover, *in silico* prediction provided initial indications that some of the bio-TPs might have an increased ecotoxicity compared with the parent compound SM. Of note are results of the bio-TPs (Table 1), which showed alerts for toxicity towards environmental bacteria (bio-TP1$_e$) and towards rainbow trouts (bio-TP1$_d$ and bio-TP1$_f$), respectively. Thus, the biotransformation may have increased the ecotoxicological potential of SM and its photo-TPs, which would deserve further experimental attention. Besides, for some transformation products, such as bio-TP1a or bio-TP1b, the selected QSAR models cannot provide a prediction, since the molecules are out of the applicability domain. Therefore, it would be worth to study these compounds in the corresponding *in vivo* or *in vitro* assays, once they are available in suitable amounts in order to corroborate the prediction and/or to fill the data gaps.

4. Conclusions

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

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

Acknowledgments

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Appendix A. Supplementary material

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

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Supplementary material for article:

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

1. Methods

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

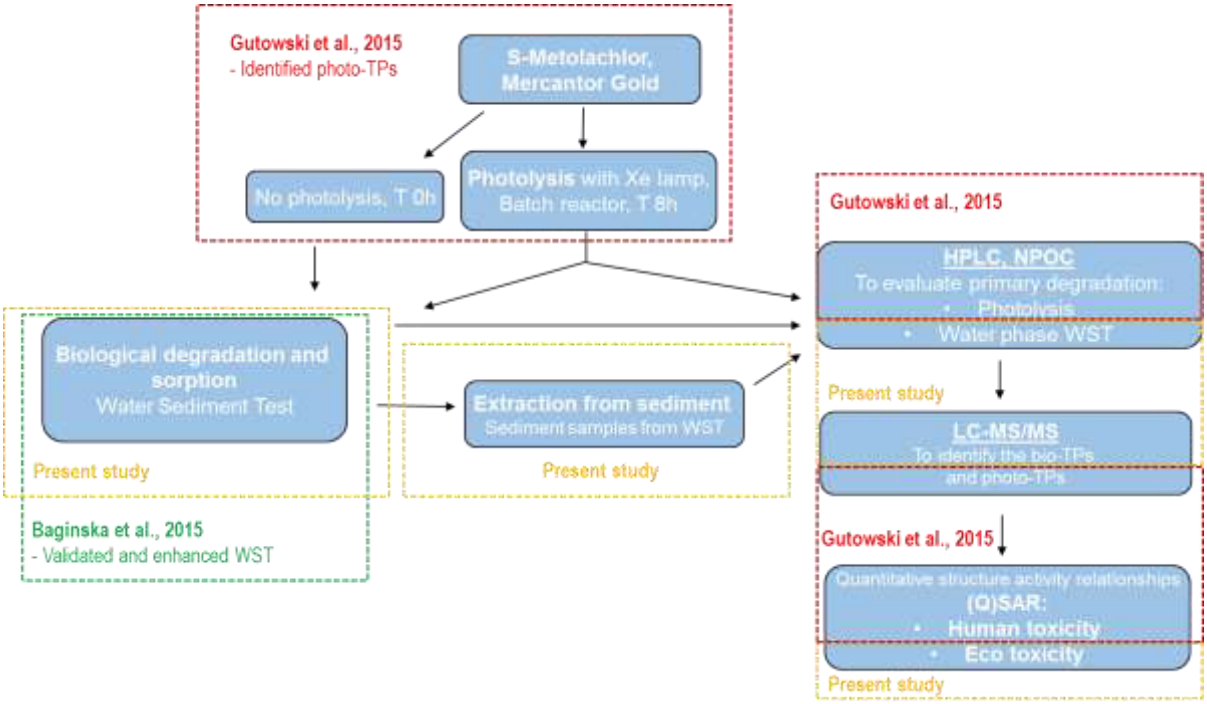


Fig. S1. Flow chart description of the experimental procedures, referred to the different published articles.

1.2. Water-sediment test conditions

Different series of the water-sediment test.

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

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

Constitutes of the artificial sediment.

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

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

1.3. Sediment conditioning (Text S1)

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

Table S3. Localization of inoculum sampling sites for the WST

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

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

The primary elimination was monitored by means of HPLC-UV (Prominence series Shimadzu, Duisburg, Germany). The chromatographic separation was achieved with RP-18 column (EC 125/4 NUCLEODUR 100-5 C18 ec, Macherey and Nagel, Düren, Germany) protected by an EC 4/3 NUCLEODUR 100-5 C18 ec guard column. The mobile phase consisted of ultrapure water (solution A) and 100% acetonitrile (solution B). For elution, the following gradient was used: 0.01 min 20% B, 3.0 min 20% B, 13.0 min 80% B, 20 min 80% B, 24 min 20% B. Sample injection volume was 20 µL and the oven temperature was set at 40 °C, flow rate was set at 0.7 mg L^{-1}. Total run time was 30 min and the wavelength was set at 220 nm. SM and MG standards (1.25, 2.5, 5, 10, 20, 40 and 80 mg L^{-1}) were used to obtain calibration curves. Retention times for SM and SM in MG were 14.20 min. Linear concentration-signal relationships were

obtained. Regression coefficients for SM and MG were r^2 = 0.999 and r^2 = 0.999; n=2, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for SM were 0.02 mg L^{-1} and 0.06 mg L^{-1}, respectively, and for SM in MG 0.07 mg L^{-1} and 0.2 mg L^{-1}, respectively. The mass spectrometer was operated in positive polarity. Flow rate was 0.7 mg L^{-1} in LC part, before MS a T cap was applied reducing the flow to the half (0.35 mg L^{-1}). Injection volume was 20 µL and oven temperature was set to 40°C. The retention time for SM was 14.25 min and molecule ion was found at 284.5 m/z. Analysis of total ion chromatogram and corresponding mass spectrum was used for structural identification of the bio-TPs. The structural identification of the biotransformation products was first based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum. Furthermore, to obtain structural elucidation the bio-TPs were isolated, used as precursor ions and further fragmented up to MS^3 using the Auto MS^n mode. The operating conditions of the source were: -500 V end plate, - 4833 V capillary voltage, 30 psi nebulizer pressure, and 12 L min^{-1} dry gas flow at a dry temperature of 350 °C. The selected lens and block voltages were: + 95.8 V capillary exit, 245.8 Vpp octopole reference amplitude and -61.0 V lens two. The scan range was determined from m/*z* 100 to 900 and the scan time was 200 ms.

2. Results:

2.1.Calculated and measured toxicity controls in WST

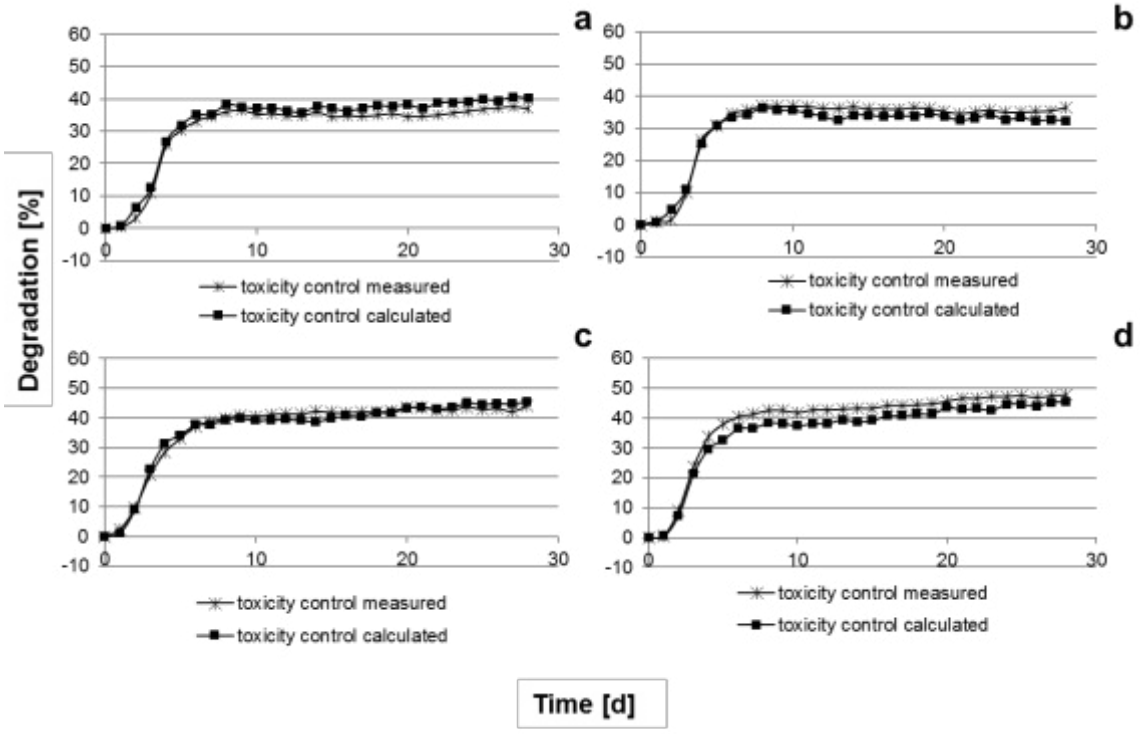


Fig. S2. Correlation between toxicity control series measured and calculated during WSTs, between samples of S-Metolachlor (SM) (a) sample from photodegradation experiment time point 0 h, and (b) sample from

photodegradation experiment time point 8 h; Mercantor Gold (MG) samples from photodegradation experiments time points 0 (c) and 8 hours (d) (Xe-Lamp).

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

2.2.Biodegradation of Mercantor Gold in WST:

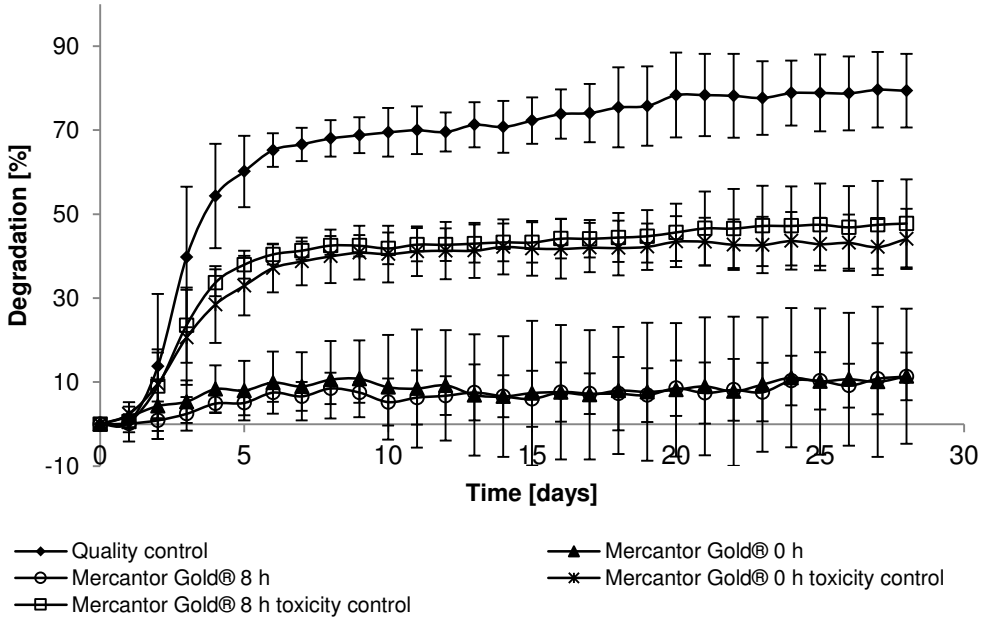


Fig.S3. Degradation of MG and its photodegradation mixture (Xe lamp irradiation and time 8.0 h), in screening water sediment test (n = 2, each bottle measured three times).

2.3. Extraction from sediment

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

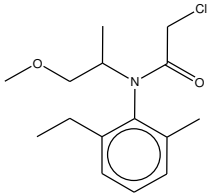
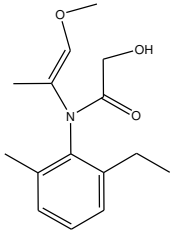
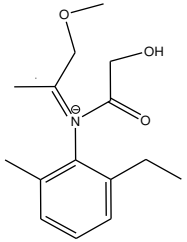
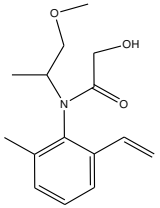
Solvent composition	pH	Extraction cycle	Recovery (%)		dt dev	
			SM	MG	SM	MG
0.01 M water solution of $CaCl_2$	7.0	3	55.4	50.0	1.5	1.1
ACN and water (9:1)	7.2	3	96.9	93.1	0.0	1.9

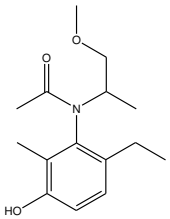
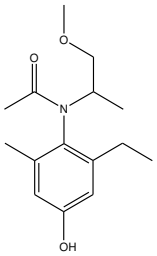
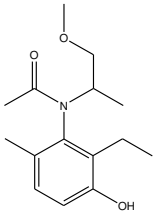
2.4.LC-MS/MS parameters of the bio-TPs

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

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

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

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

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

Article III

Photolytic transformation products and biological stability of the hydrological tracer Uranine

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Photolytic transformation products and biological stability of the hydrological tracer Uranine

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HIGHLIGHTS

- Uranine (UR) was not biodegraded in water and water-sediment system (WST).
- Only small degradation rate occurred in OECD 301 D and WST.
- Photolysis leads to incomplete mineralization of UR.
- A total of 5 stable photo-TPs were found for UR, structures were elucidated.
- Similar to the parent compound, only small biodegradation of the photo-TPs was found.

