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Identification and ecotoxicity of degradation products of chloroacetamide herbicides from UV-treatment of water

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Abstract: The widespread occurrence of chlorinated herbicides and their degradation products in the aquatic environment raises health and environmental concerns. As a consequence pesticides, and to a lesser degree their degradation products, are monitored by authorities both in surface waters and drinking waters. In this study the formation of degradation products from ultraviolet (UV) treatment of the three chloroacetamide herbicides acetochlor, alachlor and metolachlor and their biological effects were investigated. UV treatment is mainly used for disinfection in water and wastewater treatments. First, the chemical structures of the main UV-degradation products were identified using GC-MS and LC-MS. The main transformation reactions were dechlorination, mono- and multi-hydroxylation and cyclizations.

The ecotoxicity of the mixed photoproducts formed by UV-treatment until 90 % of the original pesticide was converted was compared to the toxicity of chloroacetamides using the green alga *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the marine bacteria *Vibrio fischeri* as test organisms. UV-treatment of alachlor and metolachlor increased the toxicity compared to the parent compounds while an equal toxicity was found for photolysis products of acetochlor. This suggests that toxic photodegradation products are generated from chloroacetamides under UV-treatment. An important perspective of this finding is that the photolysis products are at least as toxic as the parent compounds.

Keywords Chloroacetamide, photolysis, mass spectrometry, toxicity, photoproducts.

1. Introduction

Chloroacetamides are among the most widely used herbicides. They are used to control annual grasses and broadleaf weeds in corn, soybean, sorghum, cotton and several other crops. Their mode-of-action is to inhibit the early development of susceptible weeds by preventing biosynthesis of very long fatty acid chains thus affecting cell integrity (Böger et al., 2000; Matthes and Boger, 2002).

Among the most commonly used chloroacetamides are acetochlor (2-Chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide), alachlor (2-Chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide) and metolachlor (2-Chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide) (Li et al., 2010). The U.S. Environmental Protection Agency reported their annual use in the USA at 9.1 to 13.6 million kilograms for Acetochlor, 2.7 to 4.1 million kilograms for Alachlor and 6.8 to 9.1 million kilograms for Metolachlor (Kiely et al., 2002). Because of their widespread use and recalcitrant nature, herbicide residues are frequently detected in ground and surface waters (Gascuel-Oudoux et al., 2009; Lewis et al., 2009). Chloroacetamides are listed in the Drinking Water Contaminant Candidate List of the US Environmental Protection Agency (USEPA, 2009) and are among the most frequently detected agricultural herbicides by the National Water Quality Assessment (NAWQA) of the U.S. Geological Survey (Barbash et al., 2001). The frequencies of detection in ground water samples at all concentrations were 0.2, 2.2 and 11.7% for acetochlor, alachlor and metolachlor respectively (Cavalier et al., 1991; Barbash et al., 2001; Rocha et al., 2008). Considerable concentrations of chloroacetamides, varying according to the matrices from 0.1 to 10 µg/L, have been detected in surface water and ground water (Cavalier et al., 1991; Barbash et al., 2001; Konstantinou et al., 2006; Rocha et al., 2008). Chloroacetamides have also been detected in drinking water (Coupe and Blomquist, 2004). Hence, numerous studies have focused on control and optimization of pesticide removal from ground and surface water sources (Reichenberger et al., 2007) during drinking water treatment.

In recent years, several reviews of the occurrence and fate of transformation by-products of manmade chemicals have been made (Richardson et al., 2003; Richardson et al., 2007; Wei et al., 2010; Bull et al., 2011). Degradation products are generated during water treatment processes including oxidation. A particular emphasis has been focused on pharmaceuticals with several reviews and studies dedicated to their degradation products in the environment (Fatta-Kassinos et al., 2011a; Fatta-Kassinos et al., 2011b; Horvat et al., 2012) and in water treatment systems e.g. water works (Ternes et al., 2002; Kosjek and Heath, 2008; Kosjek et al., 2009). In these artificial systems biodegradation occurs concurrently with engineered processes like oxidation reactions with chlorine, chlorine dioxide, ozone and transformations by UV (Kosjek and Heath, 2008; Hollender et al., 2009; Kosjek et al., 2009).

In a recent review dealing with emerging contaminants from pesticide degradation, it was highlighted that degradation products were generated from transformation processes such as hydrolysis, biodegradation and chemical oxidation (Richardson, 2008). Pesticide degradation is governed by environmental factors which should theoretically attenuate their concentration in soil and water. However, unknown and potentially toxic compounds are generated. Furthermore these transformation products have been reported to be present at greater concentrations than the parent herbicides (Richardson, 2008). These transformation products were also described as exhibiting an equivalent or even greater level of toxicity than the parent

pesticides (Hladik et al., 2005a). Hladik et al. (Hladik et al., 2005b) also reported that in some environmental water samples (Chesapeake Bay) the total concentration of non-ionic degradation products of chloroacetamide herbicide were about 20 to 30 times higher than the concentration of parent chloroacetamide herbicides. According to Boxall and Sinclair, 2001, out of 89 pesticide degradation products, 70 percent were equally or less toxic than their parent compounds and 30 percent exhibited a greater toxicity. In total Hladik and co-authors identified 26 chloroacetamide degradation products in drinking water sources (Hladik et al., 2008). Although abundant information on the health risks of chloroacetamide herbicides is available, much less is known about the effects of their degradation products (Hladik et al., 2005b). This has prompted environmental authorities to include some degradation products in investigations regarding herbicide fate and transportation in aquatic ecosystems (EU, 1998; USEPA, 2009).

In this context the purpose of the present study was to identify by chemical analysis the degradation products produced by UV-treatment of Acetochlor, Alachlor and Metolachlor in aqueous solution and to describe the ecotoxicological effect of these degradation products. Direct photolysis by high pressure UV-lamp was used to degrade the herbicides as water treatment utilities are increasingly employing UV-treatment as an alternative disinfection method replacing chlorination (Cabaj et al., 1996; Hijnen et al., 2006). Since UV is employed to disinfect and not specifically degrade compounds it can only be expected that a minor fraction of a given pesticide will be degraded considering the large difference in UV dosage used for disinfection compared to targeted photolysis (Hijnen et al., 2006; Hansen and Andersen, 2012). In order to investigate which degradation products are formed following UV treatment of chloroacetamide herbicides and to determine potential toxicity the chosen herbicides were exposed to a UV dosage degrading 90 % of the parent compound. With this methodology we expected to create a mixture of products similar to those formed by UV disinfection, while further UV-treatment would increasingly form products from continued photolysis of the primary photolysis products. Afterward, chemical analysis was performed to identify the formed photolysis products in the resulting solution. For that purpose, liquid chromatography coupled to quadrupole/time-of-flight mass spectrometer (LC-Q/TOF-MS) and gas chromatography coupled to an ion trap mass spectrometer (GC-ITMS) were utilised. These two analytical techniques were chosen for their complimentary ability to detect polar and apolar compounds. An analytical strategy for the identification of degradation products was also developed. This analytical strategy has been developed and applied to allow reliable identification of numerous degradation products. Our strategy combined three complementary major steps for generating data that could be used to identify the degradation products: collision induced dissociation for fragmentation mechanism establishment, high resolution measurements for elemental composition determination and use of isotope labelled molecules for structural confirmation (Bourcier et al., 2010; Bouchonnet et al., 2011; Bouchonnet et al., 2012; Souissi et al., 2012; Coffinet et al., 2012). The labelled molecules allow the location of structural modifications induced by UV irradiation through the comparison of data with those of unlabelled molecules (Coffinet et al., 2012).

