# Ecotoxicity of carbamazepine and its UV photolysis transformation products

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 **ABSTRACT**
Carbamazepine, an anti-epileptic pharmaceutical agent commonly found in wastewater, is highly recalcitrant to standard wastewater treatment practices. This study investigated the mixture toxicity of carbamazepine transformation products formed during UV photolysis using three standard ecotoxicity assays (representing bacteria, algae and crustaceans). UV-treatment of 6 mg L$^{-1}$ carbamazepine solution was carried out over a 120 min period and samples were removed periodically over the course of the experiment. Quantification results confirmed the degradation of carbamazepine throughout the treatment period, together with concurrent increases in acridine and acridone concentrations. Ecotoxicity was shown to increase in parallel with carbamazepine degradation indicating that the mixture of degradation products formed was more toxic than the parent compound. In fact, ecotoxicity was still greater than 60 % for all three endpoints even when the carbamazepine concentration had decreased to < 1 % of the starting concentration, and acridine and acridone had decreased to < 10 % of their maximum measured concentrations. Single compound toxicity testing also confirmed the higher toxicity of measured degradation products relative to the parent compound. These results show that transformation products considerably more toxic than carbamazepine itself are likely to be produced during UV treatment of wastewater effluents and/or photo-induced degradation of carbamazepine in natural waters. This study highlights the need to consider mixture toxicity and the formation and persistence of toxicologically relevant transformation products when assessing the environmental risks posed by pharmaceutical compounds.

## INTRODUCTION

There are large knowledge gaps concerning the environmental fate and effects of most active pharmaceutical ingredients (APIs). For regulators, this translates to a high degree of uncertainty about the risks associated with pharmaceutically derived contaminants in the environment (Kümmerer, 2009a, 2009b). The quantities of APIs released to the environment may be relatively low in comparison with other types of pollutants such as pesticides and industrial pollutants, but there is a tendency for their environmental release to be continuous (via wastewater effluents) and the potential for environmental accumulation and/or chronic ecotoxicity has thus been noted (e.g. Besse and Garric, 2008; Fent et al., 2006; Ferrari et al., 2003; Hernando et al., 2006; Escher et al., 2011).

Carbamazepine, an antiepileptic pharmaceutical compound and mood stabilising drug, has attracted particular attention in recent years due to its widespread detection in municipal wastewaters (e.g. Ternes, 1998; Ollers et al., 2001, Falås et al., 2012), surface waters (e.g. Ternes, 1998, Metcalfe et al., 2003; Andreozzi et al., 2003), and drinking waters (e.g. Stackelberg et al., 2004, 2007; Togola and Budzinski, 2008; Heberer et al., 2004). It is also noted to be highly resistant to biodegradation and thus highly recalcitrant under standard biological wastewater treatment conditions (Clara et al., 2005; Kosjek et al., 2009, Falås et al., 2012). Under these circumstances, advanced oxidation processes (AOPs) may offer additional treatment value (von Gunten, 2003; Huber et al., 2005; Macova et al., 2010; Kosjek et al., 2011, Hey et al., 2012; Keen et al., 2012), however complete oxidation and mineralisation is not always achievable and stable transformation products formed during AOPs may also pose environmental risks (Aguerra et al (2005). The stability and ecotoxicity of transformation products formed during treatment and environmental transport are of major importance when assessing the suitability of water treatment options and in determining environmental risks associated with APIs in the environment.

This paper presents the results of research investigating the ecotoxicity of carbamazepine and two of its UV transformation products (Kosjek, 2007), acridine and 9(10H)-acridone. In addition to single compound testing of these compounds using three different standard test organisms (bacteria, algae, and crustacean), an experiment was also conducted to investigate the changes in ecotoxicity occurring during UV treatment of water spiked with 6 mg $L^{-1}$ carbamazepine. This experiment, conducted using a bench-top circulating flow UV-reactor system, was designed to compare the ecotoxicity of the initial carbamazepine-spiked solution with that of samples collected during the treatment process. These samples contained mixtures of carbamazepine and its UV-transformation products.

This investigation into the relative toxicity of carbamazepine and its phototransformation products is relevant to future discussions regarding the treatment, control and fate of carbamazepine and carbamazepine-derived contaminants in the environment. Knowledge about carbamazepine's UV-induced degradation pathway is also of interest from the perspective of sunlight-induced photochemical oxidation, as this may be a significant process controlling the gradual ongoing breakdown of carbamazepine released to receiving waters and natural aquatic environments. Indeed, for non biodegradable, non-sorbing APIs such as carbamazepine, sun-induced photodegradation may be the most important long-term process affecting persistence and toxicity in the environment.