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ABSTRACT

Among many fluorescence tracers, Uranine (sodium fluorescein, UR) has most widely been used in hydrological research. Extensive use of UR for tracing experiments or commercial use might cause a potential risk of long-term environmental contamination. As any organic substance released to the environment, also UR is subjected to chemical and physical reactions that can be chemical, biological and photolysis processes. These processes transform the parent compound (PC) and have not been extensively investigated for UR. This study applies two OECDs (301 D and 301 F) tests and a screening water sediment test (WST) to investigate the biodegradability of the PC. Photolysis in water was explored by Xe lamp irradiation. Subsequently, the biodegradability of the photolysis mixtures was examined. The primary elimination of UR was monitored and structures of its transformation products (TPs) were elucidated by HPLC–FLD–MS/MS. UR was found not readily biodegradable, although small degradation rates could be observed in the OECD 301 D and WST. HPLC–FLD analysis showed high primary elimination of the tracer during photolysis. However, the low degree of mineralization found indicates that the UR was not fully degraded, instead transformed to TPs. A total of 5 photo-TPs were identified. According to MS/MS data, chemical structures could be proposed for all identified photo-TPs. Likewise the parent compound it was demonstrated that photo-TPs were largely recalcitrant to microbial degradation. Although we did not find indications for toxicity, target-oriented studies on the environmental impact of these photo-TPs are warranted. Results obtained in this study show that deeper investigations are necessary to fully understand fate and risk connected to the use of UR.

1. Introduction

Fluorescent dyes are routinely used as hydrological tracers to monitor surface and subsurface water movement (Käss, 1994; Reichert and Hoetzl, 1991). One of the most important applications of tracers is to assess the flow pathways and water residence times in the area of drinking water facilities. In hydrological and hydrogeological communities Uranine (sodium fluorescein, UR) is referred to as an ideal, nearly conservative tracer for groundwater studies (Leibundgut et al., 2009, Käss, 1998, Adams and Davis, 1991; Smart and Laidlaw, 1977). UR was first used to trace subsurface flow connections in a karst system in Southwest Germany (Knop, 1878). UR gained popularity because of its low detection limits and ease of analysis at low concentrations, while it is known that it is subject to photodegradation by sunlight (Smart and Laidlaw, 1977; Käss, 1998). Recently, UR was applied as a reference substance to mimic photolytic decay of a contaminant (e.g. pesticide) in surface waters (Lange et al., 2011).

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UR has also widely been used in industry and health care applications, mainly because of its bright green color, high water solubility, moderately low costs and low toxicity (Ikeya et al., 2009; Jean et al., 2007; Smart and Laidlaw, 1977). UR is a complex organic molecule belonging to the xanthene dyes with one of the most intense fluorescence and very high quantum yield value of 0.85–0.94 (Adams and Davis, 1991; Ikeya et al., 2009; Heller et al., 1974; Schmidt, 2005).

Extensive use of UR for tracing experiments or commercial use might cause a potential risk of long-term environmental contamination. As with any organic substances released into the aquatic environment, fluorescence tracers can principally undergo non-biotic and biotic degradation processes such as photolysis, hydrolysis, oxidation and reduction. While photodegradation of UR is well known in surface waters and half-life times have been quantified ($t_{1/2}$ at concentrations of 100 and 10 mg m^{-3} are 38.1 and 33.3 min, respectively), observations of biodegradation are still speculative (Käss, 2004, Kranjc, 1997). Within soils, biodegradation was reported to be negligible, as timeframes typically span only 5–48 h in staining experiments (Alaoui et al., 2011; Anderson et al., 2009; Duwig et al., 2008).

If the degradation of an organic compound is incomplete, transformation products (TPs) are formed (Fenner et al., 2013) which can be more toxic and present at higher concentrations than their parent compounds (PCs) (Mañas et al., 2009; Olsson et al., 2013). However, only few studies have addressed TPs of fluorescence dyes so far. Gombert & Carre (2011) have highlighted formation of UR TPs and other popular tracers in lab scale simulated water treatment processes (with gaseous chlorine and UV/visible light irradiation) with their HPLC analysis of the water samples. However, they only identified one TP for Tinopal and none for UR by LC–MS.

According to Ishibashi (1965), possible photo-decomposition products of UR are pthalic acid and resorcinol. The latter one appears to be no mutagenic, as found by Heddle et al. (1983). General toxicity evaluations classify UR as a safe tracer (e.g. Leibundgut & Hadi, 1997, Behrens et al., 2001) but rarely distinguish between experiments with and without light exposure. Hence it is not clear if photolytic TPs are included or not. In 48 h tests Tonogai et al. (1978) found no acute toxicity to fish (*Oryzias latipes*) for UR and its photolytic TPs. At the same time, toxicity of the halogenated dyes increased through irradiation. For 10 days Walthall & Stark (1999) exposed *Daphnia pulex* to UR at a light–dark regimen and found chronic mortality at UR concentrations in excess of 0.25 g L^{-1}. Recently, Gombert & Carre (2011) exposed rats, *Daphnia magma* and micro-algae (*Pseudokirchneriella subcapitata*) to unidentified mixture of degradation products of UR and other fluorescent tracers at initial concentration of 1 g L^{-1} and found no acute toxicity and only moderate ecotoxicity for the tracer sodium naphthionate. Generally, concentrations used during toxicity testing are rarely reached during tracing experiments, since UR has a distinct green color already at concentrations of 1 mg L^{-1}.

Since synthetic organic dyes (e.g. monoazo, diazo, anthraquinone, triphenylmethane dyes) are prominent water pollutants, their removal from wastewater has attracted various research groups (e.g. Muhammad et al., 2012). Biodegradation by microorganisms, particularly by fungi, is an effective method (e.g. Knapp et al., 1995; Novotný et al., 2004). Most knowledge exists for azo dyes, xanthene dyes like UR are less prominent in waste water and hence underrepresented in research.

This is especially true for TP formation. During photolysis, which is omnipresent for UR, TPs may be formed following radical reactions. However, knowledge regarding their fate and properties is very limited. Furthermore, if these TPs turn out to be persistent, they will be of special interest for environmental risk assessment. Laboratory tests to identify the combined effect of photolysis and aerobic biodegradation on the formation of persistent TPs were successfully applied for two formulations of herbicide pesticides (Gutowski et al., 2014). However such, studies have not yet been performed for UR.

A combination of photolysis under simulated sunlight irradiation, two biodegradation tests (Closed Bottle test and Manometric Respiratory test, OECDs 301 D, F) and a water sediment test was carried out to evaluate the primary elimination of UR monitored by high performance liquid chromatography with fluorescence detector (HPLC-FLD). The degree of mineralization was evaluated by means of non-purgeable organic carbon (NPOC) analysis. Photo-TPs were analyzed in terms of ready biodegradability, and their structures were elucidated and identified with liquid chromatography tandem mass spectrometry (LC–FLD–MS/MS).

In the newly developed water sediment test (WST) (Baginska et al., 2015) a complex matrix (i.e., water-sediment interface) was introduced to increase reproducibility and stability of the test system. This allows one to investigate processes like biodegradation, sorption, elimination from water phase, and abiotic degradation in one set.

2. Materials and methods

2.1. Chemicals

The analytical standard of UR (98.5–100.5% chemical purity, CAS Nr. 518-47-8) was obtained from Fluka (Sigma-Aldrich, Steinheim, Germany). HPLC grade acetonitrile (CAS Nr. 75-05-8) and ammonium acetate (CAS Nr. 631-61-8) were purchased from VWR (VWR International, GmbH, Darmstadt, Germany). Aniline (CAS Nr. 62-53-3) was purchased from the same supplier; calcium carbonate (CAS Nr. 471-34-1), quartz (CAS Nr. 14808-60-7) and clay (CAS Nr. 1318-74-4) were purchased from Carl Roth, Germany. Sodium azide (CAS Nr. 26628-22-8) was purchased from Sigma-Aldrich, Germany. Peat (from *Sphagnum Moss*) was obtained from Aurich-Wiesmoor-Torfvertriebs-GMBH, Germany. All aqueous solutions were prepared using ultrapure water 18.2 MΩ cm (Ultra Clear UV TM, Barsbüttel, Germany).

2.2. Sunlight simulated photolysis experiments in aqueous solution

UR solutions were dissolved in ultrapure water the day prior to the experiment and stored in the dark. UR was subjected to the photolysis at three initial concentrations of10 mg L^{-1}, 20 mg L^{-1} and 60 mg L^{-1}. 800 ml of the test solution was transferred to the photo-reactor under gentle stirring using a magnetic stirrer. Temperature was set to 20–22 °C controlled by circulating cooler (WKL230, LAUDA, Berlin). Photolysis in water was performed in an ilmasil quartz immersion tube using a xenon lamp (TXE 150, UV consulting Peschl, Mainz, Germany) as a radiation source. The lamp emits spectra similar to natural sun light 200–800 nm with the highest intensity in the visible range (200–280 nm: 1.61 e^{-2} W/m^2, 280–315 nm: 1.16 e^{-2} W/m^2, 315–380 nm: 3.75 e^{-2} W/m^2, 380–780 nm: 5.58 e^{-1} W/m^2) (data provided by the manufacturer). Before every experiment the lamp was warmed up for 3 min to reach its maximum intensity. Photolysis experiments were performed for 8.0 h in order to mimic an average daily sunshine duration from sunrise to sunset. Samples were collected every hour for HPLC and LC–MS/MS analysis. Samples for NPOC determination were collected at the time increments of 0.0 h, 4.0 h and 8.0 h.

Samples before (0.0 h) and after (8.0 h) photolysis were collected and subsequently submitted to the ready biodegradability tests: Closed Bottle test (CBT), Manometric Respiratory test (MRT) and to screening WST. The final concentration of UR was adjusted by measuring NPOC of the tested substance (i.e. before photolysis) and photolysis treated samples, to provide required carbon content, and to reach adequate theoretical oxygen demand (ThOD), for each CBT, MRT and WST respectively (described further in Sections 2.3 and 2.4). In parallel to every experiment, a HPLC analysis was run to support the NPOC measurements and to determine primary elimination of the parent compound.

2.3. Closed Bottle test (OECD 301 D)

CBT was performed according to the guidelines of the Organization for Economic Co-operation and Development OECD (1992). This test is

characterized by low bacteria density (10^2–10^5 colony forming units (CFUs) mL^{-1}), low nutrient content, and constant temperature (20 ± 1 °C). It was kept in the dark as described elsewhere in detail (Trautwein and Kümmerer, 2011). Inoculum for the test was derived from the secondary effluent of a municipal sewage water treatment plant (SWT) (Lüneburg, Germany; population 73,500 equivalents). Two drops of inoculum were added to 1 L of mineral medium, which corresponded approximately to 500 CFUs mL^{-1}. The concentration of standard solution for UR was 2.8 mg L^{-1}, corresponding to the theoretical oxygen demand ThOD of 5 mg L^{-1}. The test consisted of four different series: (i) a blank series (containing only the mineral medium and inoculum), (ii) quality control (containing readily biodegradable sodium acetate as the only relevant carbon source apart from the inoculum), (iii) a test series (containing the target compound) and (iv) toxicity control (containing target compound and sodium acetate as carbon source). The amount of sodium acetate for each series corresponded to ThOD of 5 mg L^{-1}. All tests were run in duplicates.

The whole process was monitored by measuring dissolved oxygen concentration in the test vessels with Fibox 3 (Fiber-optic oxygen meter connected with Temperature sensor PT 1000) (PreSens, Precision Sensing GmbH, D-93053 Regensburg, Germany). This is in accordance with the international standard (ISO, 1990; OECD, 1992) for the 28th day period (Friedrich et al., 2013). A compound is qualified as "ready biodegradable" when 60% of ThOD expressed as percentage of oxygen consumption is consumed within a period of 10 days after the oxygen uptake reached 10% of ThOD. Samples from the beginning (day 0) and the end of the test (day 28) were collected and stored at −20 °C until analysis with HPLC-FLD and LC–M/MS.

