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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.

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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.

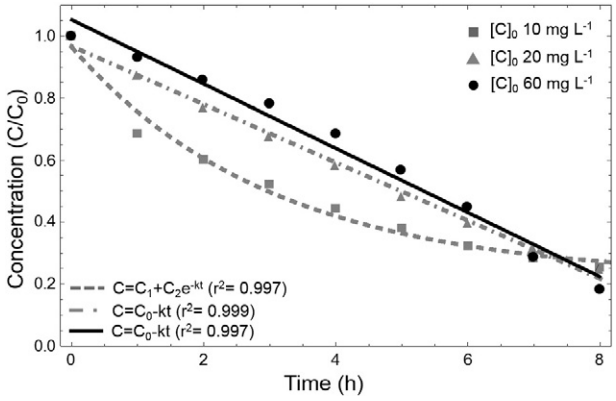

Fig. 1. First-order and zero-order photolysis kinetics of UR at 10, 20 and 60 mg L^{-1}, photolysis with Xe lamp for 8.0 hours. All values represent the means ± SD (n = 2).

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

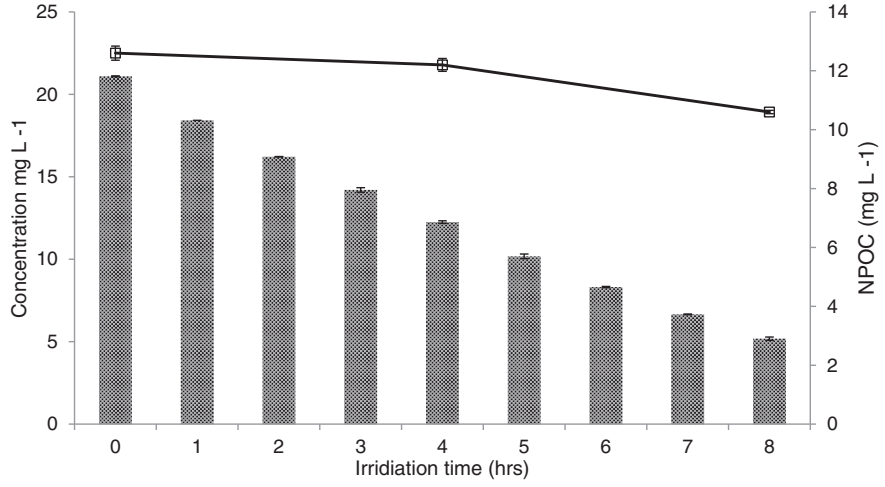

Fig. 2. Elimination of UR during the irradiation with Xe lamp for 8.0 hours. Secondary y-axis represents evaluation of non-purgeable organic carbon. All values represent the means ± SD (n = 2).