This study provides an example of the 'effect-driven approach' for determining the risks associated with pollutant transformation products. The 'effect driven approach', described by Escher and Fenner (2011) in their paper on advances in environmental risk assessment of transformation products, focuses on ecotoxicity testing of reaction mixtures in which a parent compound is undergoing degradation (e.g. during UV-treatment). This approach facilitates prioritisation of APIs and their transformation products for risk assessment purposes without requiring the prior identification and quantification of individual transformation products. In 'effect driven' assessments, compounds are prioritised for transformation product identification and further study if the reaction mixture increases in toxicity in parallel with decreasing parent compound concentration.

## 1. METHODS
### 2.1 Standards, Solvents, and Other Chemicals.
Carbamazepine (99%, CAS 298-46-4) and N-Methyl-N- [tert-butyldimethyl-silyl]trifluoroacetimide (MTBSTFA) were purchased from Acros Organics. Acridine (97%, CAS 260-94-6) and 9(10H)-acridone (99%, CAS 578-95-0) were obtained from Sigma-Aldrich.

### 2.2 Chemical analyses
Solid phase extraction (SPE) was used for sample preparation for all chemical analyses. Concentrated solutions (i.e. stock solutions and selected concentration check samples from the acute toxicity test dilution series) were initially diluted as appropriate (dilution factors ranged from 17 to 600) before loading at neutral pH onto Oasis® HLB reversed-phase sorbent SPE cartridges (Waters, Corp., Milford, MA, USA). These cartridges were also used for preconcentration of the 150 mL subsamples taken throughout the carbamazepine UV-treatment experiment. SPE cartridges were conditioned with 3 mL of ethyl acetate, 3 mL methanol, and 3 mL of tap water. Sample aliquots were loaded on the SPE columns at a flow-rate of 4–5 mL min$^{-1}$. Each cartridge was then washed with water (3 mL), dried for 30 min under vacuum and eluted with 1 mL acetone, 1 mL of 7/3 ethyl acetate / acetone mixture and 1 mL of ethyl acetate. The combined eluant was evaporated to dryness under a gentle nitrogen stream and reconstituted with 0.5 mL ethyl acetate.

Acridone and carbamazepine were transformed into the tert-butyldimethyl-silyl ether derivatives by adding 30 μL MTBSTFA and maintaining the samples at 60°C for 12 hours. A Varian 3800 gas chromatograph (GC) interfaced with an ion trap Saturn 2000 mass spectrometer (MS) was used for analysis. 10 μL samples were injected (split-splitless) using a PTV injector at 80 °C for 0.30 min before being increased by 200°C/min to 300°C and held for 5 min. A Zebron ZB-5 HT INFERNO 30 m × 0.25 mm × 0.25 μm (Phenomenex) column was used for separation. The GC oven temperature programme was originally held at 80°C for 1 min, then increased by 25°C/min to 225°C and held for 1 min; increased by 1°C/min to 231°C; increased by 10°C/min to 280°C; increased by 45°C/min to 320°C and held at 320°C for a further 3 min. The total runtime was 22.59 min. The mass analyser was operated in electron ionisation (EI) mode, and the following fragment ions were monitored for quantitation: *m/z* 179 for acridine, and *m/z* 252 and *m/z* 193 for the acridone-MTBS and carbamazepine-MTBS derivatives respectively.

For each sample, two parallel subsamples were extracted, derivatised and analysed according to the above protocol. Furthermore, each of these subsamples was injected and analysed twice, with the mean value taken as the relevant concentration. Method performance analyses showed satisfactory linearity ($r^2 > 0.98$) and good repeatability for all three analytes. Blank control samples were also prepared and analysed.

**2.3 Ecotoxicity testing**

Three internationally standardised aquatic ecotoxicity tests (Table 1) were used to investigate the ecotoxicity of carbamazepine, acridine, and acridone. All measurements were conducted in triplicate. The selected ecotoxicity tests used organisms from different trophic levels and included the following short-term toxicity tests:

- inhibition of bioluminescence in the marine bacterium *Vibrio fischeri* (Biotox testing kit; ISO 11348-3, 1998);
- growth inhibition of the green algae *Pseudokirchneriella subcapitata* (ISO 8692, 2004);
- immobilisation of the crustacean *Daphnia magna* Straus (Cladocera, Crustacea) (ISO 6341, 1996).