2.4. Manometric Respiratory test (OECD 301 F)

The MRT works with higher bacterial density (5–10 × 10^6 CFUs mL^{-1}) and diversity as the CBT thus increasing the probability for biodegradation. This test was also performed according to the OECD guidelines (OECD, 1992) in the dark at room temperature (20 ± 1 °C) under gentle stirring. CO_2 production as the parameter of the endpoint biodegradation was measured indirectly by the OxiTop OC110-system (WTW, Weilheim, Germany). This system uses pressure heads to seal the test vessel. By biodegradation, process oxygen is consumed and carbon dioxide formed. Carbon dioxide is removed by a reaction with sodium hydroxide to form sodium carbonate. This results in a drop of pressure inside the test vessel which is proportional to the degree of mineralization of the test compound. The concentration of standard solution for UR was 16.7 mg L^{-1}, corresponding to the theoretical oxygen demand ThOD of 30 mg L^{-1}. Inoculum was derived from the municipal sewage treatment plant (Lüneburg, Germany; population 73.500 inhabitants). Aliquots (measuring) of 80 ml of inoculum were added to 1 L of mineral medium. The validity criteria are the same as for the CBT.

2.5. Water sediment test (WST)

The recently developed screening water sediment biodegradation test (WST) (Baginska et al., 2015) was applied in this study. This test combines the relative easiness in handling characteristic for screening tests on the one hand and a complex matrix characteristic (i.e., water–sediment interface) for simulation tests on the other hand. Furthermore, an artificial matrix was introduced to achieve higher reproducibility and stability of the test system. All components of the artificial medium (sediment, inoculum, mineral medium) were standardized and based on OECD guidelines for testing of chemicals (methods 218, 301 D and 302 C) (OECD, 1981, 1992, 2004).

Briefly, the WST consisted of five different series (details can be found in Baginska et al., 2015): blank, quality control, test, toxicity control and sterile control (Table 1); each run in three parallels. Each of the series was placed in glass bottles (1 L) equipped with two septum sealed bottle nozzles. With water phase (500 mL) and artificial sediment (230 g) volumetric ratio was 1:5. Table 2 shows the individual sediment constitutes constituting the artificial sediment. The aniline (used as quality control) and test substance concentrations were prepared in a way that they corresponded to 40 mg L^{-1} of theoretical oxygen demand (ThOD). The nominal concentrations were 17.2 and 24.4 mg L^{-1} for aniline and UR, respectively. To obtain abiotic conditions in the sterile control, sodium azide was added in a concentration of 400 mg L^{-1} in water phase and 800 mg kg^{-1} in sediment. All assays were incubated in the dark at 20 °C in closed vessels. Test duration was 28 days as in related OECD tests. The water phase in the bottles was gently stirred to improve water exchange between water and sediment without disturbing the sediment. During the experiment, pressure change inside the vessels was monitored by pressure sensors (OxiTop®, WTW Weilheim, Germany).

Table 1
Screening water sediment test vessels content accordingly to test series.

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

In order to avoid false negative results of bacterial toxicity of test compounds against the inoculum, the oxygen consumption was measured in the toxicity control and subsequently compared with the predicted level computed from the oxygen consumption in the quality control and in the test series. A substance was considered to be toxic if measured toxicity control was lower than 25%, which corresponded to less than 50% degradation of aniline. If the measured toxicity control was lower than calculated, a substance was assumed to have inhibitive or toxic impact on the inoculum. More information can be found in the Text S1 in supplementary information (SI). The full method and preparation steps are described in detail by (Baginska et al., 2015).

2.6. Kinetics and half-life of UR under photolysis

In order to check whether the photolysis was pseudo zero-order or pseudo first-order rate, the experimental data was as plotted as normalized concentration C/C_0 versus t time and different zero and first-order models equations were fitted with the aid of Software Wolfram Mathematica® 7.0 by means of nonlinear model fit regressions. The statistical analysis of the fitting was performed by means of ANOVA. The half-life of UR was determined by using numerical solution to the equation above by means of the FindRoot option on the Software Mathematica® 7.0, which finds a numerical value of t when the initial concentration (C_0) is reduced in 50%, i.e. the half-life of UR under photolysis.

The observed kinetic constants (k_{obs}) of photolysis were obtained by subtracting the exponents of different degradation curves presented as apparent kinetic constants (k_{app}) and degradation factors such as volatilization, hydrolysis and biodegradation (as dark experiment, k_{dark}). k_{obs} can then be expressed as follows:

$$k_{obs} = k_{app} - k_{dark} \quad (1)$$

Table 2
Composition of the artificial sediment used in screening water sediment test.

Constituent	Characteristics	Content [% dry weight]
Peat	From sphagnum moss	2
Clay	Kaolin type	5
Quartz sand	Grain size 0.8–0.2 mm	93
Calcium carbonate	Powder	0.01

where the estimated half-lifes can refer to the actual experiments, without the contribution of other factors.

2.7. Analysis of UR and TPs by HPLC-FLD and LC–MS/MS

The primary elimination was monitored by means of HPLC-FLD (Prominence series Shimadzu, Duisburg, Germany). The chromatographic separation was achieved with RP-18 column (EC 125/4 mm NUCLEODUR 100–5 μm C18 ec, Macherey and Nagel, Düren, Germany) protected by a EC 4/3 mm NUCLEODUR 100–5 μm C18 ec guard column. Mobile phase consisted of 10 mM ammonium acetate (solution A) and 100% acetonitrile (solution B). For elution, the following gradient was used: 0.01 min 10% B, 5.0 min 30% B, 10.0 min 60% B, 13.0 min 10% B. Sample injection volume was 5 μL and the oven temperature was settled at 30 °C, flow rate was 1.0 mL min^{-1}. Retention time for UR was 6.0 min. The total time of chromatographic run was 16 min. The excitation and detection wavelengths were set to 476 and 515 nm, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for UR were 1.0 μg L^{-1} and 3.0 μg L^{-1}, respectively.

The identification and elucidation of the TPs were performed with the LC–MS/MS Bruker Daltonic Esquire 6000 plus ion-trap mass spectrometer (IT-MS) equipped with the Bruker data analysis system (Bruker Daltonic GmbH, Bremen, Germany). The mass spectrometer was connected to a HPLC system (Agilent Technologies, Böblingen, Germany, HPLC 1100 series). The analytical separation was carried out using the same C18 column and the same gradient method as applied in above HPLC analysis. Flow rate was 0.7 mL min^{-1} in the LC part, before the eluent entered the MS a T cap was applied reducing the flow to the half (0.35 ml min^{-1}). Injection volume was 5 μL and oven temperature was set to 30 °C. The retention time for UR was 6.3 min. The MS was operated in a positive mode polarity and a molecular ion $[M + H]^+$ was found at 333.1 m/z. Analysis of total ion chromatogram and corresponding mass spectrum was used for structural identification of TPs. By means of AutoMS(n) mode, each m/z of TPs identified in the TIC was used as precursor ion and further fragmented up to MS^3. More information about the LC–MS/MS can be found in SI (Text S2).

3. Results and discussion

3.1. Photolysis

In general, the rate of decrease in UR concentration was a function of concentrations and of the absorbance. As a result, the modified exponential decay and linear decay relationship of C/C_0 *vs* t (from 0.0 h to 8.0 h) were applied. According to Oppenländer (2002), photochemical reactions do not have a specific reaction order, but they are strongly dependent on the absorbance conditions. Thus, if the absorbance ($A = \log I_0/I$) is >2, the degradation of the UR follows a linear decay (or pseudo zero-order), as expressed for the equation $C = C_0 - kt$, where k is the kinetic constant, t is time, and C_0 and C are the concentrations of UR. On the other hand, an exponential decay (or pseudo first-order) occurs when the total absorbance is ≪1, following the equation $C = C_0 e^{-kt}$.

Nevertheless, for 10 mg L^{-1} of UR initial concentration a pseudo first-order did not fit the experimental data. Thus, a modified exponential decay model was applied as proposed by Martins et al. (2010). The modified exponential decay equation reads as follows

$$C = C_1 + C_2 e^{-kt} \quad (2)$$

where C_1 is the non-primary eliminated fraction and C_2 is the primary eliminated fraction of UR, respectively.

As can be seen in Fig. 1, Eq. (2) closely fitted the experimental data with an r-squared higher than 0.99. It demonstrates that UR photolysis followed two different kinds of degradation according to its initial concentration during the photolytic process. A modified pseudo-first order rate of the photolysis took place at the lowest concentration studied (10 mg L^{-1}), and a pseudo zero-order photolysis rate at the high concentrations studied (20 mg L^{-1} and 60 mg L^{-1}). The rate constants and half-lives for UR are given in Table 3.

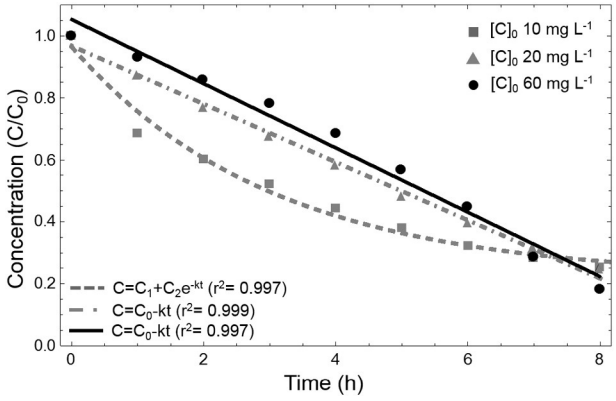

Fig. 1. First-order and zero-order photolysis kinetics of UR at 10, 20 and 60 mg L^{-1}, photolysis with Xe lamp for 8.0 hours. All values represent the means ± SD (n = 2).

Typical half-life times for UR (for first-order decay) in hydrological textbooks (Leibundgut et al., 2009) are in the range of 11 hours. However, these depend on the experimental conditions (concentration, light source, experimental setup etc.). Moreover, at low concentrations and hence lower absorbance values, the decomposition of UR follows a first order decay only (Kamiya and Iwaki, 1966; Leibundgut et al., 2009). In general, the kinetic results obtained in this study for the photolysis of UR are not in accordance with the findings of Wang et al. (2008), who reported a pseudo-first order degradation rate and a 4.3 h half-time for an initial 30 mg L^{-1} mixture of UR and Phloxine B irradiated under visible light for 8 h. The difference in the kinetics might be due to another type of lamp, glass beakers used instead of a batch reactor or due to the simultaneous photolysis of the two substances, which could result in a different degradation rate for a single compound. Generally, the divergence of the results calls for standardization of photolytic experiments.

The HPLC analysis showed UR degradation of about 75.4% to 83.0%, varying on the initial concentration. The degree of NPOC removal was measured in parallel with each experiment to monitor the mineralization of UR during the photolysis (Fig. 2). The results presented NPOC removal from 8.2% to 17%, depending on the UR concentration (Table 3). This indicated that the tested substance was not fully mineralized, instead transformed to TPs, more resistant than UR to photolysis under Xe lamp irradiation. The monitoring of the pH showed, that at the beginning of the experiment the UR solution had pH of 7.6 (0.0 h) and at the end (8.0 h) the pH was 6.3. This indicates that hydrolysis reactions did not play an important role during the photolysis. Dissociation of carboxylic groups would be of relevance at pH between 4 and 6, thus the dye remained fluorescent at the end of irradiation (Smart and Laidlaw, 1977). pH has strong but reversible effect on UR peak intensity (Käss, 1998, Adams and Davis, 1991): Maximum fluorescence is reached at pH 8.5 but decreases down to 80% at pH 7.0.

3.2. Identification and elucidation of UR photo-TPs

Formation of new peaks was detected in the samples collected during photolysis by means of LC–MS/MS. The retention time for UR was 6.3 min and the molecular ion $[M + H]^+$ of m/z 333. It was less (43 Da) than the UR sodium salt (376.2 Da) and it was due the change of two Na ions for H. The TP peaks were gradually increasing with the irradiation time to reach the maximum intensity after 8.0 h. This demonstrates the formation of first generation photo-TPs, without further decomposition. Hence, the primary investigation was based on suspected-target approach by comparing the chromatograms from the

Table 3
Summary of UR photolysis results at various concentrations.

Concentration (mg L^{-1})	UR removal (%)	NPOC removal (%)	k (h^{-1})	C_1, C_2	C_0	Half-time (h)	R-squared
10	74.7	8.2	0.330	0.22, 0.75	–	2.97	0.997
20	75.4	15.9	0.094	–	0.97	4.99	0.999
60	83.0	13.2	0.104	–	1.05	5.33	0.999

beginning of the experiment (0.0 h) with the samples taken at each time point (every 60 min) until 8.0 h.

Fig. 3(A) shows the total ion chromatogram (TIC) of UR and its TPs in ultra-pure water obtained at the time point 8.0 h. Fig. 3 (B–D) shows extracted ion chromatograms of newly formed photo-TPs (TP_{1a-b}, TP_2, and TP_{3a-b}) resulting from photolysis after 8.0 h. Aforementioned TPs tend to be of higher polarity than their PC. The kinetics of appearance of photo-TPs which were formed during the photolysis are provided in detail in SI (Figs. S1, S2, and S3).