Following degradation product identification, ecotoxicity of these three chloracetamides was compared with mixtures of photo-transformation products formed by their respective UV-irradiation. Ecotoxicity assessment of photolyzed and non-photolyzed spiked water samples were carried out using three standardized short-term bioassays with the crustacean, *Daphnia*

magna, the algae, *Pseudokirchneriella subcapitata*, and the bioluminescent bacteria, *Vibrio fischeri*.

2. Materials and methods

2.1. Chemicals and standards

Acetochlor, Alachlor, Metolachlor and deuterated analogues: Acetochlor-d₁₁ (99% atom D), alachlor-d₁₃ (99% atom D) and metolachlor-d₆ (98% atom D) were purchased from Sigma-Aldrich (St. Quentin Fallavier – France). Chromatographic-grade solvents – methanol, acetonitrile and methylene chloride - were also purchased from Sigma-Aldrich and used as received (99% purity).

2.1.1. Photolysis experiments

A photoreactor equipped with a high-pressure mercury lamp (HPL-N 125W/542 E27 SG, Philips, 94856 Ivry-sur-Seine, France) was constructed for the photolysis experiments. The lamp is intended for water treatment and emits light in a broad spectra ranging from 200 to 1100 nm. Six quartz cylindrical containers were placed on a circular trajectory around the lamp. During the radiation the solutions were stirred with a sonicator (Bioblock Scientific 91631, Illkirch, France). This setup allowed irradiation of several solutions simultaneously. The lamp was placed into the inner part of the reactor cooled by water circulation at 25 ± 2 °C to avoid uncontrolled heating of the irradiated solution. The quartz containers were filled with 30 mL of stock solutions and irradiated. The reactor was covered by aluminium foil to optimize solution irradiation, and avoid light emission outside the reactor. For toxicity experiments a solution of 100 mg·L⁻¹ of each herbicide was prepared in Milli-Q water (Milli-Q, Millipore, France). Subsamples of this stock solution were irradiated with UV for 90 min corresponding to degradation of approximately 90% of each of the herbicides with concurrent formation of photoproducts. For each treatment experiment a “dark control” solution was also prepared. The dark controls consisted of parent herbicides for which all the experimental steps were performed except for the UV-irradiation. The dark control solution and 90 min irradiated solutions were diluted in the appropriate test media for the ecotoxicity assays to attain the final test concentration series.

2.1.2. Solid phase extraction

Solid-phase extractions were carried out prior to chemical analysis of the dark control and photolyzed solutions. Each reaction sample was extracted using a SPE cartridge (Atoll column 30 ATH, 200 mg resin in 3 mL cartridge, Interchim, Montluçon, France). The cartridge was conditioned with 3 mL of methanol followed by 3 mL of distilled water. 4 mL of a 5:95 methanol:water mixture was used for washing the SPE cartridge. After elution of 10 mL of sample at a rate of approximately 60 drops per min, the cartridge was vacuum-dried and eluted with 8 mL of methanol and 4 mL of acetonitrile with 0.1% formic acid, successively. The extracts were concentrated at ambient temperature under a gentle stream of nitrogen until dryness. The dried samples were then re-diluted the corresponding mobile phase according to the analytical technique and transferred into a glass vial. For the injection in GC-MS, it was re-

diluted in 100 µl of methylene chloride. For LC/MS analysis, the mobile phase was 200 µL of H₂O/CH₃CN 0.1% FA (60/40).

The selected elution solvent for SPE extraction and mobile phases for LC/MS and GC/MS, analysis were determined based on comparison of many common solvents. The selection was based achieving the highest number of peaks detected in each method and the largest area of chromatographic peaks.

2.2. Chemical analysis of the herbicides and their transformation products using chromatography coupled with mass spectrometry

Degradation products with low polarity were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a “CP3800” gas chromatograph system equipped with a “CP8400” autosampler and coupled with a “Saturn 2000” ion trap mass spectrometer (Varian, Les Ulis - France). The chromatographic separation was performed on a 60 m “Factor four VF-10-MS” (10% phenyl, 90% methylpolysiloxane) capillary column (internal diameter: 0.25 mm, film thickness: 0.25 µm) from Varian. The chromatographic separation and mass spectrometric analysis were performed as described by (Bouchonnet et al., 2011).

Liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF MS/MS) (2690 liquid chromatography module from Waters, Q-ToF Premier, Waters) was used for the identification of polar degradation products. Reverse phase chromatographic separations were performed using a "Pursuit XRs Ultra" column 2.8 µm C18 50 x 2.0 mm from Varian. Chromatographic and spectrometric conditions were adopted as in our previous work (Bourcier et al., 2010).

Further details on the chromatographic methods and mass spectroscopy conditions applied for both LC and GC analysis are given in Supporting Information and by Coffinet et al., 2012.

Table 1. Experimental conditions for algae, daphnia and bacteria standard ecotoxicity tests performed on chloracetamides and the mixtures of photolysis products.

Species	Test		
	Algae	Crustacean cladoceran	Bacteria
	<i>Pseudokirchneriella subcapitata</i>	<i>Daphnia magna</i>	<i>Vibrio fischeri</i>
Temperature (°C)	20	20	15
Optimal pH	7.8 ± 0.2	8.0 ± 0.3	7.0 ± 0.2
Light source	Light intensity of 10,000 lux	Darkness	-
Media volume	4 ml/ vial	25 ml/ beaker	200 µl/ vial
Organisms stocked	5·10 ³ cells/ml at start	5 animals/beaker	-
Aeration	No aeration (air exchange via a hole in the lid of the growth vial)	No aeration (beakers are covered by a watch glass)	-
Test duration	72 h	48 h	30 min
Replicates	3 with 5 control replicates	4	2
Assessment endpoint	Biomass growth rate inhibition	Daphnia immobilization	Bioluminescence inhibition
Measurement endpoint	Chlorophyll fluorescence	immobile daphnia	luminescence
International Standard Organisation Reference	ISO 8692, 2004 (ISO8692, 2004)	ISO 6341, 1996 (ISO6341, 1996)	ISO 11348-3, 1998 (ISO11348-3, 1998)

2.3. Ecotoxicity evaluation

The toxicity of the chloroacetamides and photolysis products solutions of the chloroacetamide, produced by UV treatment, was evaluated with three standardized ecotoxicity assays (ISO6341, 1996; ISO8692, 2004; ISO11348-3, 1998). A summary of experimental conditions for the algal, daphnia and bacterial methods is given in Table 1. The daphnia and algal experiments were conducted under the same temperature and lighting conditions as the culture conditions. Full details of the experimental conditions of the ecotoxicity experiments are presented in the Supplementary Information.

3. Results and discussion

3.1. Identification of the photo-degradation products

For each herbicide, the initial concentration was reduced by approximately 90% by UV irradiation in order to produce a mixture of photo degradation products. To achieve structural characterization of the photo degradation products experiments were performed with a combination of GC-MSⁿ (gas chromatography followed by multiple stage mass spectrometry) and LC-HR/MS-MS (liquid chromatography followed by high resolution tandem mass spectrometry). The first stage in the strategy of photo degradation product identification was to compare chromatograms of irradiated solutions with those of reference solutions.