Table 1: Summary of experimental conditions for bacteria, algae, and daphnia ecotoxicity assays

| | Bacteria | Algae | Cladoceran |
|---|---|---|---|
| *Test species* | *Vibrio fischeri (Photobacterium phosphoreum)* | *Pseudokirchneriella subcapitata (Selenastrum capricornatum)* | *Daphnia magna* |
| *Temperature (°C)* | 15 | 20 | 20 |
| *Optimal pH* | $7.0 \pm 0.2$ | $7.8 \pm 0.2$ | $8 \pm 0.3$ |
| *Light source* | - | Light intensity of 10,000 lux supplied by cool white fluorescent tubes | Darkness |
| *Media volume* | 200 μl/ vial | 4 ml/ vial | 50 ml/ vial |
| *Number of organisms exposed* | Variable | $5\text{-}10 \cdot 10^3$ cells/ml | 5 juvenile *Daphnia* per beaker |
| *Aeration* | Test vial is left uncovered during the test | Constant air exchange through a hole in the lid of each growth vial | Limited aeration (beakers are covered by a watch glass during the test) |
| *Test duration* | 30 min | 48h | 48h |
| *Replicates* | 3 - 6 | 3 - 6 | 2 - 3 |
| *Assessment endpoint* | Inhibition of bioluminescence | Biomass growth | *Daphnia* immobilisation |
| *Measurement endpoint* | Luminescence | Chlorophyll fluorescence | Number of immobilised daphnia |
| *International Standard Organisation (ISO) Reference* | ISO 11348-3, 1998 | ISO 8692, 2004 | ISO 6341, 1996 |

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For the Biotox test, 950 µL of sample solution and 50 µL of *V. fischeri* culture were mixed and bioluminescence was measured after 5, 15 and 30 minutes exposure to the test solution. Algal biomasses were determined using acetone pigment extractions as described by Mayer et al., (1997). For all three ecotoxicity tests, range finding tests were initially conducted for each compound and test species, prior to definitive tests combining ecotoxicity testing and test compound quantification. The aim was to obtain Effective Concentration values (i.e. $EC_{10}$ and $EC_{50}$) for each individual compound. In cases where complete inhibition/immobilisation of the test organism/endpoint did not occur within the solubility limits of the test compound, the assay was re-run with six replicates and LOEC/NOEC values were determined.

The same three assays were also used to monitor changes in ecotoxicity during UV treatment of the 6 mg $L^{-1}$ carbamazepine solution. All bioassays were conducted under static conditions with no renewal of the test solution. Dissolved oxygen and pH were measured at the beginning and end of the testing period to check compliance with standard test conditions. Quality control tests with potassium dichromate were also performed for all organisms. Furthermore, in addition to standard control samples, DMSO carrier controls were also included.

## 2.4 Preparation of solutions for ecotoxicity testing

Stock solutions for ecotoxicity testing of individual compounds were prepared in dimethylsulphoxide (DMSO). An aliquot of each stock solution was then diluted in ultra-pure water (MilliQ) to prepare the top concentration for ecotoxicity testing and further diluted using the appropriate growth medium to prepare the full concentration series for each test. The highest concentrations of solvent in the test solutions were always < 0.1 % of the total volume of test solution. The stock solution for the 6 mg $L^{-1}$ carbamazepine UV-treatment test was prepared in methanol. The concentration of methanol in the starting solution was 0.1 %.

## 2.5 UV treatment of carbamazepine and related ecotoxicity assessments

UV treatment of a 6 mg $L^{-1}$ carbamazepine solution (prepared with MilliQ water) was conducted using a bench scale UV photoreactor with a circulating flow system. This experiment was carried out in triplicate. The apparatus consisted of a steel container (8 L), a waterpump (85 L $h^{-1}$), and a medium pressure metal-halogen UV lamp (690 W). The UV lamp (Bau 42, Scan Research A/S, Denmark) emitted a polychromatic light ($\lambda$ = 185-400 nm) with enhanced emission in the relevant range for photochemical treatment (i.e. 190-250 nm). The emission spectrum for this lamp has been previously reported (Kosjek et al., 2009).