The generated MS/MS fragmentation pattern was based on the photo-TPs peak intensity to achieve structural elucidation, results are shown in Table 4. A total of 5 UR photo-TPs were identified. Fig. 4 shows proposed photolysis pathway for UR. For structural elucidation each peak was isolated and further fragmented (Table 4) by means of AutoMS(n).

Hydroxylation reactions are common for photolysis processes (Oppenländer, 2002). Therefore, detected photo-TPs were assumed to be possibly mono- and di-hydroxylated derivatives of UR. The hydroxylation could take place in any of the UR′ aromatic rings. The mass fragmentation results do not provide information about the exact hydroxylation position. The postulated MS^2 fragmentation pattern and obtained mass spectra of photo-TPs can be found in SI (Figs. S4–S13). It is interesting to mention that only one photo-TP with *m/z* 264.9 had a lower mass compared with the PC. The extracted ion chromatograms of *m/z* 264.9 showed that these compounds were present at two different retention times. Consequently, these products are labeled as TP_{1a} and TP_{1b} (R_t = 1.5 and 5.1 min). Formation of isomers due to photolysis of Thalidomide with similar difference in retention times was reported by Mahmoud et al. (2014). In most cases these TPs exhibited similar MS^2 fragmentation pathways. Product ions of this compound lose 16 and 55 Da to provide ions of 248 and 209, respectively, indicating formation of constitutional isomers (Table 4). The two different retention times of this TP were probably due to position of the OH group, which could be added to 10 possible sites of the aromatic rings and rendered the molecules more polar. However, on the basis of the MS fragmentation, the identification of the exact position of the hydroxyl group was not feasible. Formation of this compound could occur due to decarboxylation with the ring opening, followed by oxidation and hydroxylation. A similar mechanism was reported during photolysis of other fluorescent dyes and aromatic compounds (Belov et al., 2014; Chiang et al., 1997; Kamiya et al., 2007).

The product with *m/z* 349.0 (TP_2) (Fig. 4) differs only 16 Da from the PC, indicating that generation of this compound could occur due to addition of a hydroxyl group to the one of UR′ aromatic rings. Addition of the OH group to the structure might happen at ten possible sites of the molecule. The fragment ion of this compound loses 17 and 44 Da provide fragment ions of *m/z* 331 and 315, respectively.

Two peaks were detected with same nominal mass of *m/z* 377.0 but two different retention times (R_t = 5.3, 5.8 min). The photoproduct with *m/z* 377.0 ($TP_{3a,b}$) (Fig. 4) differs 44 Da from the parent compound suggesting that COOH– group could be added to the UR structure, possibly due to the photocarboxylation (Ito et al., 1988). Addition of COOH– group could also explain higher polarity of $TP_{3a,b}$ compared to UR. A possible explanation could be the high grade of primary elimination and the low degree of mineralization of the parent compound. Both TPs deliver similar fragmentation pattern since all lose 18 and 44 Da which is in accordance with carboxyl moiety addition. Taking above in consideration the mass of 377.0 *m/z* observed at two different retention times could be interpreted as positional isomers.

The fragmentation patterns confirmed that $TP_{1a,b}$ and TP_2 are hydroxylated products whereas $TP_{3a,b}$ belong to the carboxylated compounds. However, due to many possible addition sites to the aromatic ring of UR, it is difficult to know the exact position of either the hydroxyl or the carboxyl groups (Fig. 4).

3.3. Biodegradation in CBT, MRT and WST

Detailed results for the biodegradation tests can be found in supplementary information (Fig. S14 and Fig. S15). The validity criteria for CBT according to the OECD guideline (>60% ThOD of the quality control — sodium acetate is required to be degraded within 14 days) were met (OECD, 1992). No toxic effects on bacteria (biodegradation in toxicity

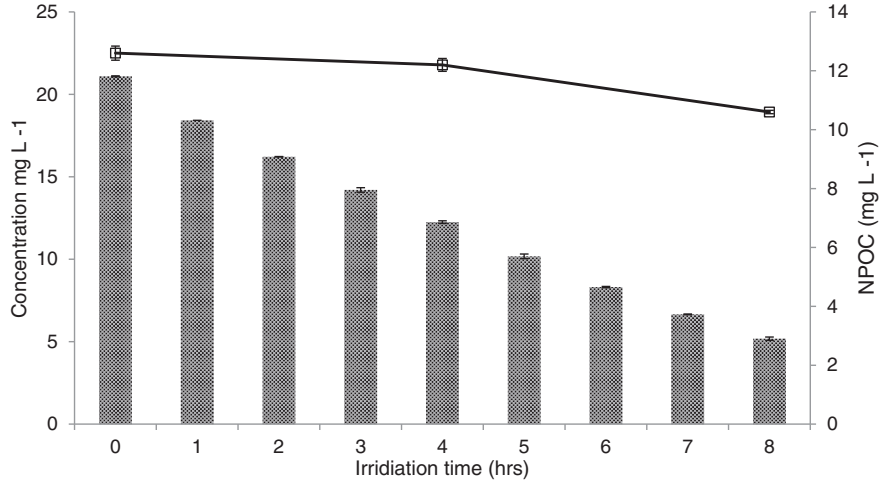

Fig. 2. Elimination of UR during the irradiation with Xe lamp for 8.0 hours. Secondary y-axis represents evaluation of non-purgeable organic carbon. All values represent the means ± SD (n = 2).

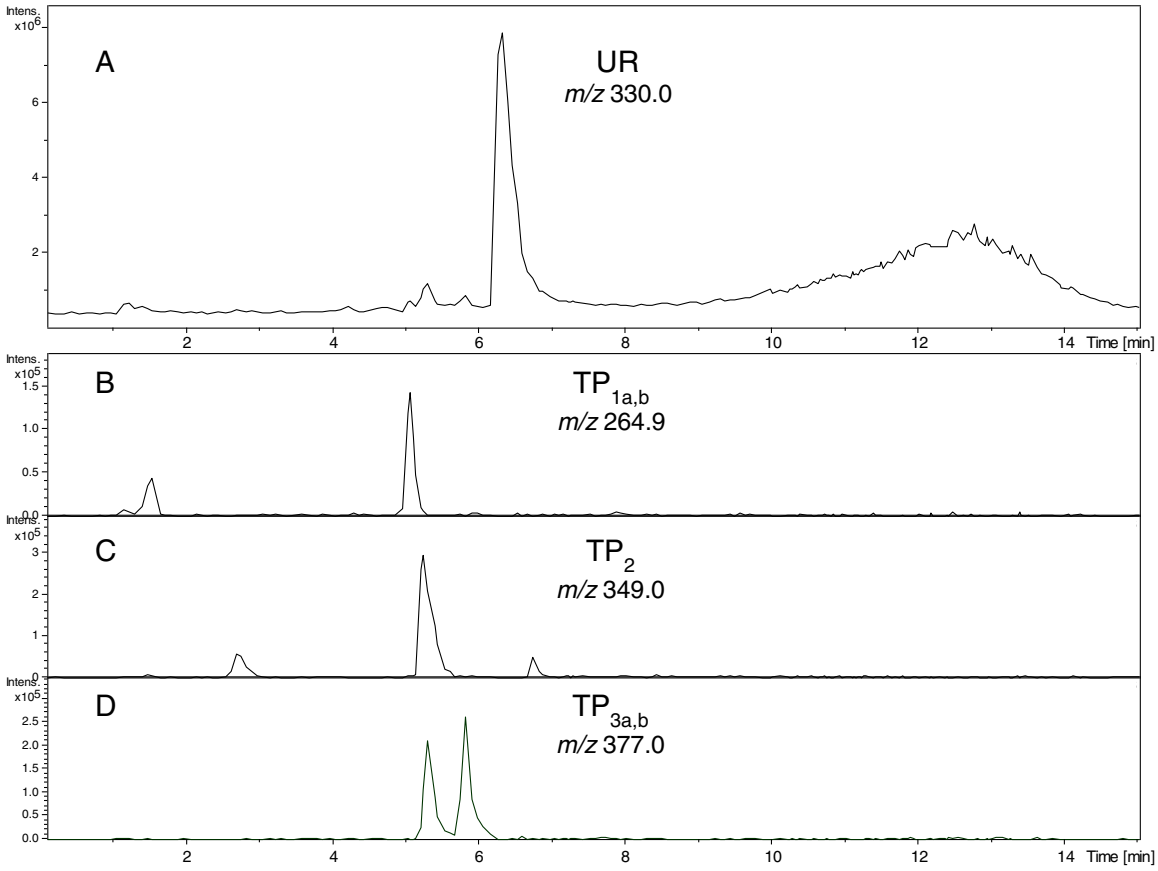

Fig. 3. The total ion chromatogram of UR after photo-treatment for 8.0 h. (B–D) extracted ion chromatograms of Xe lamp generated transformation products. Note that the scale varies.

control > 25%, Fig. S14, A and B) were observed by any tested substance in the toxicity control bottles. No biodegradation has been observed for UR in the CBT. The average biodegradation value after 28 days for UR itself (0.0 h photolysis time) monitored by measurement of the oxygen concentration was 7.6% (Fig. S14, A). On the background of the typical variation of such biodegradability results this has to be classified as no biodegradation.

For samples after 8.0 h photolysis the average biodegradation value was 13.2% on the 28th day (Fig. S14, B). These values classify UR and UR-TPs as *not readily biodegradable*. Similarly to the CBT the UR was *not readily biodegradable* in the MRT. The validity criteria were met — 60% of the quality control substance was biodegraded within 10 days. No toxic effects on bacteria were observed in the toxicity control as well as no degradation was observed in the sterile control. Generally it is assumed to expect higher degradation rate in MRT compared to CBT due to higher inoculum density and bacterial diversity. However obtained biodegradation values at the end of MRT were lower compared with the end results of CBT. For UR (0.0 h photolysis time) was −0.1% (Fig. S15, A), likewise no biodegradation has been observed for the photo-TPs. In the samples after 8.0 h photolysis the average biodegradation value was −8.4% on the 28th day (Fig. S15, B). The reason for the negative values in MRT might be interpreted as high degradation in the blank controls and should be considered could be due to some background in the blanks and can be considered as 0% degradation of the test substance.

During the WST (Fig. 5), the inoculum was of sufficient activity ('quality control' 79 ± 5% biodegradation). No significant difference was observed between biodegradation of UR and its photolysis mixture. The biodegradability of UR was slightly higher and reached 28 ± 16% compared with photo-degraded sample 18 ± 6% (Fig. 5). However, both are not significant. The explanation of slightly higher degradation rates in WST in comparison to MRT could be the higher bacterial diversity of the inoculum used for this test, which in fact was a mixture of bacterial cultures from several natural water bodies and secondary effluent from sewage treatment plant. Both, UR and its photo-TPs were not toxic to the inoculum as biodegradation in 'toxicity control' reached 53 ± 7% and 49 ± 10%, respectively. Correlation between 'toxicity control calculated' and experimental values of the 'toxicity control' can be found in SI, (Fig. S16).

To sum up, results presented here classify UR and its TPs as *not readily biodegradable*. Moreover, observed biodegradation is very low. On the one hand, these results are in accordance with the findings of Smart and Laidlaw (1977) who reported that UR is resistant to biodegradation. On the other hand, Käss (2004) stated that biodegradation of

Table 4
Chromatographic parameters of UR and its transformation products analysis by LC/MS–MS (R_t — retention time, m/z — mass to charge ratio, relative abundance in brackets).