In GC/MS chromatograms (as shown in figure S4), the degradation products peaks were visually distinguishable. In LC/MS chromatograms, the presence of degradation products was suspected through the appearance of a new chromatographic profile in the photolyzed solution compared to the reference solution of each chloracetamide. Thus, the extraction of the ions and their ion daughter from the total ion flow (Figure S5) was done simultaneously in both solutions as a confirmation of the fact that those compound are newly generated DPs.

As some products might not be directly visible in the chromatogram a long investigation of the chromatograms that carefully compares the chromatograms of irradiated with non irradiated solution were performed. Thirty one degradation products were detected for the three herbicides. Products that occurred at very low concentrations (relative abundance of peaks less than 1%) were not investigated further. The relative abundance and suggested structures of the photo degradation products are presented in Table 2. The methodology for characterization of degradation products is fully described in previous published studies (Bourcier et al., 2010; Bouchonnet et al., 2011; Bouchonnet et al., 2012; Souissi et al., 2012; Coffinet et al., 2012) in which we described how establishment of characteristic dissociation pathways of molecular ions permitted systematic structure elucidation of degradation products. The detailed structure elucidation of the photoproducts of metolachlor is described in Coffinet et al., 2012 and the similar identification of the same products of alachlor and acetochlor is supported in the the data and the mechanistic interpretations of the mass spectras are supported by the data shown in supporting information namely figures S6, S7 and S8 as well as tables S2 and S3.

Table 2. Molecular weight, structure and relative abundance for each chloracetamide (P1 for Acetochlor, P2 for alachlor, P3 for metolachlor) and the suggested structure of those photoproducts that occurred with >1% relative abundance in LC or GC chromatograms from samples created by UV-treatment of each chloracetamide. (R or R' = CH₃ for Alachlor, H for Acetochlor and Metolachlor, X=OCH₃ for Alachlor, OCH₂CH₃ for Acetochlor and CH₂OCH₃ for Metolachlor, X' = H for Alachlor and Acetochlor and CH₃ for Metolachlor).

	Compound	MW	Structures	RA ^a (%)		Compound	MW	Structures	RA ^a (%)		Compound	MW	Structures	RA ^a (%)
P ₁	Acetochlor	269		-	P ₂	Alachlor	269		-	P ₃	Metolachlor	283		-
A	Alachlor Acetochlor Metolachlor	251 251 265		26 7 8	B	Alachlor Acetochlor Metolachlor	235 235 -		8 4 -	C	Alachlor Acetochlor Metolachlor	191 177 -		2 3 -
D ^b	Alachlor Acetochlor Metolachlor	233 233 247		14 16+11 1+2	E ^b	Alachlor Acetochlor Metolachlor	189 175 -		5 3+3 -	F	Alachlor Acetochlor Metolachlor	189 175 -		3 4 -
G ^c	Alachlor Acetochlor Metolachlor	- - 265		- - 33+23+ 8+4+2	H	Alachlor Acetochlor Metolachlor	- - 263		- - 2	I	Alachlor Acetochlor Metolachlor	251 251 297		10 6 1
J	Alachlor Acetochlor Metolachlor	237 223 -		34 10 -	K	Alachlor Acetochlor Metolachlor	- 251 -		- 28 -	L ^d	Alachlor Acetochlor Metolachlor	- 269 -		- 5 -

^a Relative abundances for photoproducts were evaluated integrating peak areas on MH⁺ ion currents in ESI-MS (LC-MS) and CI-MS (GC-MS), ^b Two isomers are possible for Acetochlor and Metolachlor; R (H) and R' (CH₃) switch together, ^c Six isomers are possible: Hydroxylation can occur in the positions (1 to 6), ^d Two isomers are possible: the second hydroxylation can take place in positions 1 and 2.

Moreover, we assumed that the generated photoproducts retained the parent skeleton and common moieties with the original structure. Thus the presence of characteristic ion fragments facilitated the structure assignments. Hydroxylations and cyclizations (ring formations) were the main observed reactions in our study. A general scheme suggesting the main photolysis reaction pathways based on the major observed degradation products are proposed in Figure 1. The main degradation product structures suggest that irradiation initially leads to the cleavage of the C-Cl (carbon-chlorine) bond eliminating a Cl[•]-radical to form a radical of the dechlorinated chloroacetamide [**M-Cl**][•]. This radical leads to the formation of four families of compounds. The first one includes monohydroxylated compounds directly formed by the addition of a hydroxyl radical onto [**M-Cl**][•] resulting in photoproduct **A** (Figure 1; Table 2, second row) which was detected with both GC-MS and LC-MS for all three herbicides. The dechlorinated radical [**M-Cl**][•] formed from any of the chloroacetamides may also undergo cyclization before hydroxylation leading to the formation of **I**, obtained for all three pesticides, and **K** obtained only for Acetochlor. **I** loses an ethyl or a methyl radical for Alachlor and Acetochlor, respectively, to give **J**. From the bicyclic radical, an alternative reaction route was suggested involving the alpha cleavage of the alkyl substituent on the nitrogen atom leading to **F** (detected by GC-MS for Alachlor and Acetochlor). From [**M-Cl**][•], another cyclization pathway implying one of the two alkyl chains of the aromatic ring to form **D1** and **D2** isomers is observed for the three pesticides. **D1** and **D2** can either eliminate the alkyl chain on the nitrogen atom to form **E1** and **E2** for Alachlor and Acetochlor or react with water to give the hydroxylated structure **H** for Metolachlor, **L1** and **L2** for Acetochlor. Finally, [**M-Cl**][•] can react with water to give **B** which was observed for Alachlor and Acetochlor. **B** loses the alkyl substituent on the nitrogen atom to provide **C** in the case of Alachlor and Acetochlor. A hydroxyl addition also occurs onto the aromatic ring (**G1** to **G3**) and onto one of the alkyl chains (**G4** to **G6**). Except for the compounds **G1** to **G6**, all the hydroxylated degradation products are mainly observed in LC-MS due to their polarity. (Figure 1; Table 2).

Photolysis products and pathways described in this work differ from those reported in our previous work concerning ozonation of the same chloroacetamides (Bouchonnet et al., 2012). In UV induced photolysis the chlorine atom is systematically eliminated whilst during ozonation it remains in the structures of all degradation products. Moreover, photolysis mainly induces hydroxylation processes, whereas ozonation mainly induces carboxylation of chloroacetamides.

Figure 1. The suggested mechanism of chloracetamides (M) photodegradation . (R or R' = CH₃ for Alachlor, H for Acetochlor and Metolachlor, X = OCH₃ for Alachlor, OCH₂CH₃ for Acetochlor and CH₂OCH₃ for Metolachlor, X' = H for Alachlor and Acetochlor and CH₃ for Metolachlor).

3.2. Comparative toxicity of the three herbicides and their photo degradation products

Toxicity assessment of photodegradation products formed by both solar radiation and UV-treatment is a major ecotoxicity and toxicity concern as generated intermediates could exhibit either loss of toxicity or greater toxicity than the parent compounds. Recent examples in the literature have indicated increased toxicity of photolysis products of extensively used herbicides and pesticides (Osano et al., 2002; Friedman et al., 2006; Bonnet et al., 2007; Mestankova et al., 2011; Virág et al., 2007).