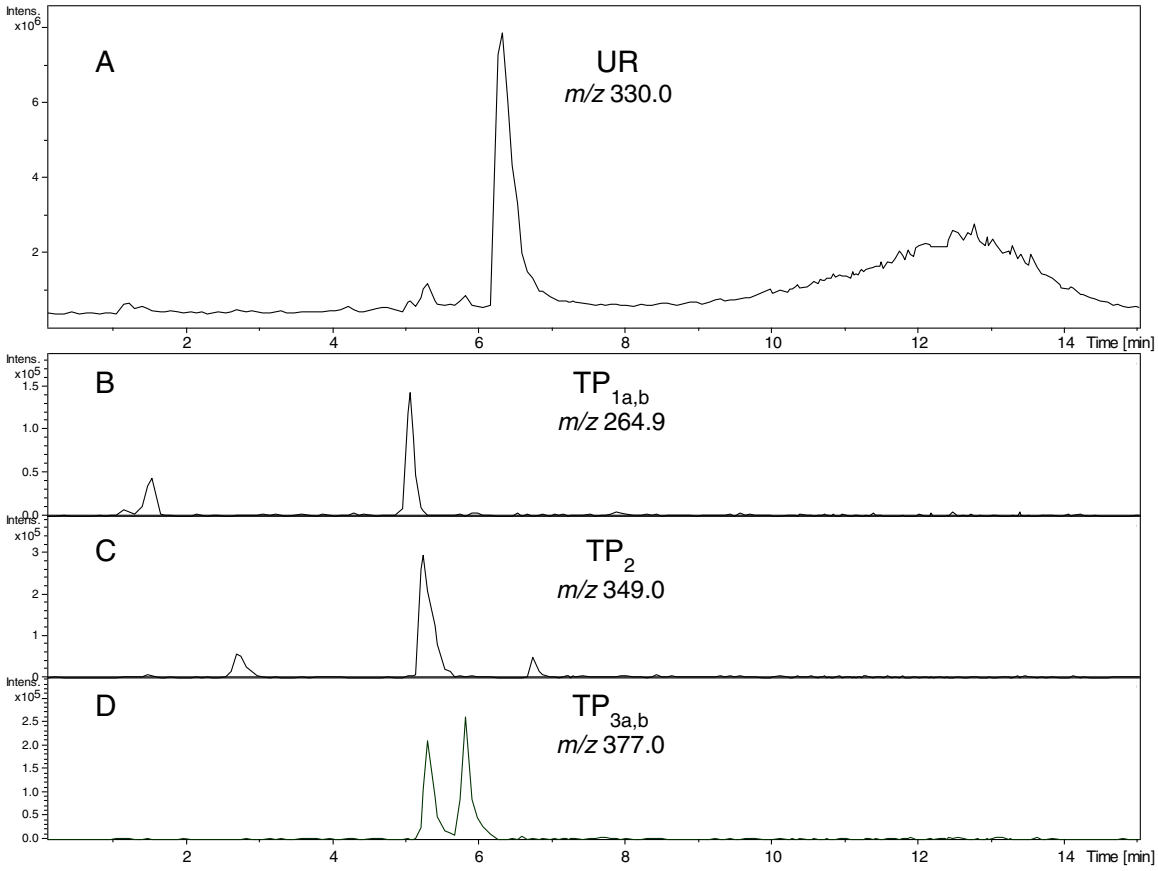

Fig. 3. The total ion chromatogram of UR after photo-treatment for 8.0 h. (B–D) extracted ion chromatograms of Xe lamp generated transformation products. Note that the scale varies.

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),

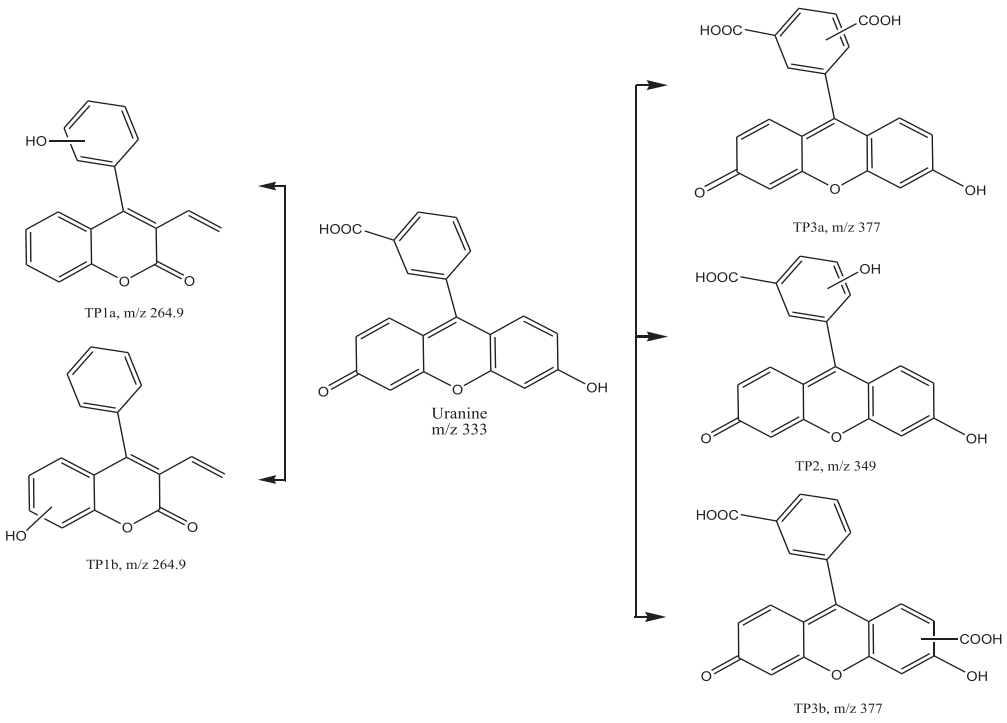

Fig. 4. Proposed photo transformation products of UR identified by means of LC–MS/MS.

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).

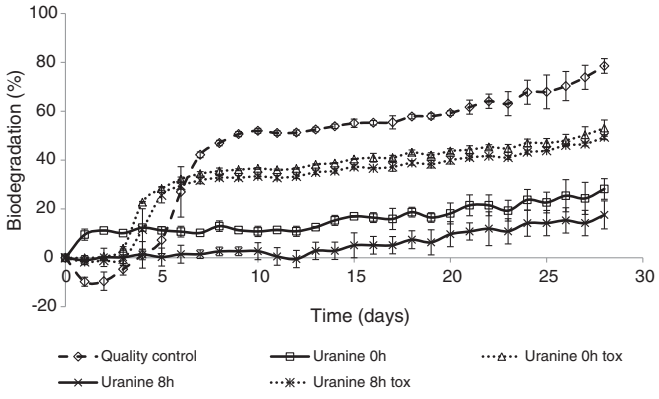

Fig. 5. Degradation of UR (Xe lamp irradiation time 0.0 h) and photolysis mixture (Xe lamp irradiation time 8.0 h), in screening water sediment test (average from two independent tests).

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.

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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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