Compound	R_t (min)	Main precursor ion (*m/z*)	Product ions (*m/z*), % of relative abundance in brackets
UR	6.3	333.0	315.0 (100), 287.9 (60), 271.0 (64.9),
TP_{1a}	1.5	264.9	248.9 (100.0), 245.8 (82.6), 234.0 (21.6), 209.2 (32.6), 188.9 (8.6)
TP_{1b}	5.1	264.9	248.9 (75.1), 245.9 (39.7) 235.4 (100.0), 208.9 (6.0), 172.0 (21.6)
TP_2	5.2	349.0	331.8 (50.7), 315.0 (44.0), 305.2 (70.9), 302.8 (100.0), 183.7 (18.8)
TP_{3a}	5.3	377.0	359.0 (100), 330.9 (4.5), 314.8 (9.1), 274.9 (3.5), 214.8 (1.0)
TP_{3b}	5.8	377.0	359.0 (22.0), 332.0 (1.63), 330.9 (7.86), 315.8 (0.5), 275.0 (4.5),

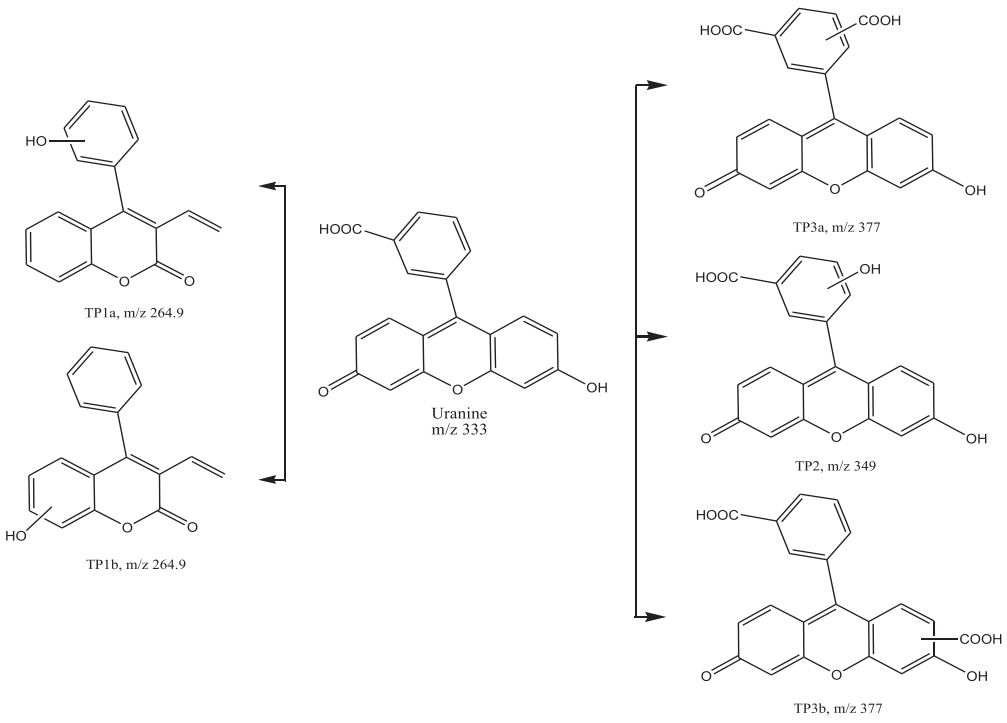

Fig. 4. Proposed photo transformation products of UR identified by means of LC–MS/MS.

UR in the environment cannot be excluded. Especially, the results of the WST confirm theseobservations and one can state that a small part of the parent compound as well as of its photo-TPs might be degraded in the natural environment. However, such a small difference in biodegradation rate (CBT, WST) is within the natural variation of biological systems, especially when biodegradation was evaluated on the base of an indirect measurement (i.e., pressure measurement). The measurements with HPLC-FLD confirmed that no elimination of UR and the photoproducts occurred during the CBT and MRT. However, at the end of WST the HPLC-FLD analysis showed elimination of 2.4 mg L^{-1} (11.7%) of initial UR concentration from the water phase. This might be a result of partial sorption to the sediment particles or due to the bacterial metabolism. In the WST, 93% of sediment mixture consisted of quartz sand, while the rest was clay and peat. Hence sorption cannot be excluded, although Kasnavia et al. (1999) found only small sorption of UR for negatively charged media (e.g., sand and sandstones).

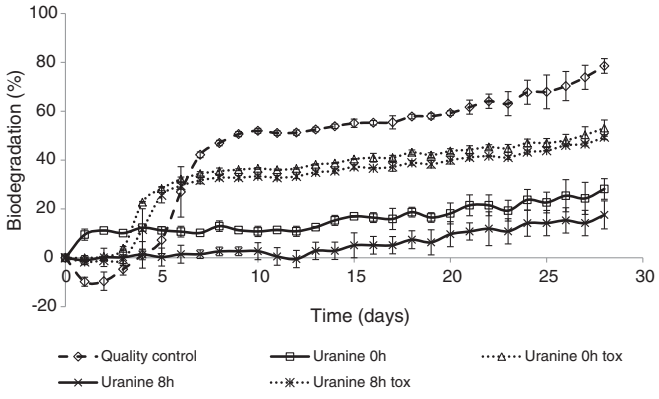

Fig. 5. Degradation of UR (Xe lamp irradiation time 0.0 h) and photolysis mixture (Xe lamp irradiation time 8.0 h), in screening water sediment test (average from two independent tests).

4. Conclusions

This study demonstrates that by a well-selected combination of laboratory tests, a deeper insight into the nature and environmental fate of TPs derived from water tracer UR can be gained. We focused on the combined effect of two processes (direct photolysis and biodegradation) as appropriate tool for the first screening of a substance's behavior in aquatic environment and a good starting point providing the information that allows one to plan direction of further research.

UR and its photo-TPs were not readily biodegraded in all performed tests, yet small extent of biotic degradation of UR cannot totally be excluded in the aquatic environments. This study is therefore another demonstration that photolysis should be considered as the main degradation pathway for UR in surface water systems. However, only a small part of the UR is entirely mineralized and it should be considered as a compound that potentially forms persistent photo-TPs in aquatic environments. No indication for toxicity was found, which tests is in accordance with previous toxicity studies. Still, target-oriented investigations on long term impacts of photo-TPs from UR are warranted.

Results obtained in this study demonstrate that deeper investigations are necessary to fully understand fate and risk connected to the use of UR. Therefore, it is highly recommended that ecological impact of the PC and especially its photo-TPs should be further investigated and should be taken into account for a detailed risk assessment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.07.002.

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Supplementary information to the article:

"Photolytic transformation products and biological stability of the hydrological tracer Uranine"

1. Methods

1.1. Sediment conditioning (Text S1)

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

Table S3. Localization of inoculum sampling sites for the WST

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

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

The mass spectrometer was operated in positive polarity. Analysis of total ion chromatogram and corresponding mass spectrum was used for structural identification of the photo-TPs. The structural identification of the transformation products was first based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum. Furthermore, to obtain structural elucidation the photo-TPs were isolated, used as precursor ions and further fragmented up to MS^3 using the Auto MS^n mode. The operating conditions of the source were: -500 V end plate, - 3250 V capillary voltage, 30 psi nebulizer pressure, and 12 L min^{-1} dry gas flow at a dry temperature of 350 °C. The selected lens and block voltages were: + 229.2 V capillary exit, 300.0 Vpp octopole reference amplitude and -59.5 V lens two. The scan range was determined from m/*z* 40 to 600 and the scan time was 200 ms.

2. Results

2.1. *Kinetic profiles of the photo-TPs*

Fig. S1, S2 and S3 shows the course appearance of peak area of the photo-TPs (relative abundance above 1%) measured by LC-EC-MS in positive mode (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of UR at 0 min.) (Initial concentration of UR = 60 mg L^{-1}).

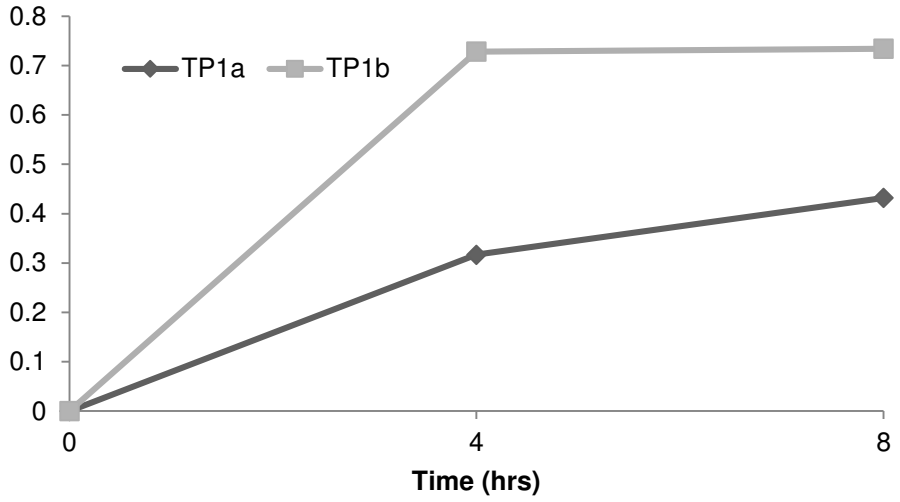

Fig.S1. The course appearance of peak area of the photo-TPs1a-b (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of UR at 0 min.) (Initial concentration of UR = 60 mg L^{-1}).

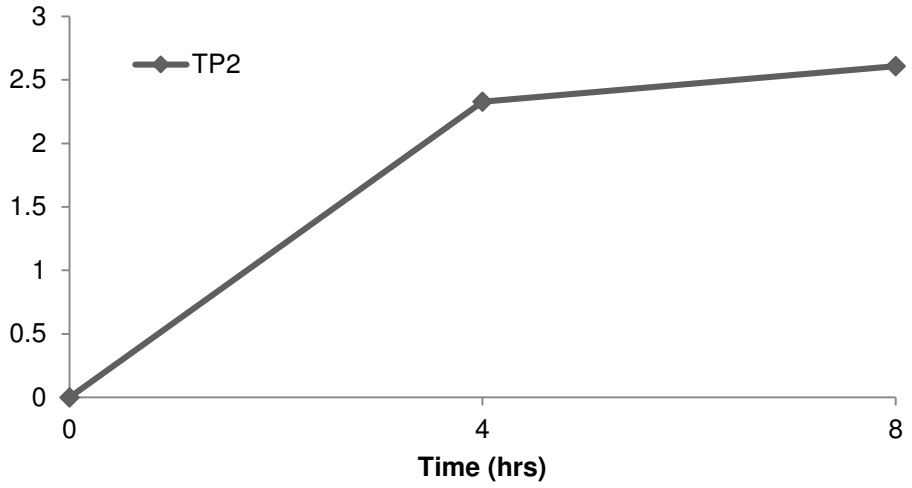

Fig.S2. The course appearance of peak area of the photo-TP2 (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of UR at 0 min.) (Initial concentration of UR = 60 mg L^{-1}).

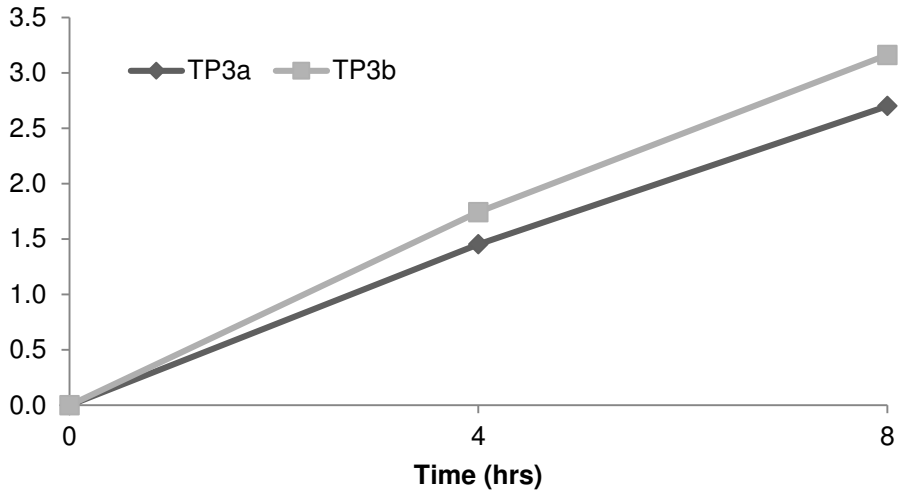

Fig.S3. The course appearance of peak area of the photo-TPs3a-b (A/A_0 as A is the peak area of photo-TPs and A_0 is the peak area of UR at 0 min.) (Initial concentration of UR = 60 mg L^{-1}).

2.2. Elucidation of the transformation products:

Transformation product TP 1_a, m/z 264.9, RT 1.5 min.

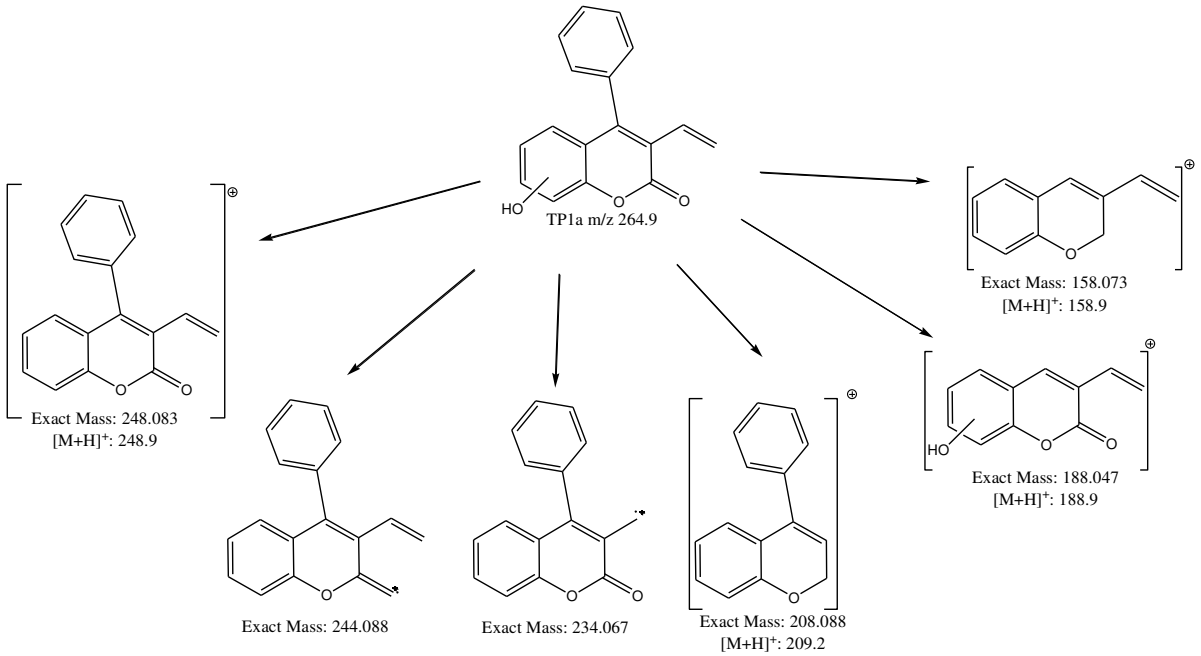

Fig.S4. Proposed fragmentation pattern for TP1_a.