Experimental median effective concentration (EC_{50}) values for the three selected standard ecotoxicity assays were obtained for solutions of Metolachlor, Alachlor and Acetochlor as well as for mixtures of their photolysis products obtained by UV treatment (until 90 % of the parent chloroacetamides were degraded) and are presented in Table 2. The dose response curves behind each EC_{50} can be found in supporting information figures S1, S2 and S3. Using the calculated values of EC_{50} , a comparison can be made between the toxicity of the initial herbicides solution and the mixture of the herbicides photodegradation products. A lower EC_{50} indicates that less of a compound is needed to give the same toxic effect e.g. the formation of products more toxic than the parent compound.

Table 3. Measured fifty percent effective concentrations (EC_{50} s) of the investigated pesticides and their photolysis products with the three standard ecotoxicity assays

Species	Sample	EC_{50} ^a (µg/L)	95% Confidence Interval (µg/L)
<i>Pseudokirchneriella subcapitata</i>	Metolachlor	Parent compound	44.3
		Photolysed products	25.6
	Acetochlor	Parent compound	2.50
		Photolysed products	2.12
	Alachlor	Parent compound	6.69
		Photolysed products	2.52
<i>Daphnia magna</i>	Metolachlor	Parent compound	$22.3 \cdot 10^3$
		Photolysed products	$16.6 \cdot 10^3$
	Acetochlor	Parent compound	$6.29 \cdot 10^3$
		Photolysed products	$6.15 \cdot 10^3$
	Alachlor	Parent compound	$7.50 \cdot 10^3$
		Photolysed products	$5.36 \cdot 10^3$
<i>Vibrio fischeri</i>	Metolachlor	Parent compound	$17.1 \cdot 10^3$
		Photolysed products	$7.33 \cdot 10^3$
	Acetochlor	Parent compound	^b > $200 \cdot 10^3$
		Photolysed products	> $200 \cdot 10^3$
	Alachlor	Parent compound	$155 \cdot 10^3$
		Photolysed products	$96 \cdot 10^3$

^a EC_{50} = the median effective concentration, which is the concentration causing a response in 50% of the test parameter

^b Higher than highest tested concentration).

The variability in species sensitivity for the three chloroacetamides was high with green algae (*P. subcapitata*) being much more sensitive (lower EC_{50}) than bioluminescent bacteria (*V. fischeri*) or the crustacean (*D. magna*).

Previous studies on Metolachlor toxicity to *P. subcapitata* reported EC_{50} values ranging from 55 to 84 µg/L (Fairchild et al., 1997; Fairchild et al., 1998; Battaglinand, 2002; Kock et al., 2010), which are comparable to our obtained 72h EC_{50} of 44 µg/L. The same concurrence on experimental EC_{50} values for algae was obtained for Alachlor and Acetochlor standard samples compared to literature reported values. Our experimental EC_{50} values were 2.5 µg/L and 6.7 µg/L for Acetochlor and Alachlor, respectively, which compares with literature values of 0.5-1.4 µg/L ((Battaglinand, 2002; Belden Jason B, 2007; EFSA, 2008) and 6-12 µg/L (Fairchild et al., 1997; Fairchild et al., 1998; Battaglinand, 2002; Köck et al., 2010). EC_{50} values for the three herbicides obtained with the two other bioassays were also concurrent with reported values in the literature as presented in Table 3 and Table S1. The EC_{50} for the photolyzed samples

represent the nominal concentration of chloroacetamide. Meaning that for Metolachlor the mixture of degradation products is almost twice as toxic to algae as the parent compound alone, with a nominal concentration of Metolachlor degradation products of only 25.6 µg/L needed in comparison to 44.3 µg/L of the parent compound to cause the same 50 % inhibition of the test parameter.

The solutions of Acetochlor and UV photolysed Acetochlor had almost the same toxicity in the algae tests (table 3, Figure S2). Thus, the generated photoproducts from Acetochlor exhibit the same toxicity as Acetochlor itself. The algal bioassay for Metolachlor and Alachlor (Figure S2) and samples of resultant photolysis products showed statistically significant lower EC₅₀ values for UV-treated samples. This indicates that at least one photolysis product formed by UV-treatment from both Metolachlor and Alachlor is more toxic than the parent chloroacetamides. Theoretically, the results could also be explained by synergistic toxic effects between the degradation products and/or the parent chloroacetamides.

The standardized and extensively used *V. fischeri* based bioassay known as Microtox® was chosen to represent prokaryotic microorganisms. Cytotoxic compounds or inhibitors of any of the enzymes in the bioluminescence mechanism will result in a reduction in luminescence (Dunlap, 1999). Toxicity towards bioluminescent bacteria increased for Metolachlor and Alachlor in irradiated solutions compared to the un-irradiated samples (Table 3), which manifests as significantly lower EC₅₀ values for the UV treated herbicides compared to the two parent compounds.

It was not possible to obtain toxicity data with the bioluminescent bacteria assay for Acetochlor as the highest tested concentration (200 mg/L) did not exhibit any inhibitory effect. Contrary to this finding, Virag and co-workers (Virág et al., 2007) reported that UV degradation products of Acetochlor have a broad inhibitory effect to various soil microorganisms.

Toxicity assessments using the *D. magna* bioassay for all three chloroacetamides showed slightly lower EC₅₀ values for the UV-treated samples compared to the parent chloroacetamides. This indicates increased toxicity associated with photolysis products compared to the parent chloroacetamides. In contrast, for the daphnia assay the decreases in EC₅₀ values of photolyzed samples compared to the respective parent parent chloroacetamides were so small that the 95 % confidence intervals overlap indicating that the toxicity increase by the UV-treatment is insignificant for all three chloroacetamides.

3.3. Comparing toxicity and structures of products of the photo degradation

Comparison of toxicity development following UV treatment of chloracetamides and the determined molecular structures of photolysis products can be used to draw conclusions about toxicity of the various photodegradation products of chloroacetamides.

The most remarkable result is that all the detected photolysis products of the three chloroacetamides are dechlorinated yet still a very low EC₅₀ towards algae is retained. This indicates that the specific activity of chloroacetamides towards photosynthesizing organisms remains active when the chlorine atom is replaced with a hydroxygroup (photoproduct A). This result is very significant since it implies a high specific toxicity towards photosynthesising organisms can persist in water following UV treatment due to transformation products of the parent chloroacetamides. This phenomenon would not be revealed by LC-MS or GC-MS based

analytical methods aimed at parent chloroacetamides, as is commonly employed for monitoring purposes.

UV treatment of Acetochlor generally caused the lowest increase in toxicity observed. The explanation could be that the three most abundant photodegradation products of Acetochlor were photoproducts D, J and K (see Table 2) which are formed by cyclizations, eliminating the acetamide structure as the acetate becomes part of the formed ring. Furthermore these three most abundant photoproducts do not contain a phenolic functional group contrary to several of the photodegradation products (G and H) formed by UV treatment of Metolachlor for which the highest increase in toxicity was found.

The enhancement of toxicity for photolysis products of Metolachlor towards both algae and bacteria can be attributed to the relatively high abundance of hydroxylated congeners (G1 to G6, see RA in Table 2.) including phenolic compounds. Minor product H, which was detected only in association with Metolachlor is the only other detected phenolic photodegradation product from all three chloroacetamides.

Chloroacetamide based degradation products obtained through anodic Fenton treatment were reported to be resistant to biodegradation and to be toxic (Friedman et al., 2006). Among the major detected degradation products in the Friedman et al. study were phenols and aldehydes, to which this enhanced toxicity was attributed (Friedman et al., 2006). Toxicity of phenols includes respiratory uncoupling and polar narcosis (Shijin, 2003), whereas aldehydes exhibit electrophilic toxicity via the covalent binding to nucleophilic biological macromolecules (Dimitrov et al., 2004).