The fate and behaviour of carbamazepine and its transformation products were studied over a 120 min irradiation period. A stock solution of 60 mg carbamazepine in 10 mL of methanol was prepared and gradually added to 10 L of MilliQ water to give a 6 mg $L^{-1}$ carbamazepine solution. This was allowed to circulate through the flow system for 10 minutes prior to the beginning of the test in order to allow for thorough mixing of the test solution. The first 150 mL sample (Time 0) was then taken. Subsequent samples were removed after 2, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 minutes of UV exposure. For each sampling event the lamp was switched off following the predetermined exposure period and the system left to circulate for a further 5 minutes before sampling. The temperature of the test solution increased during treatment, however ice was used to blanket the UV treatment system to

maintain the temperature below 50 °C at all times in order to limit thermal degradation. Carbamazepine, acridine and acridone concentrations were quantified throughout the UV treatment process using the SPE and GC-MS method described above. All samples were stored at 4 ℃ prior to chemical and ecotoxicological analyses.

**2.6 Dose-response calculations and other statistical analyses**
Dose-response data for the *V. fischeri* bioassays were fitted using a log–logistic distribution, and $EC_{10}$, $EC_{50}$ values and their 95 % confidence intervals were calculated according to the method of Barnes et al. (2003). For the *P. subcapitata* bioassays, algal growth rate was calculated from the logarithmic increase in cell density in each individual algae culture, assuming exponential growth during the 48-h duration of the assay. Algal data analysis, including calculation of probit and Weibull dose–response models, $EC_{50}$ and $EC_{10}$ values and their 95 % confidence intervals was carried out using a Windows-based dose–response regression tool programmed by K.O.Kusk which functions according to the method described in Christensen et al., (2009). *D. magna* bioassay results were analysed using the Toxicity Data Analysis and Database Software (Toxcalc$^{TM}$ v5.031), which generates median effective concentrations and their 95% confidence intervals and probit values. All other statistics presented in this paper were calculated using Microsoft Office Excel.

**2. RESULTS**
**3.1 Ecotoxicity of carbamazepine, acridine and acridone**
Acute toxicity data for the individual compounds, expressed either as median effective concentrations (EC10/EC50) or NOECs and LOECs are presented in Table 2. The results of all three acute toxicity assays showed the parent compound, carbamazepine, to be significantly less toxic than either of the tested transformation products. Furthermore, of the two transformation products tested, acridone was consistently less toxic than acridine across all three assays. Table 3 presents a comprehensive list of acute/short-term toxicity results for aquatic based bioassays that have previously appeared in the literature for these three compounds. As a whole these values support the results presented above.

Table 2: Ecotoxicity testing results for carbamazepine, acridine and acridone (all values in mg l$^{-1}$). Results are based on measured solution concentrations.

| Ecotoxicity Assay | Carbamazepine | Acridine | Acridone |
|---|---|---|---|
| Bacteria, *Vibrio fischeri* (5, 15, 30 min) | NOEC = 5.59 LOEC = 8.16 | $EC_{10}$ = 0.78 – 0.98 $EC_{50}$ = 5.34 - 6.90 | NOEC = 0.99 - 2.10 LOEC = 1.44 - 3.07 |
| Algae, *Pseudokirchneriella subcapitata* (48 h) | $EC_{10}$ = 39 | $EC_{10}$ = 0.11 $EC_{50}$ = 0.61 | $EC_{10}$ = 0.87 |
| Cladoceran, *Daphnia magna* (48 h) | $EC_{10}$ > 30 | $EC_{10}$ = 0.39 $EC_{50}$ = 0.71 | $EC_{10}$ = 0.82 $EC_{50}$ = 1.49 |

Table 3: Published acute toxicity testing for carbamazepine, acridine, and acridone. The table includes a range of different aquatic species as well as results for benthic invertebrates. EC50/LC50 values relate either to solution concentrations or porewater concentrations as relevant to the particular test. All vales are given in mg L$^{-1}$.