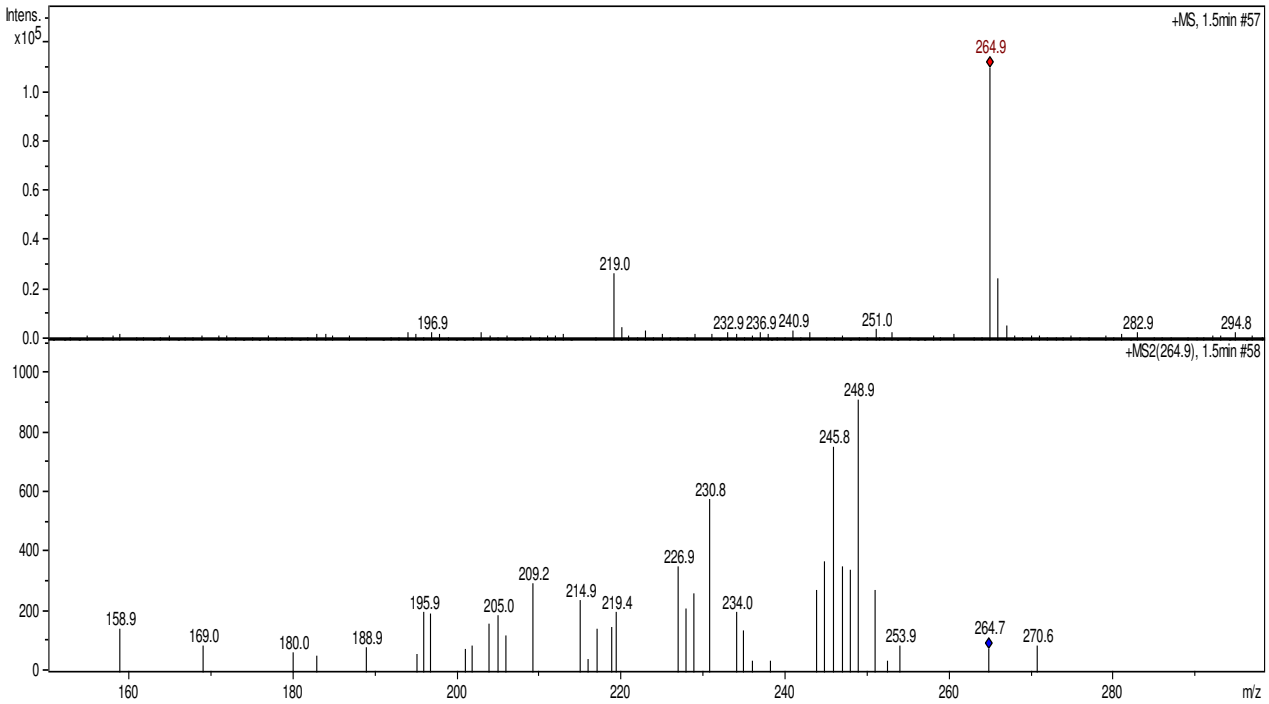

Fig.S5. Obtained mass spectrum for$TP1_a$.

Transformation product $TP1_b$, m/z 264.9, RT 5.0 min.

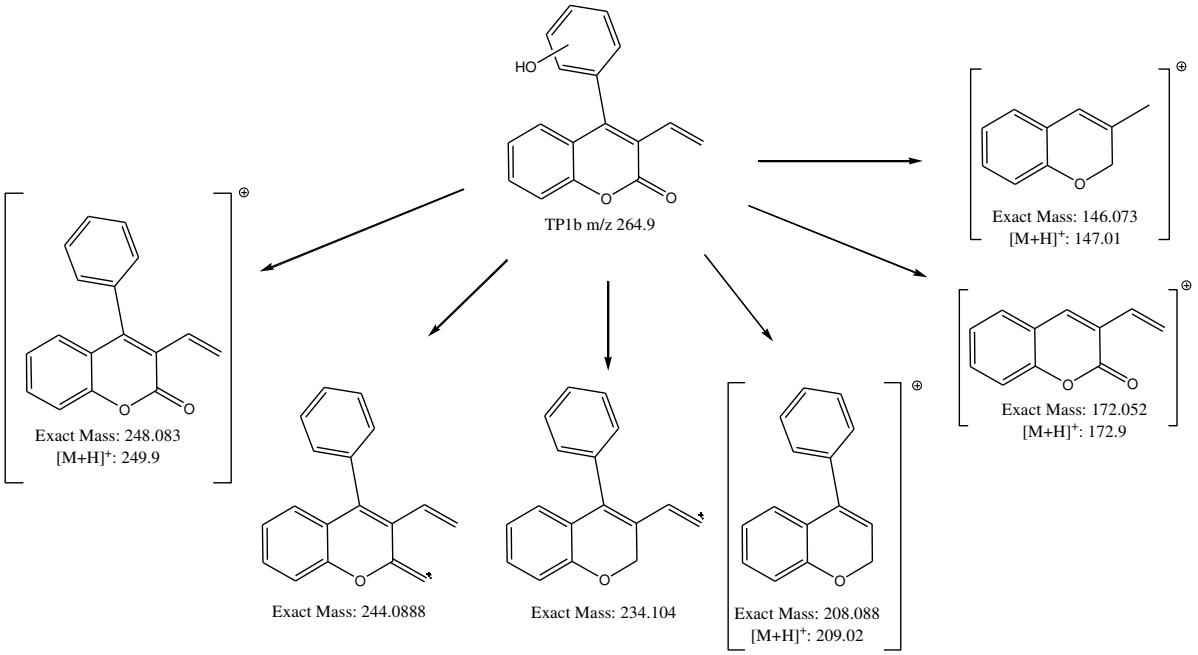

Fig.S6. Proposed fragmentation pattern for $TP1_b$.

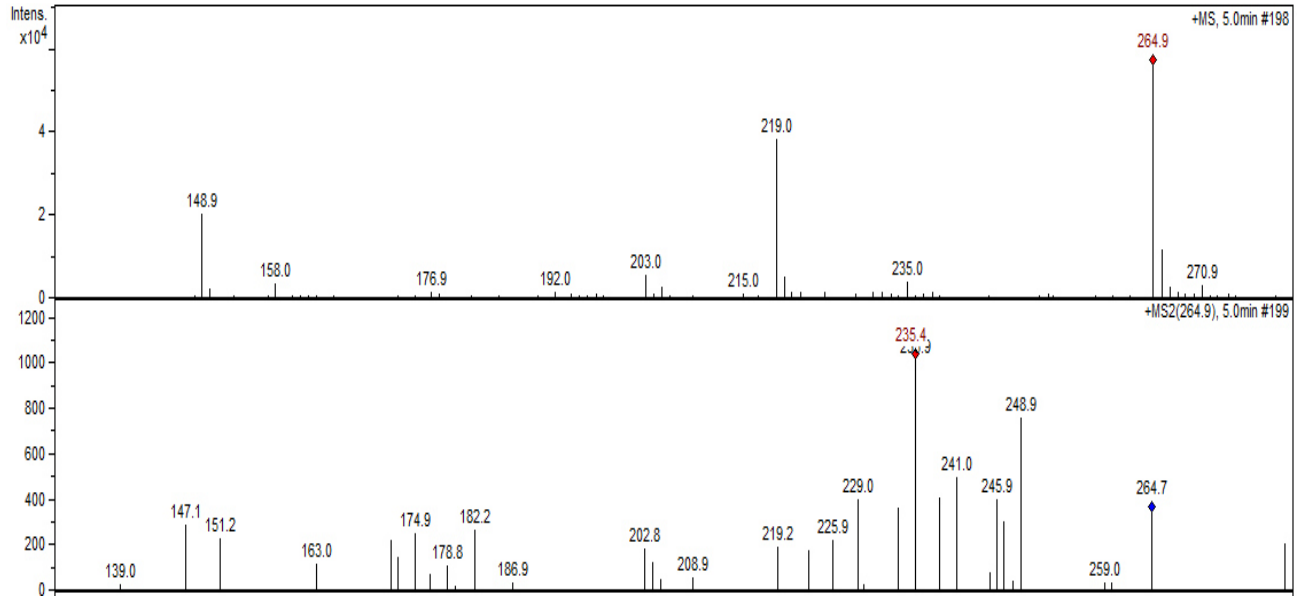

Fig.S7. Obtained mass spectrum for $TP1_b$.

3. Transformation product TP2, m/z 349.0, RT 5.2 min.

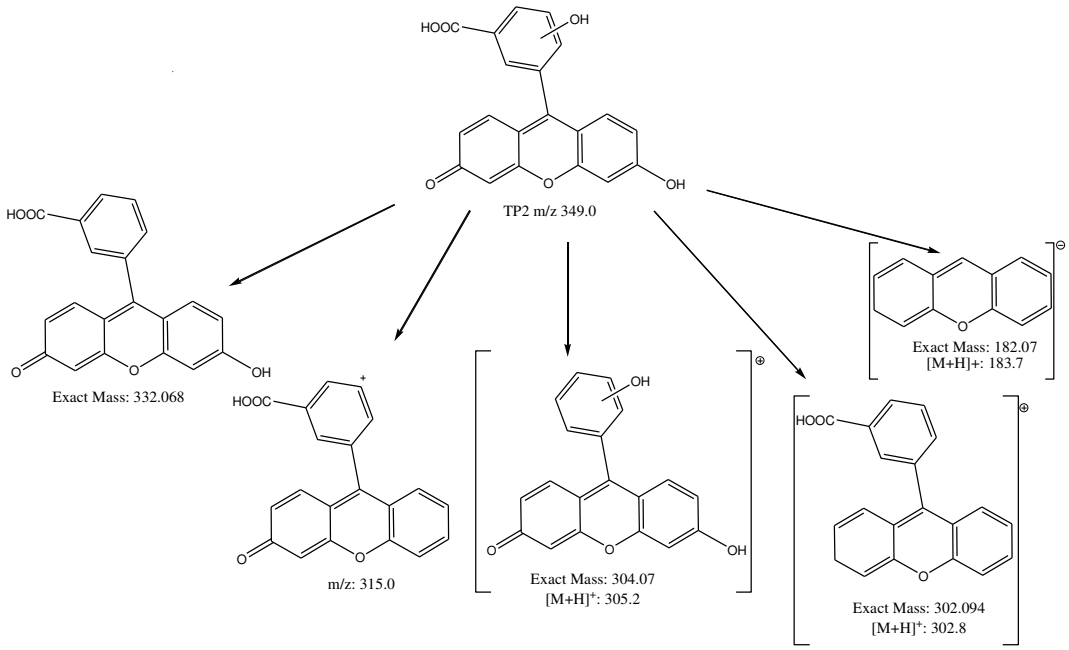

Fig.S8. Proposed fragmentation pattern for TP2.