An increased toxicity of solar photolysis products of Metolachlor towards *V. fischeri* was also reported by Dimou et al (Dimou et al., 2005). These authors attributed the higher toxicity of the photolyzed sample to the formation of 2-ethyl-6-methylaniline as this photoproduct reached its highest concentration in accordance with the toxicity increase. 2-ethyl-6-methylaniline was apparently not formed by the UV-treatment procedure used in our study as it was not found however should be detectable with the applied analytical approach used.

If the increased toxicity of photoproducts from Metolachlor to both algae and bacteria can be ascribed to the phenolic photodegradation product, one could interpret that the marked increase in algal toxicity observed with Alachlor derived degradation products was caused by photoproduct A, which is primarily formed by Alachlor (Table 2 and 3). One can speculate that the specific toxicity of photoproduct A to algae occurs via the same mechanism as the parent compound since the electronegativity of a chlorine substituent is similar to a hydroxyl group in an organic molecule.

It was reported elsewhere that the decrease of aromaticity during photooxidative degradation of diuron, a substituted urea herbicide, leads to a significant decrease of toxicity (Mestankova et al., 2011). All identified photoproducts in the present study remained aromatic. Thus, the maintained toxicity may be attributed to the persistence of the aromaticity in the photodegradation products .

It was also reported that stable aniline degradation products of chloroacetamides may contribute considerably to environmental risks of pesticide application and that genotoxic effects may arise upon degradation (Osano et al., 2002). Alachlor and Metolachlor solar photodegradation products 2,6-diethylaniline and 2-ethyl-6-methylaniline, which were not

detected in this study were reported to be 20.9 and 6.3 times more acutely toxic than their corresponding parent compounds, respectively (Osano et al., 2002).

4. Conclusion

This work investigated the formation of photoproducts of chloroacetamides that may occur in connection with the use of UV for disinfection of water.

It was found that UV-treatment of chloroacetamides generally produces dechlorinated photodegradation products which were hydroxylated and/or formed through cyclization processes eliminating the acetamide structure.

The mixture of photoproducts formed by UV treatment of each of the three investigated chloroacetamide herbicides; acetochlor, alachlor and metolachlor, were generally found to be more toxic in all three ecotoxicity assays used, but to different degrees and not all at a statistically significant level.

This increase of toxicity can be ascribed to the formation of UV-degradation products of a more toxic nature than the parent compounds. The hydroxyl group which replaces the lost chlorine atom during photolysis seems to enhance toxicity by substitution into the same position of the molecule or substitution into the aromatic ring to form phenolic degradation products.

The high specific toxicity of photodegradation products towards photosynthesising organisms suggests their inclusion in monitoring methods for chloroacetamides together with their parent structures in order to give a more complete picture of contamination levels and risks of toxicity.

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Supporting Information

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References

1. Chemical analysis conditions

1-1- GC-MS experimental conditions:

All experiments were carried out by automatically injecting 1.0 μL of sample in the splitless mode at a rate of 50 $\mu\text{L}\cdot\text{s}^{-1}$. Helium gas was used as a carrier at a flow rate of 1.4 $\text{mL}\cdot\text{min}^{-1}$. The oven temperature started at 50 $^{\circ}\text{C}$, was held for 0.5 min, then increased incrementally 10 $^{\circ}\text{C}\cdot\text{min}^{-1}$ up to 320 $^{\circ}\text{C}$ where it was held for 7.5 min. The total duration of GC analysis was 35 min. The manifold, ion trap electrodes and transfer line temperatures were set at 120 $^{\circ}\text{C}$, 220 $^{\circ}\text{C}$ and 300 $^{\circ}\text{C}$, respectively. Calibration of the mass spectrometer was automatically performed using ions resulting from electron ionization (EI) of perfluorotributylamine. Chemical ionization (CI) experiments were carried out using methanol as the reagent gas. Spectra were recorded using the automatic gain control (AGC) function with a target value of 20.000 in EI, 5000 in CI. In all experiments emission current was set to 10 μA and the electron multiplier voltage to 1850 V. Full scan mass spectra were recorded on a scanning range from m/z 50 to m/z 450. An isolation window of one mass unit was employed for multiple stage mass spectrometry (MS^n) experiments. In MS^n , precursor ions were stored with a q_z stability parameter of 0.30, and fragmented by collision induced dissociation with different activation energy values ranging from 0.2 to 1.0 V using the resonant excitation mode.

1-2- LC-MS experimental conditions:

The CH_3CN + formic acid (0.1%) (A) / H_2O + formic acid (0.1%) (B) gradient used was the following: 40% A / 60% B from 0 to 11.0 min, 100% A from 11.1 to 20.0 min and 40% A / 60% B from 20.1 min to 30.0 min. A constant flow of 0.2 $\text{mL}\cdot\text{min}^{-1}$ was used. The volume of injected samples was 2 μL . The electrospray interface was operated under the following conditions: capillary voltage 2.6 kV, cone voltage 20 V, source temperature 100 $^{\circ}\text{C}$, desolvation temperature 450 $^{\circ}\text{C}$, and collision energy 5 and 15 eV. Accurate mass measurements (four decimal places) were carried out at a resolution higher than 5000 (full width at half maximum) using an independent reference spray (phosphoric acid) via the Lockspray interface to ensure accuracy. For accurate mass measurements, analyses were acquired using W-mode and using an independent reference spray via the LockSpray interface to ensure accuracy. Phosphoric acid was used as the lock mass at a flow rate of 10 $\mu\text{L}\cdot\text{min}^{-1}$. The LockSpray frequency was set at 10 s and data for the reference compounds were averaged over 10 spectra min^{-1} . The accurate mass and the elemental composition for all ions are obtained using instrument software MassLynx. The ions used for the mass correction were m/z 294.9685 and 196.9616 depending on the ions. All the measured mass were determined with an error < 5ppm.

2. Ecotoxicity bioassays conditions

2-1- *Daphnia magna* acute immobilization tests

This aquatic animal is extensively used as a test organism in aquatic toxicology due to their small size, short life cycle and amenability to lab culture. Static acute 24–48 h assays were conducted according to internationally accepted Standard Methods (ISO6341, 1996). A healthy culture of *Daphnia magna* is used as the source of mother organism. The day before the start of the test, the mother cultures were cleaned up and all animals except the mother animals were removed. Thus, the neonates that were born in the night and were less than 24h were used for the test. Immobility at 24 h and 48 h is the bioassay endpoint, assumed to be equivalent to mortality. In each test a control and 5 (or 6) test concentrations were included in each sample. Each concentration was tested using four replicate vessels with 5 neonates per replicate. *Daphnia* neonates exposed to the control and different concentrations were incubated in darkness at 20°C. After 24 h and 48 h of exposure the number of immobilized organisms were determined. The EC50 is determined as the concentrations producing 50% of immobilization as described below.