| Organism, test duration, and ecotoxicological endpoint | Carbamazepine EC$_{50}$/LC$_{50}$ | Acridine EC$_{50}$/LC$_{50}$ | Acridone EC$_{50}$/LC$_{50}$ |
|---|---|---|---|
| Bacteria (Vibrio fischeri); 15 min; bioluminescence (Kim et al., 2007) | 52.2 | | |
| Bacteria (*Vibrio fischeri*); 30 min; bioluminescence (Ferrari et al., 2003) | > 81 | | |
| Bacteria (*Vibrio fischeri*); 5, 15, 30 min; bioluminescence (Jos et al., 2003) | 64 - 79 | | |
| Algae (*Chlorella vulgaris*); 48 h; growth inhibition (Jos et al., 2003) | 37 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Kim et al., 2007) | >100 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Ferrari et al., 2003) | > 13.8 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Jos et al., 2003) | 98 | | |
| Cladoceran (*Daphnia spp.*); 48 h; immobilisation (Cleuvers, 2002) | > 100 | | |
| Cladoceran (*Ceriodaphnia dubia*); 48 h; immobilisation (Ferrari et al., 2003) | 77.7 | | |
| Cnidarian (*Hydra attenuata*); 96 h; tulip phase morphology; (Quinn et al., 2008) | 29.4 | | |
| Bivalve, Zebra mussel (*Dreissena polymorpha*); 96 h; cell cytoxicity; haemocytes, gill and digestive gland cells; (Parolini et al., 2011) | 5.1 – 6.8 | | |
| Algae (*Desmodesmus subspicatus*); 24 h; growth inhibition (Eisentraeger et al., 2008) | | 2.1 | |
| Algae spp. (7 species tested); 96 h; growth inhibition (Dijkman et al., 1997) | | 0.08 – 0.79 | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Eisentraeger et al., 2008) | | 4.6 | |
| Cladoceran (*Daphnia pulex*); 48 h; mortality (first instar) (Parkhurst et al., 1981) | | 2.3 | |
| Cladoceran (*Daphnia pulex*); 28 d; reproduction (adult) (Parkhurst et al., 1981) | | 0.8 | |
| Cladoceran (*Daphnia pulex*); 24 h; immobilisation (adult) (Southworth et al., 1978) | | 1.71 | |
| Benthic invertebrate, midge | | 0.07 | |

| | | | |
|---|---|---|---|
| (*Chironomus riparius*); 96 h; survival (first instar larvae) (Bleeker et al., 1998) | | | |
| Bivalve, Zebra mussel (*Dreissena polymorpha*); 48 h; filtration rate (Kraak et al., 1997) | | 0.96 | |
| Calonoid copepod (*Diaptomus clavipes*); 14 h; mortality (first naupliar) (Cooney and Gehrs, 1984) | | 1.55 | |
| Benthic invertebrate, midge (*Chironomus riparius*); 28 d; survival (emergence) (Paumen et al., 2008) | | 0.37 | 1.02 |
| Benthic invertebrate, midge (*Chironomus riparius*); 96h; survival (first instar larvae Bleeker et al (1999). | | 0.40 | > 4.8 |
| Benthic oligochaete (*Chironomus riparius*); 28 d; mortality (Paumen et al., 2009) | | 0.25 | 0.75 |
| Benthic oligochaete (*Lumbriculus variegatus*); 28 d; reproduction (Paumen et al., 2009) | | 0.23 | 0.1 |

### 3.2 Carbamazepine UV treatment experiment

UV-treatment of 6 mg $L^{-1}$ carbamazepine solution was carried out over a 120 min period and samples were removed periodically over the course of the experiment for ecotoxicity testing. The starting concentration of 6 mg $L^{-1}$ is higher than measured environmental concentrations (Lacey *et al*., (2012), but facilitated the collection of measureable toxicity data from beginning to end of the UV-treatment experiment, even though the concentration of carbamazepine had reached < 1 % of the starting concentration by the end of the experiment. Quantification results confirmed the degradation of carbamazepine throughout the treatment period, together with concurrent increases in acridine and acridone (Figure 1). Toxicity was shown to increase in parallel with these changes, indicating that the mixture of transformation products formed was more toxic than the parent compound by itself (Figures 2, S1, and S2; Table S1).

Figure 1 shows the results of chemical quantification analyses performed on duplicate samples taken during one of the 6 mg $L^{-1}$ carbamazepine UV-treatment tests. The concentration of carbamazepine decreased steadily during the UV exposure experiment (i.e. tenfold decrease in concentration within the first 60 min exposure), but was still detectable after 120 minutes. Quantifiable transformation products (i.e. those with commercially available standards) reached a peak level after about 15 minutes exposure and then decreased. Concentration profiles of the two transformation products were both below the detection limit after 120 minutes of UV treatment. Although methanol, a potential radical scavenger, was used as the solvent