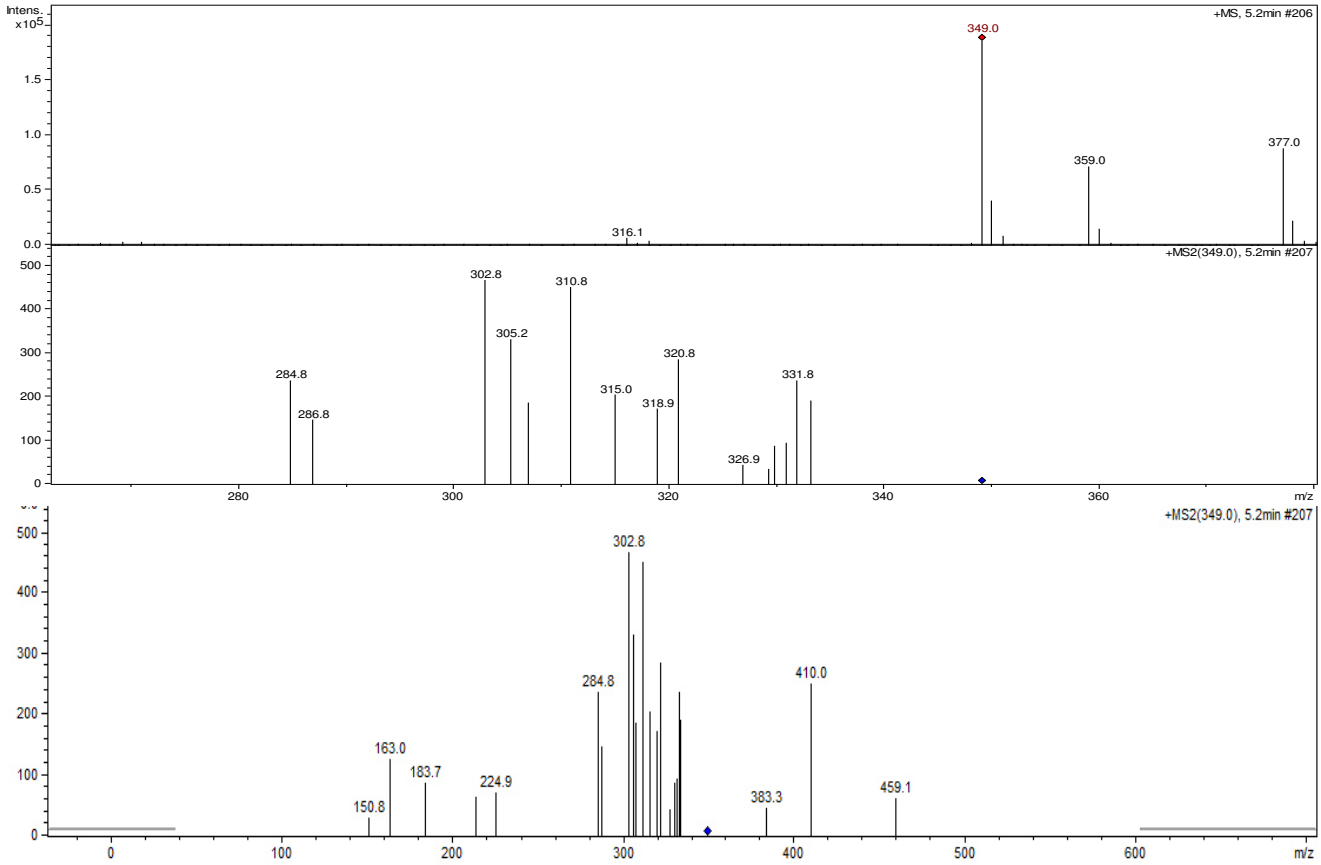

Fig.S9. Obtained mass spectrum for TP2.

4. Transformation product TP 3_a, m/z 377.0, RT 5.2 min.

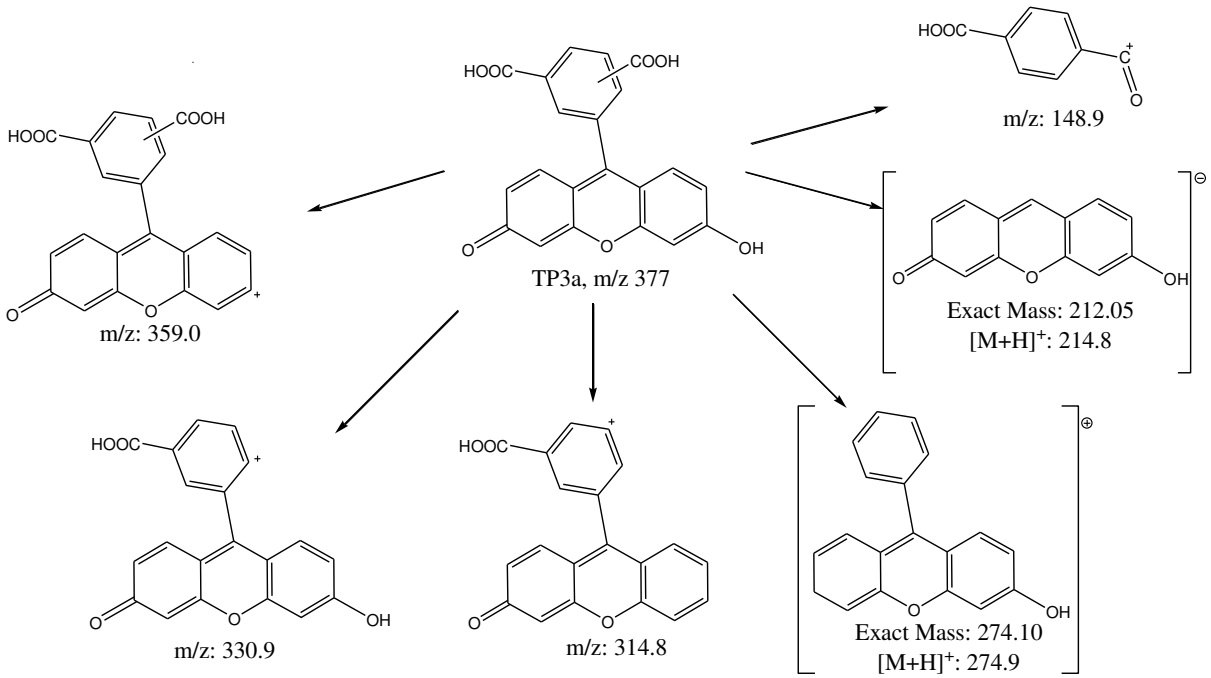

Fig.S10. Proposed fragmentation pattern for TP3_a.

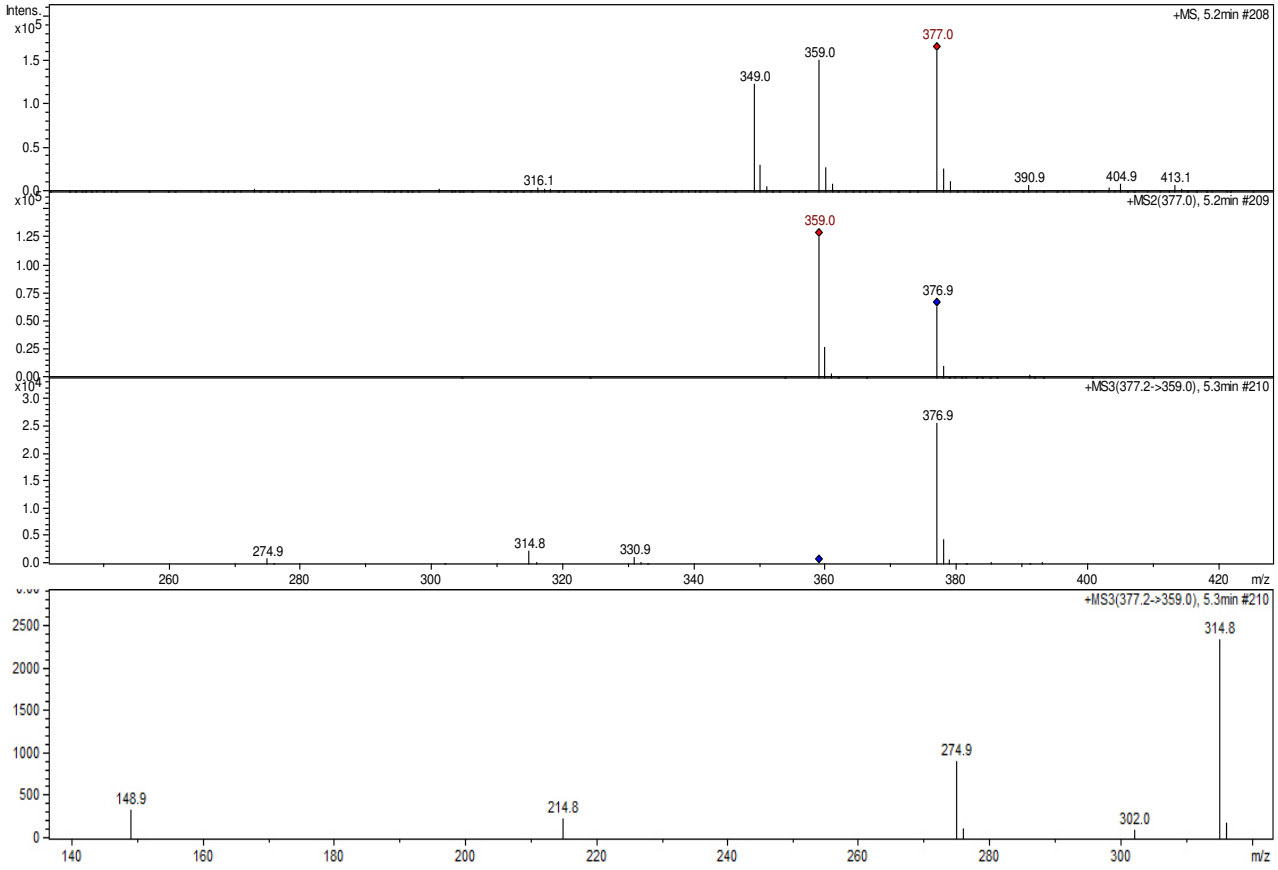

Fig.S11. Obtained mass spectrum for TP3_a.

5. Transformation product TP 3_b, m/z 377.0, RT 5.8 min.

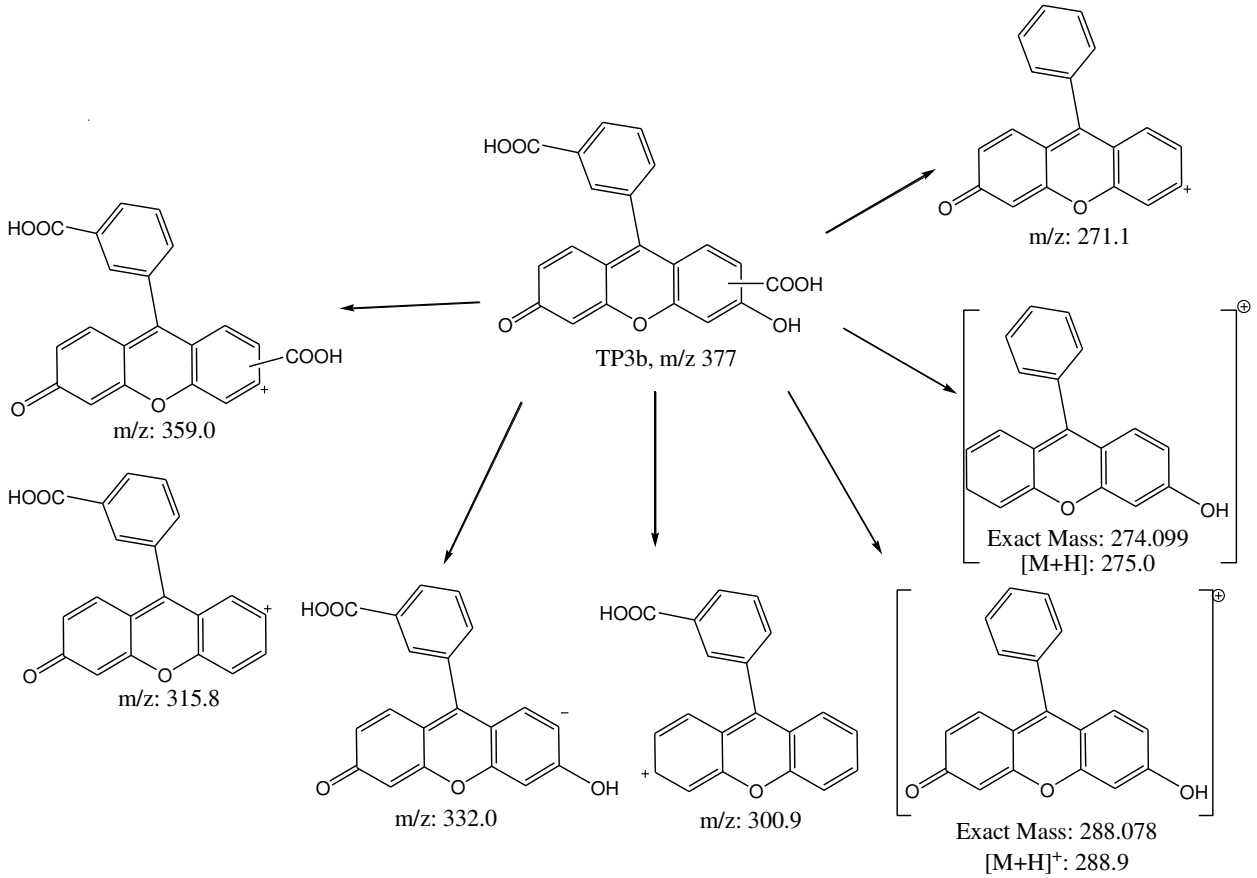

Fig.S12. Proposed fragmentation pattern for $TP3_b$.