2-2- *Pseudokirchneriella subcapitata* growth inhibition test

A growth inhibition test using the green algae *Pseudokirchneriella subcapitata* was performed according to ISO 8692 (ISO8692, 2004). A series of dilutions of parent compound and photolyzed solution samples were prepared. 10-ml algal cultures in 20-ml growth vials capped with lids were used with a small hole drilled to allow equilibration with atmospheric CO₂ while minimizing any volatilization losses from solution. The sample dilutions and the controls were inoculated with the microalgae from the concentrated suspension to obtain 5-10·10³ cells/mL as the start algae density. Tests were conducted in triplicate with six control replicates and a pH of approximately 7.8. The samples were incubated for 72 h at 20°C, with a continuous illumination of 10,000 lux from fluorescent tubes. The algal biomass was measured by fluorescence on acetone extracts of samples taken immediately after addition of the algae (0 h), after 24, 48 and 72 h of exposure with a Hitachi F-2000 Fluorescence Spectrophotometer (Hitachi, Japan) with excitation light at 430 nm and emission light at 671 nm. Samples from the vials were extracted with acetone 24 h prior to the fluorescence measurements. The fluorescence was used directly for calculation of growth rates in each vial.

2-3- Bioluminescence inhibition test of *Vibrio fischeri*

The experimental procedure for conducting the bacterial bioluminescence assay is based on the ISO protocol (ISO11348-3, 1998). The analysis was carried out with all dilution and reagents maintained at 15°C. A working solution of luminescent bacteria was prepared by reconstituting a vial of frozen lyophilized *Vibrio fischeri* cells using 12.5 ml of the reagent diluent provided by the manufacturer. The reconstituted solution was equilibrated for a minimum of 30 minutes at 4°C. The hydrated cell suspension is usable for several hours when kept chilled. The osmolality is adjusted in order to obtain 2% w/v NaCl in each solution or sample. Bacterial reagents are reconstituted just prior to the analysis and the pre-incubation times follow standard protocols. In all measures, the percent of inhibition (% I) was determined by comparing the relative response

of the control to that of the diluted sample. Each dilution was tested in duplicate and performed at 15°C.

A dilution series of the samples to be analysed were prepared in sodium chloride solution (2% NaCl). Different concentration intervals were used for the tested chemicals depending on the expected EC50. For the photolyzed solutions, a larger concentration range was adopted as the EC50 values were unknown. The sample pH values were approximately 7. A fixed amount of bacteria (100 µl of the reconstituted cell suspension) was added to the dilution vials. Luminescence was measured at time zero (before addition of test solution) and then after 5, 15 and 30 min and compared to the measured value of a bacterial control. The concentration of toxicants in the test which causes a 50% inhibition of bioluminescence after exposure for 5, 15 or 30 min was designated as the EC50 value. The measurements of bioluminescence were made using the luminometer Luminoskan TL Plus (Thermo lab system).

2-4- Statistical analysis

Due to the different nature of the endpoints (continued responses in algal and biotox tests and quantal responses in the *Daphnia* test), the ecotoxicity test results were treated with different statistical methods.

Specific algal growth rates were calculated from the exponential increase in cell density in each replicate using the standard formula shown in ISO 8692 (ISO8692, 2004) assuming exponential growth during the 72-h duration of the assays. The inhibition of growth rates are calculated as percentages of the mean growth rate of the controls and plotted against the logarithm of the concentration. Further data treatment and calculation of log-norm and Weibull concentration–response curves and estimation of EC50 and EC10 with 95% confidence intervals were carried out on a Windows-based computer program (Christensen et al., 2009).

The relative inhibition of *Vibrio fischeri* bioassays were determined using equations (1) and (2) corresponding respectively to the correction factor for each measuring time (KF5, KF15 and KF30) and the relative response (REL% xmin). The dose–response curves exhibiting the bioluminescence inhibition were generated by the same statistical program as the algae growth inhibition. Further treatment of data was performed with the program previously mentioned (Christensen et al., 2009).

$$KF_x = IC_x / IC_0 \quad (1)$$

where IC_x = Average luminescence of control samples after contact time (x min)

$$REL\% \text{ xmin} = [IT_x / KF_x \times IT_0] \times 100 \quad (2)$$

where IT_x = Luminescence intensity of test sample after contact time *Daphnia magna* toxicity assay results were analyzed statistically with a probit procedure using Toxcalc® (version 5.0, ToxCalc. 2001. Tidepool Scientific, LLC. McKinleyville, CA, USA). Median effective concentrations and 95% fiducial limits as well as probit values were calculated.

Tables and Figures related to exotoxicity

Table S1 Literature survey of fifty percent effective concentration EC₅₀s of the investigated herbicides with the three used ecotoxicity tests

	Alachlor		Acetochlor		Metolachlor	
Bioassay	EC 50	Reference	EC 50	Reference	EC 50	Reference
<i>Pseudokirchneriella subcapitata</i> EC ₅₀ µg/L	10	Köck et al., 2010	0.52	EFSA, 2008	55	Köck et al., 2010
	6 4-9	Fairchild et al., 1997	1.4	Belden Jason B 2007	57	Battaglinand , 2002
	5.9	Battaglinand 2002	1.43	Battaglinand, 2002	84 72-95	Fairchild et al., 1998
	10	Fairchild et al., 1998	2.50	Own results	44.3	Own results
	6.69	Own results				
<i>Daphnia magna</i> EC ₅₀ mg/L	10	Köck et al., 2010	7.4	EFSA, 2008	23.5	Köck et al., 2010
	7.50	Own results	8.2	Belden Jason B 2007	22.3	Own results
			6.29	Own results		
<i>Vibrio Fischeri</i> EC ₅₀ 15 min mg/L	160	Köck et al., 2010			17	
	206±39	Bonnet et al., 2007				Köck et al., 2010
	105.4	Lapertot et al., 2008		NA*	17.1	Own results
	155	Own results				

*NA: Not Available

Figure S1 Response-dose curves obtained for the three herbicides and their photodegradates obtained with *Daphnia magna* toxicity test. Line in blue indicate the upper and lower 95% fiducial limits, line in black represents the optimal fit.

Effect curve fit : Metolchlor Reference Solution

Effect curve fit : Acetochlor Reference Solution

Effect curve fit : Alachlor Reference solution

Effect curve fit : Metolchlor Photolyzed Solution

Effect curve fit : Acetochlor Photolyzed Solution

Effect curve fit : Alachlor Photolyzed solution

Figure S2 Response-dose curves and confidence intervals obtained for the three herbicides and their photodegradates obtained with *Pseudokirchneriella subcapitata* growth inhibition test.

Figure S3 Response-dose curves and confidence intervals for reference and photolyzed metolachlor and alachlor solutions with *Vibrio fischeri* bioluminescence inhibition test.

Details on structure determination of photolysis products

Figure S4: Chromatograms of photolysed and non-photolyzed Alachlor (A) and acetochlor (B) solutions obtained in GC-MS analysis. The similar chromatograms on metolachlor is published in Coffinet et al., 2012.

Figure S5: Chromatograms obtained in LC-MS experiments: Extracted photoproducts and acetochlor ions chromatograms for (a) acetochlor reference solution (b) irradiated (5 min) acetochlor and (c) irradiated (60 min) acetochlor solution.

Figure S6: Structure of deuterated alachlor and acetochlor showing the positions that were deuterated.

Figure S7: Fragmentation pathway for compound A in LC-MS.

Compound E

Figure S8: The fragmentation pathway of compounds E(left) and F(right; Ala m/z 189 and Ace m/z 175) established on the basis of main MS^n transitions for the degradation products and their analogues from the deuterated pesticides. The m/z of the corresponding deuterated analogues used as one of the tools to elucidate the structures is indicated in parenthesis.