Figure 1. Measured concentrations of carbamazepine, acridine, and acridone in samples removed periodically from a 6 mg L$^{-1}$ carbamazepine UV-treatment experiment. Measurements were carried out in duplicate and both results are plotted.
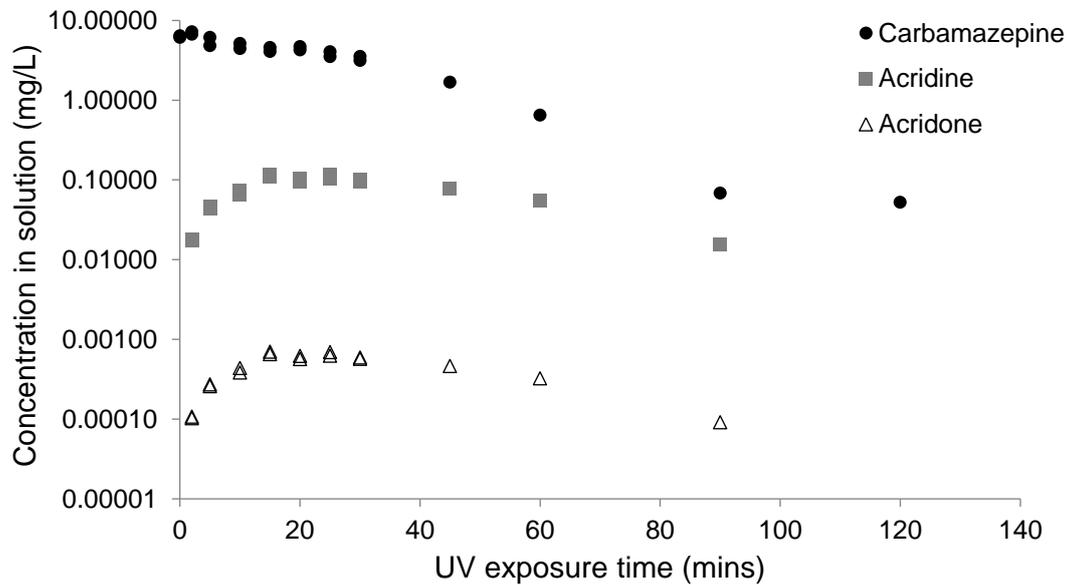


Figure 2. Measured toxic response of *Vibrio fischeri* (inhibition in bioluminescence, 15 min), *Pseudokirchneriella subcapitata* (growth inhibition, 48 h), and *Daphnia magna* (immobilisation, 48 h) exposed to solutions sampled from triplicate runs of the 6 mg L$^{-1}$ carbamazepine UV-treatment experiment. Error bars represent the standard error of the mean.
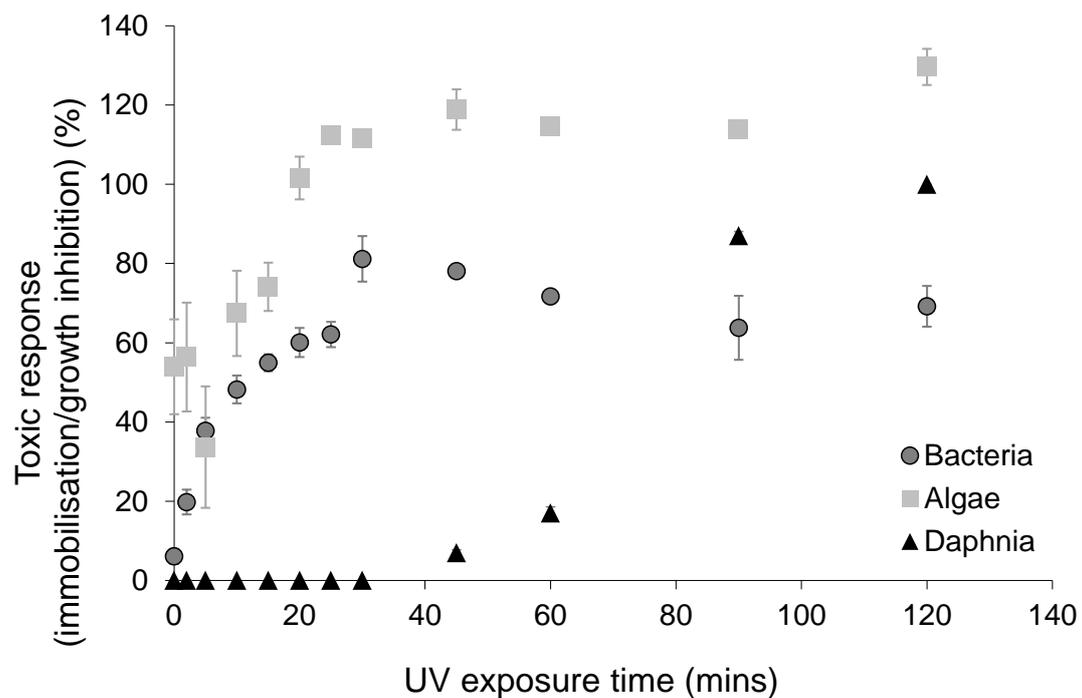
for the carbamazepine stock solution, Kosjek et al. (2009) showed that the concentration of methanol used in this experiment should not have inhibited the decay of carbamazepine or the identified transformation products during treatment. Their investigation used the same benchtop UV-treatment system as the current experiment and showed no significant difference in carbamazepine decay in the presence of 1.2, 2.5 and 6.2 % methanol. As the experimental design for the study reported in this paper included only 0.1 % methanol by volume, the presence of methanol is not believed to have affected the chemical analysis results obtained.