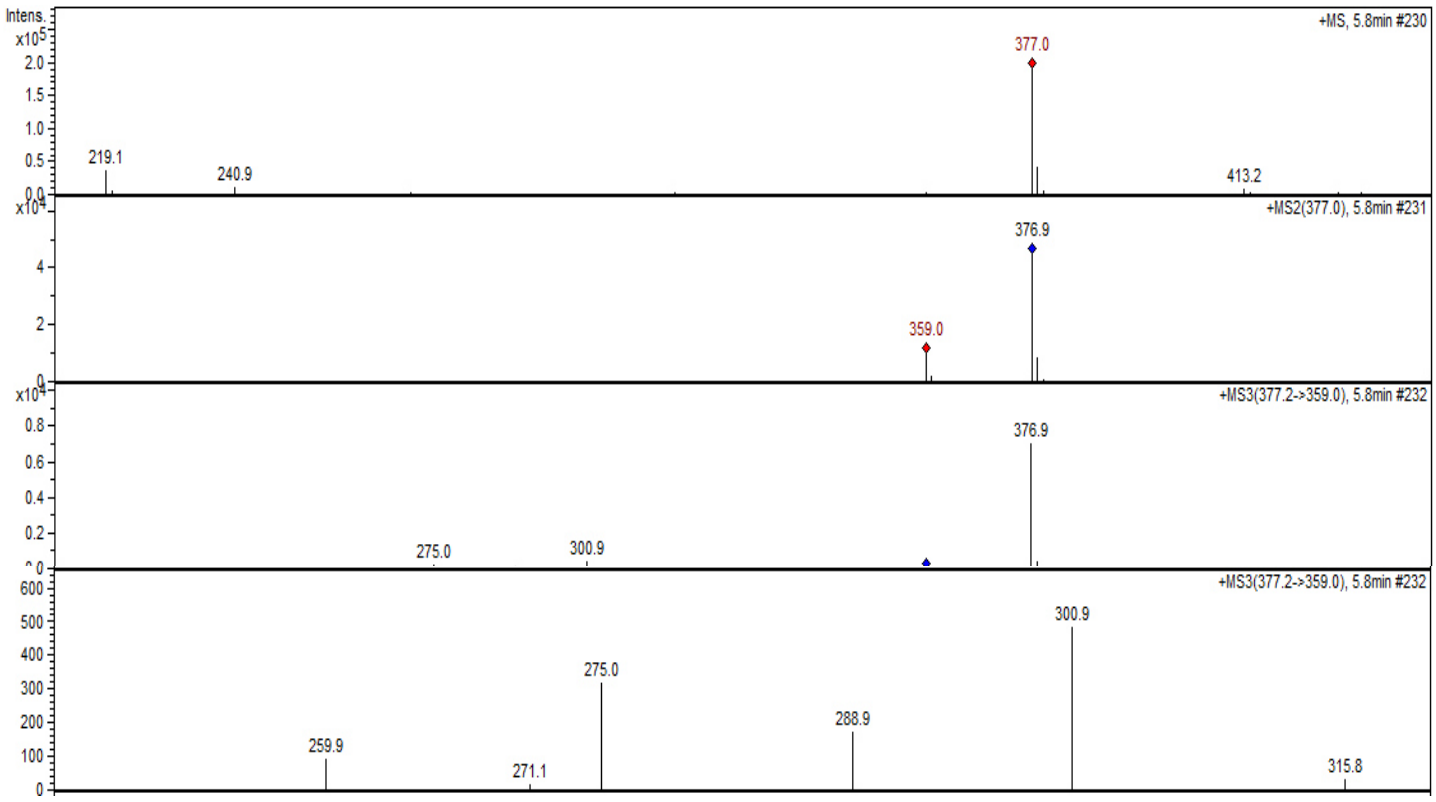

Fig. S13. Obtained mass spectrum for$TP3_b$.

2.3. Biodegradation of UR in the MRT.

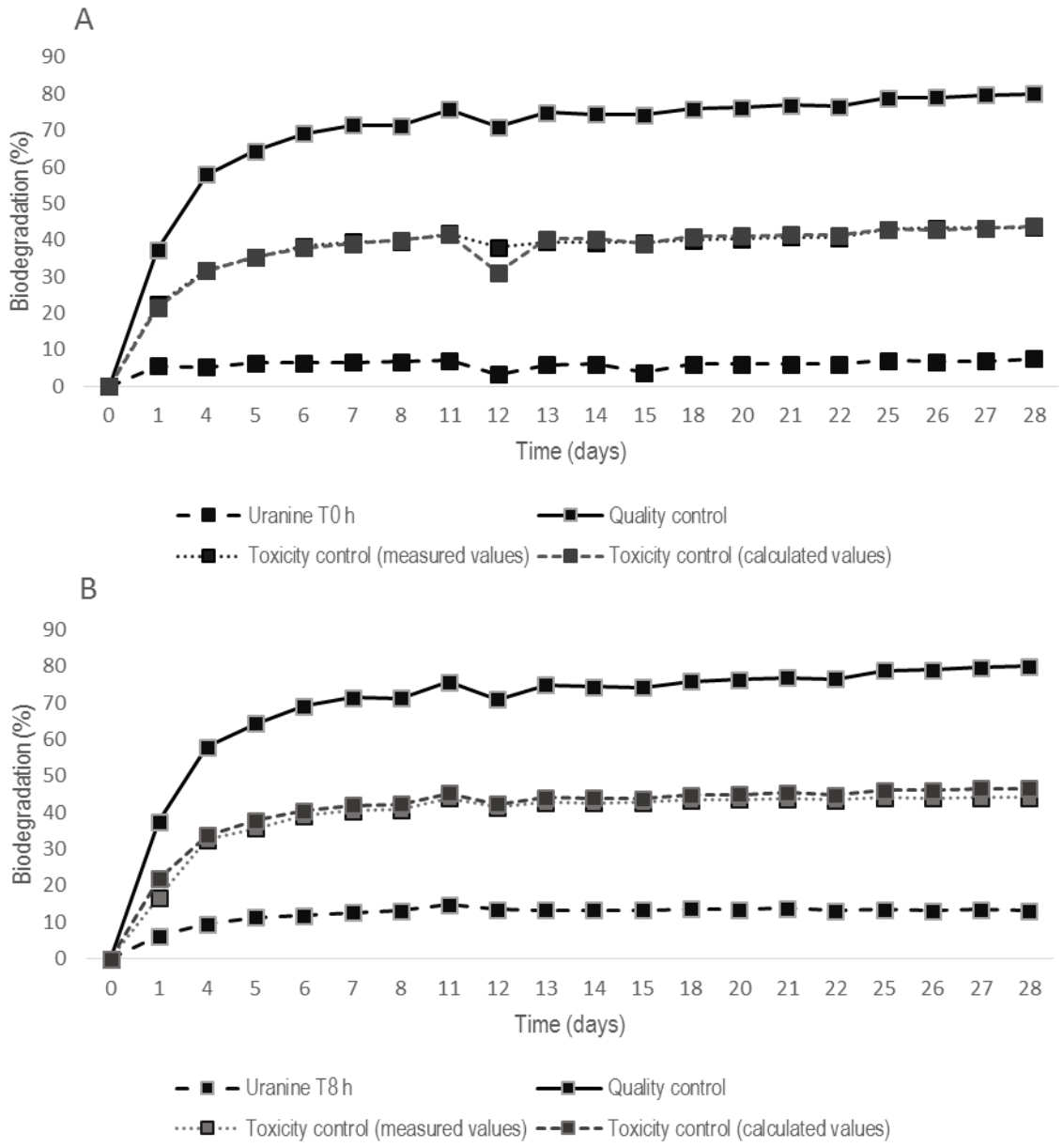

Fig. S14. Biodegradation in Closed Bottle test of UR A) at the time point 0.0 h (without phototreatment), B at the time point 8.0 h (after phototreatment).

2.4. Biodegradation of UR in the MRT.

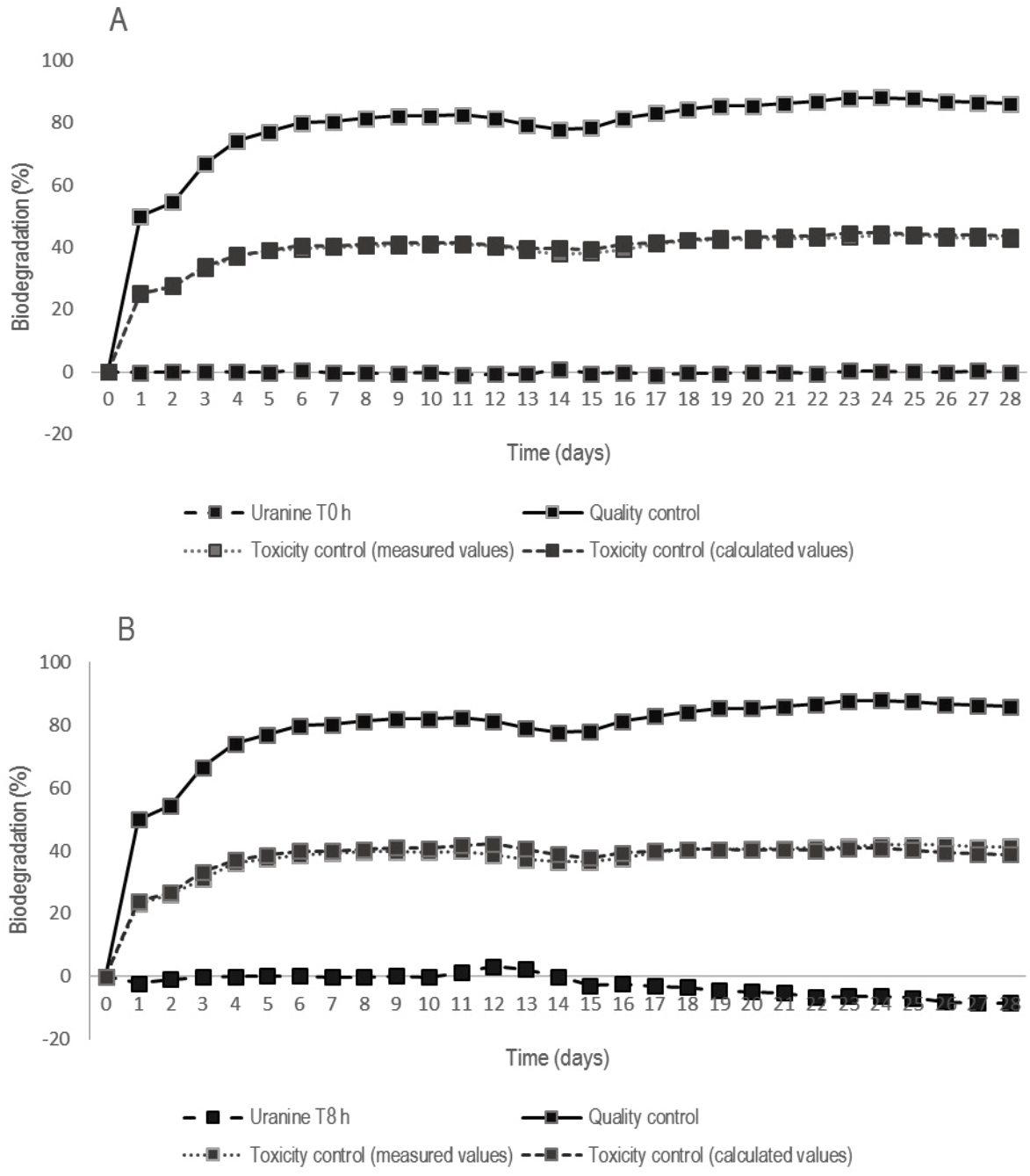

Fig.S15. Biodegradation in Manometric Respiratory test of UR A) at the time point 0.0 h (without phototreatment), B at the time point 8.0 h (after phototreatment).

2.5. Calculated and measured toxicity controls in WST.

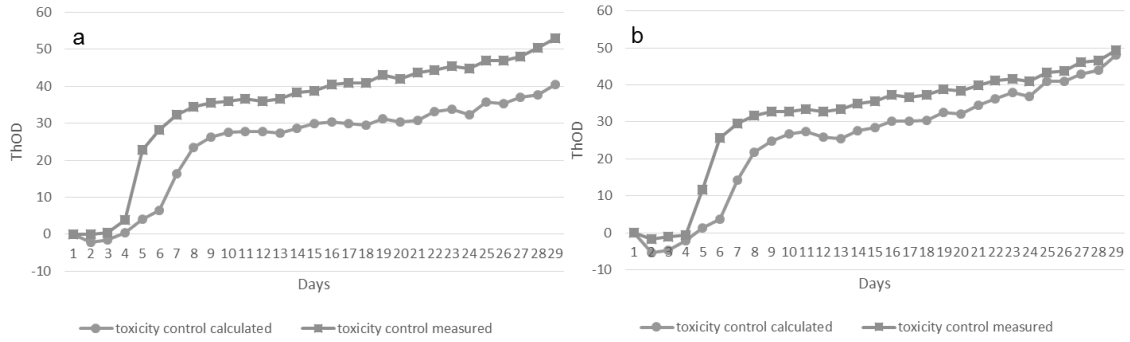

Fig. S16. Correlation between toxicity control series measured and calculated during WSTs, between samples of Uranine (UR) (a) sample from photodegradation experiment time point 0 h, and (b) sample from photodegradation experiment time point 8 h; (Xe lamp).

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