Table S2: GC/MS Molecular weight, retention time, chemical structure and main MSⁿ transition obtained by GC-MS in chemical ionization (CI) and electron ionization (EI) for alachlor photoproducts with the shift in masses measured on the deuterated analogue indicated.

	Molecular weight	Retention time (min)	Shift Ala-d ₁₃	Principale transitions in CI-MS ⁿ (m/z)	Principale transitions in CI-MS ⁿ (m/z) Ala-D ₁₃	Principale transitions in EI-MS ⁿ (m/z)	Principale transitions in EI-MS ⁿ (m/z) Ala-D ₁₃	Structure
<u>A</u>	251	27,3	13	252 → 234 (-18) 252 → 220 (-32) 220 → 162 (-58) 220 → 188 (-32)	265 → 247 (-18) 265 → 233 (-32) 233 → 175 (-58) 233 → 200 (-33)	251 → 219 (-32) 219 → 161 (-58)	264 → 232 (-32) 232 → 174 (-58)
<u>B</u>	235	24,8	13	236 → 204 (-32) 204 → 162 (-42) 162 → 134 (-28)	249 → 217 (-32) 217 → 175 (-42) 175 → 142 (-33)	235 → 203 (-32) 203 → 160 (-43)	248 → 215 (-33) 215 → 172 (-43)
<u>C</u>	191	23,5	13	192 → 150 (-42) 192 → 164 (-28) 192 → 174 (-18)	205 → 163 (-42) 205 → 173 (-32) 205 → 187 (-18)	191 → 176 (-15) 176 → 148 (-28) 148 → 120 (-28) 191 → 162 (-29) 191 → 149 (-42) 149 → 120 (-29) 149 → 134 (-15)	204 → 189 (-15) 189 → 161 (-28) 161 → 129 (-32) 204 → 170 (-34) 204 → 162 (-42) 162 → 128 (-34) 162 → 144 (-18)
<u>D</u>	233	25,1	12	234 → 202 (-32) 202 → 160 (-42) 202 → 174 (-28) 174 → 146 (-28) 160 → 132 (-28)	246 → 214 (-32) 214 → 172 (-42) 214 → 186 (-28) 186 → 154 (-32) 172 → 140 (-32)	233 → 202 (-31) 202 → 160 (-42)	245 → 214 (-31) 214 → 172 (-42)
<u>E</u>	189	25,9	12	190 → 174 (-16) 174 → 146 (-28) 190 → 160 (-30) 160 → 132 (-28)	202 → 183 (-19) 183 → 155 (-28) 202 → 167 (-35) 167 → 139 (-28)	189 → 160 (-29) 189 → 174 (-15) 174 → 146 (-28) 189 → 161 (-28)	201 → 167 (-34) 201 → 183 (-18) 183 → 155 (-28) 201 → 173 (-28)
<u>F</u>	189	24,3	13	190 → 174 (-16) 174 → 132 (-42) 190 → 148 (-42)	203 → 184 (-19) 184 → 142 (-42) 203 → 161 (-42)	189 → 160 (-29) 160 → 132 (-28) 189 → 147 (-42)	202 → 168 (-34) 168 → 136 (-32) 202 → 160 (-42)

Table S3: Molecular weight, retention time, chemical structure and main daughter ions for the degradation product for alachlor in LC-MS.

Compound	Retention Time (min)	MH ⁺ m/z	Shift in u.m.a (Ala-D13)	Experimental mass	Theoretical mass	Molecular formula	Error (mDa)	Error (PPM)	Main daughter ions (CID)
A	3,4	252	+13	252.1571	252.1600	C ₁₄ H ₂₂ NO ₃	-2,9	-11,5	252-220-222-190-162
B	4,8	236	+13	236.1638	236.1651	C ₁₄ H ₂₂ NO ₂	-1,3	-5,5	236-204-162
D	2,9	234	+12	234.1488	234.1494	C ₁₄ H ₂₀ NO ₂	-0,6	-2,6	234-202-174
E	6	190	+12	190.1248	190.1232	C ₁₂ H ₁₆ NO	1,6	8,4	190-172-148

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Supporting Information

**Identification and ecotoxicity of degradation products of
chloroacetamide herbicides from UV-treatment of water**

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1. Chemical analysis conditions

1-1- GC-MS experimental conditions:

All experiments were carried out by automatically injecting 1.0 μL of sample in the splitless mode at a rate of 50 $\mu\text{L}\cdot\text{s}^{-1}$. Helium gas was used as a carrier at a flow rate of 1.4 $\text{mL}\cdot\text{min}^{-1}$. The oven temperature started at 50 $^{\circ}\text{C}$, was held for 0.5 min, then increased incrementally 10 $^{\circ}\text{C}\cdot\text{min}^{-1}$ up to 320 $^{\circ}\text{C}$ where it was held for 7.5 min. The total duration of GC analysis was 35 min. The manifold, ion trap electrodes and transfer line temperatures were set at 120 $^{\circ}\text{C}$, 220 $^{\circ}\text{C}$ and 300 $^{\circ}\text{C}$, respectively. Calibration of the mass spectrometer was automatically performed using ions resulting from electron ionization (EI) of perfluorotributylamine. Chemical ionization (CI) experiments were carried out using methanol as the reagent gas. Spectra were recorded using the automatic gain control (AGC) function with a target value of 20.000 in EI, 5000 in CI. In all experiments emission current was set to 10 μA and the electron multiplier voltage to 1850 V. Full scan mass spectra were recorded on a scanning range from m/z 50 to m/z 450. An isolation window of one mass unit was employed for multiple stage mass spectrometry (MS^n) experiments. In MS^n , precursor ions were stored with a q_z stability parameter of 0.30, and fragmented by collision induced dissociation with different activation energy values ranging from 0.2 to 1.0 V using the resonant excitation mode.

1-2- LC-MS experimental conditions:

The CH_3CN + formic acid (0.1%) (A) / H_2O + formic acid (0.1%) (B) gradient used was the following: 40% A / 60% B from 0 to 11.0 min, 100% A from 11.1 to 20.0 min and 40% A / 60% B from 20.1 min to 30.0 min. A constant flow of 0.2 $\text{mL}\cdot\text{min}^{-1}$ was used. The volume of injected samples was 2 μL . The electrospray interface was operated under the following conditions: capillary voltage 2.6 kV, cone voltage 20 V, source temperature 100 $^{\circ}\text{C}$, desolvation temperature 450 $^{\circ}\text{C}$, and collision energy 5 and 15 eV. Accurate mass measurements (four decimal places) were carried out at a resolution higher than 5000 (full width at half maximum) using an independent reference spray (phosphoric acid) via the LockSpray interference to ensure accuracy. The accurate mass and the elemental composition for all ions are obtained using instrument software MassLynx. The ions used for the mass correction were m/z 392.9154, m/z 294.9385 and m/z 196.9616.

2. *Ecotoxicity bioassays conditions*

2-1- *Daphnia magna* acute immobilization tests

This aquatic animal is extensively used as a test organism in aquatic toxicology due to their small size, short life cycle and amenability to lab culture. Static acute 24–48 h assays were conducted according to internationally accepted Standard Methods (ISO6341, 1996). A healthy culture of *Daphnia magna* is used as the source of mother organism. The day before the start of the test, the mother cultures were cleaned up and all animals except the mother animals were removed. Thus, the neonates that were born in the night and were less than 24h were used for the test. Immobility at 24 h and 48 h is the bioassay endpoint, assumed to be equivalent to mortality. In each test a control and 5 (or 6) test concentrations were included in each sample. Each concentration was tested using four replicate vessels with 5 neonates per replicate. *Daphnia* neonates exposed to the control and different concentrations were incubated in darkness at 20°C. After 24 h and 48 h of exposure the number of immobilized organisms were determined. The EC50 is determined as the concentrations producing 50% of immobilization as described below.