The pH of the carbamazepine UV test solution decreased during treatment from ~ pH 5.8 to pH 3.6. Therefore, it was necessary to amend all samples to a standardised pH prior to toxicity testing in order to prevent pH-induced toxic responses (i.e. hydrogen ion toxicity) from confounding the ecotoxicity results. Samples were therefore amended to the appropriate pH range for each test organism (i.e. ~ pH 7 for *V. fischeri*; pH 7.8 for *D. magna*; pH 8 for *P. subcapitata*). Figure 2 summarises key results from the three ecotoxicity bioassays, and full results for all three organisms are given in Figures S1, S2 and Table S1. The results indicate increasing toxicity over time during UV-treatment in parallel with the formation of carbamazepine degradation products. Peak toxicity to *V. fischeri* occurred around 25 minutes after the onset of UV-treatment when concentrations of acridine and acridone were also at their peaks, however the inhibition of *V. fischeri* bioluminescence remained around 70 % for the remainder of the experimental period, indicating that other transformation products must also be contributing to the ongoing toxic effect. This is also supported by the results of the *P. subcapitata* bioassay presented in Figure S2. In this case growth inhibition reached almost 100 % within 20 minutes of the onset of UV treatment, and greater than 100 % growth inihibition (i.e. cell death) was recorded in subsequent samples. By contrast, *D. magna* was less sensitive to the mixture toxicity of the UV treated carbamazepine solution (Figure 2; Table S1). This bioassay only demonstrated a measurable toxic effect (i.e. immobilisation of the organisms) after 45 minutes of UV treatment, but in keeping with the other bioassays this effect was still evident at the end of the experiment, indicating that toxic transformation products still remained in the solution at the end of the treatment period.

## 3. DISCUSSION
The presented results clearly indicate that the photodegradation products, acridine and acridone, are considerably more toxic than the parent compound carbamazepine across multiple trophic levels. Although the sensitivities of the different test organisms differed, all species showed greater toxicity of the tested photodegradation products compared with the parent compound carbamazepine and this result was further reflected in the increasing toxicity of the carbamazepine spiked water throughout the UV treatment experiment. This is the first time that these compounds have been tested simultaneously under the same experimental conditions and using the same suite of assays, however we note that the results compared favourably with existing acute toxicity data (Table 3).

Recent efforts to quantify concentrations of carbamazepine in surface waters suggest that current environmental levels are well below the concentrations likely to cause acute toxicity to aquatic organisms. For instance, Lacey *et al*., (2012) referenced ten different studies measuring carbamazepine concentrations in wastewater influents and effluents across Europe and the USA, and reported a maximum effluent concentration

across those studies of 6.5 μg L$^{-1}$. This is three orders of magnitude smaller than the lowest carbamazepine effect concentration measured in this experiment (*V. fischeri*, LOEC 8.16 mg L$^{-1}$). However, studies which have investigated chronic/sublethal impacts of pharmaceutical compounds, including carbamazepine, have typically observed physiological effects at much lower contaminant concentrations than the measured threshold concentrations for acute toxicity. For example, Lurling *et al.*, (2006) conducted chronic ecotoxicity tests using *Daphnia pulex* and observed significantly reduced rates of population growth at carbamazepine concentrations of 200 μg L$^{-1}$ due to retardation of juvenile somatic growth and subsequent delays in maturation and time to first reproduction. Similarly, Ferrari *et al.*, (2003) reported carbamazepine NOEC and LOEC values of 25 μg L$^{-1}$ and 100 μg L$^{-1}$ respectively for a chronic 7-day test using reproduction of *Ceriodaphnia dubia* as the endpoint. These studies show that chronic carbamazepine exposure effects may occur at concentrations at least 3 orders of magnitude lower than the EC$_{50}$ typically reported for acute *Daphnia* assays (e.g. this paper; Cleuvers, 2002; Jos *et al.*, 2003; Kim *et al.*, 2007), and indicate that, based on estimates of 'predicted no effect concentrations' and 'predicted environmental concentrations' (Ferrari et al., 2003), carbamazepine poses a potential risk to aquatic environments. Given the current findings regarding the comparative ecotoxicities of carbamazepine and its UV-degradation products, this risk could actually be considerably greater than was previously supposed.