2-2- *Pseudokirchneriella subcapitata* growth inhibition test

A growth inhibition test using the green algae *Pseudokirchneriella subcapitata* was performed according to ISO 8692 (ISO8692, 2004). A series of dilutions of parent compound and photolyzed solution samples were prepared. 10-ml algal cultures in 20-ml growth vials capped with lids were used with a small hole drilled to allow equilibration with atmospheric CO₂ while minimizing any volatilization losses from solution. The sample dilutions and the controls were inoculated with the microalgae from the concentrated suspension to obtain 5-10·10³ cells/mL as the start algae density. Tests were conducted in triplicate with six control replicates and a pH of approximately 7.8. The samples were incubated for 72 h at 20°C, with a continuous illumination of 10,000 lux from fluorescent tubes. The algal biomass was measured by fluorescence on acetone extracts of samples taken immediately after addition of the algae (0 h), after 24, 48 and 72 h of exposure with a Hitachi F-2000 Fluorescence Spectrophotometer (Hitachi, Japan) with excitation light at 430 nm and emission light at 671 nm. Samples from the vials were extracted with acetone 24 h prior to the fluorescence measurements. The fluorescence was used directly for calculation of growth rates in each vial.

2-3- Bioluminescence inhibition test of *Vibrio fischeri*

The experimental procedure for conducting the bacterial bioluminescence assay is based on the ISO protocol (ISO11348-3, 1998). The analysis was carried out with all dilution and reagents maintained at 15°C. A working solution of luminescent bacteria was prepared by reconstituting a vial of frozen lyophilized *Vibrio fischeri* cells using 12.5 ml of the reagent diluent provided by the manufacturer. The reconstituted solution was equilibrated for a minimum of 30 minutes at 4°C. The hydrated cell suspension is usable for several hours when kept chilled. The osmolality is adjusted in order to obtain 2% w/v NaCl in each solution or sample. Bacterial reagents are reconstituted just prior to the analysis and the pre-incubation times follow standard protocols. In all measures, the percent of inhibition (% I) was determined by comparing the relative response of the control to that of the diluted sample. Each dilution was tested in duplicate and performed at 15°C.

A dilution series of the samples to be analysed were prepared in sodium chloride solution (2% NaCl). Different concentration intervals were used for the tested chemicals depending on the expected EC50. For the photolyzed solutions, a larger concentration range was adopted as the EC50 values were unknown. The sample pH values were approximately 7. A fixed amount of bacteria (100 µl of the reconstituted cell suspension) was added to the dilution vials. Luminescence was measured at time zero (before addition of test solution) and then after 5, 15 and 30 min and compared to the measured value of a bacterial control. The concentration of toxicants in the test which causes a 50% inhibition of bioluminescence after exposure for 5, 15 or 30 min was designated as the EC50 value. The measurements of bioluminescence were made using the luminometer Luminoskan TL Plus (Thermo lab system).

2-4- Statistical analysis

Due to the different nature of the endpoints (continued responses in algal and biotox tests and quantal responses in the *Daphnia* test), the ecotoxicity test results were treated with different statistical methods.

Specific algal growth rates were calculated from the exponential increase in cell density in each replicate using the standard formula shown in ISO 8692 (ISO8692, 2004) assuming exponential growth during the 72-h duration of the assays. The inhibition of growth rates are calculated as percentages of the mean growth rate of the controls and plotted against the logarithm of the concentration. Further data treatment and calculation of log-norm and Weibull concentration–

130 response curves and estimation of EC50 and EC10 with 95% confidence intervals were carried
131 out on a Windows-based computer program (Christensen et al., 2009).

132 The relative inhibition of *Vibrio fischeri* bioassays were determined using equations (1) and (2)
133 corresponding respectively to the correction factor for each measuring time (KF5, KF15 and
134 KF30) and the relative response (REL% xmin). The dose–response curves exhibiting the
135 bioluminescence inhibition were generated by the same statistical program as the algae growth
136 inhibition. Further treatment of data was performed with the program previously mentioned
137 (Christensen et al., 2009).

$$138 \qquad \qquad \qquad KF_x = IC_x / IC_0 \qquad \qquad \qquad (1)$$

139 where ICx = Average luminescence of control samples after contact time (x min)

$$140 \qquad \qquad \qquad REL\% \text{ xmin} = [IT_x / KF_x \times IT_0] \times 100 \qquad \qquad \qquad (2)$$

141 where ITx = Luminescence intensity of test sample after contact time *Daphnia magna* toxicity
142 assay results were analyzed statistically with a probit procedure using Toxcalc® (version 5.0,
143 ToxCalc. 2001. Tidepool Scientific, LLC. McKinleyville, CA, USA). Median effective
144 concentrations and 95% fiducial limits as well as probit values were calculated.

145

Tables and Figures

Table S1 Literature survey of fifty percent effective concentration EC₅₀s of the investigated herbicides with the three used ecotoxicity tests

Bioassay	Alachlor		Acetochlor		Metolachlor	
	EC 50	Reference	EC 50	Reference	EC 50	Reference
<i>Pseudokirchneriella subcapitata</i> EC ₅₀ µg/L	10	Köck et al., 2010	0.52	EFSA, 2008	55	Köck et al., 2010
	6.4-9	Fairchild et al., 1997	1.4	Belden Jason B 2007	57	Battaglinand, 2002
	5.9	Battaglinand 2002	1.43	Battaglinand, 2002	84.72-95	Fairchild et al., 1998
	10	Fairchild et al., 1998	2.50	Own results	44.3	Own results
	6.69	Own results				
<i>Daphnia magna</i> EC ₅₀ mg/L	10	Köck et al., 2010	7.4	EFSA, 2008	23.5	Köck et al., 2010
	7.50	Own results	8.2	Belden Jason B 2007	22.3	Own results
			6.29	Own results		
<i>Vibrio Fischeri</i> EC ₅₀ 15 min mg/L	160	Köck et al., 2010				
	206±39	Bonnet et al., 2007			17	Köck et al., 2010
	105.4	Lapertot et al., 2008		NA*	17.1	Own results
	155	Own results				

*NA: Not Available

150

151 Figure S1 Response-dose curves obtained for the three herbicides and their photodegradates obtained with *Daphnia magna* toxicity test. Line in blue

152 indicate the upper and lower 95% Fiducial limits, line in black represents the optimal fit.

Effect curve fit : Metolchlor Reference Solution

Effect curve fit : Acetochlor Reference Solution

Effect curve fit : Alachlor Reference solution

Effect curve fit : Metolchlor Photolyzed Solution

Effect curve fit : Acetochlor Photolyzed Solution

Effect curve fit : Alachlor Photolyzed solution

155 **Figure S2** Response-dose curves and confidence intervals obtained for the three herbicides and their photodegradates obtained
 156 with *Pseudokirchneriella subcapitata* growth inhibition test.

Figure S3 Response-dose curves and confidence intervals for reference and photolyzed metolachlor and alachlor solutions with *Vibrio fischeri* bioluminescence inhibition test.

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