As stated previously, carbamazepine is continually released to the environment at low concentrations due to its ubiquitous presence in wastewater effluents. It is assumed that removal of carbamazepine from surface waters will occur largely via the photodegradation pathway as sorption of carbamazepine is very limited (Ternes et al., 2002; Scheytt et al., 2005; Clara et al., 2004) and it is resistant to biodegradation (Clara et al., 2004; Kosjek et al., 2009). Hence, the formation of stable photodegradation products is not only relevant to wastewater treatment processes, where UV treatment may be implemented to assist with microbial disinfection (e.g. inactivation of Cryptosporidium); it is also relevant to the breakdown of carbamazepine in natural river environments. With this in mind it is important to note the recalcitrance of the degradation products formed during this experiment. Even though progressive mineralisation could not be monitored by means of TOC analysis due to the presence of methanol in the spiking solution, the persistent toxicity at the end of the UV-treatment experiment provides evidence that complete mineralisation did not occur. Previous research (Kosjek *et al.*, 2009) has shown that at least six photochemical derivatives are likely to form during the UV degradation of carbamazepine. It should also be noted that Li *et al.*, (2011) reported that oxycarbazepine, a keto analogue of carbamazepine which is increasingly used as a substitute, also degrades to form essentially the same suite of UV-degradation products as carbamazepine. These authors also reported a toxic response in *D. magna* upon exposure to UV-treated oxycarbazepine solutions. By contrast, Rizzo et al., (2009) observed decreasing toxicity when 5 mg/L carbamazepine solution was treated by TiO$_2$ photocatalysis, but it should be noted that the degradation pathway under that treatment regime (see Calza et al., 2012) would be different to the uncatalysed photolysis pathway relevant in this experiment.

The UV dose applied in the current experiment is higher than that typically used for UV disinfection of water. A typical disinfection dose is equivalent to the UV dose delivered in less than 1 minute in the current experimental setup. Hence, even though

some degradation products may begin to form during UV-disinfection (note that toxic effects were observed in samples taken only 2 min after the onset of UV treatment) UV-disinfection doses are unlikely to significantly degrade carbamazepine. By contrast, if UV were to be purposely used as an end of pipe of treatment to photolyse chemicals the UV dose would be much higher, although not necessarily high enough to remove all toxic degradation products within a feasible timeframe. The feasibility of applying the UV lamp used in this study for degradation of both carbamazepine and other pharmaceuticals and estrogenic chemicals was evaluated in previous studies (Hansen et al., 2007a; Hansen et al., 2007b). Recalculating the doses from that study to the current laboratory setup indicates that 90 % removal of steroid estrogens would be equivalent to 2-5 min treatment (Hansen et al., 2007a), whereas some pharmaceuticals would require the equivalent of 20-40 min UV treatment to be degraded by 90 % (Hansen et al., 2007b).

In this study, mixture toxicity was still high (> 60 % toxicity for all endpoints) at the end of the UV-treatment experiment (Figure 2), even though carbamazepine degradation had reached > 99 % and acridine and acridone concentrations had declined to < 10 % of their peak concentrations. These results highlight the need to further consider the identity, stability, and environmental relevance of recalcitrant carbamazepine degradation products. Researchers have already noted that other pharmaceuticals, such as prednisone (DellaGreca et al., 2003) and diclofenac (Schulze et al., 2010), may also form transformation products more toxic than the parent compound. Failing to account for toxicologically relevant transformation products can easily lead to substances being incorrectly identified as posing negligible environmental risks, when in fact they are significant. However, systematic ecotoxicity testing of treatment transformation products is complicated by the complexity of the relevant degradation pathways and the large numbers of intermediate compounds of varying stability which can be formed (e.g. Agüera et al., 2005; Kosjek et al., 2009; Calza et al., 2012; Li et al., 2012). Moreover, degradation pathways may differ depending on the particular wastewater treatment regime or natural degradation pathway in question (e.g. activated sludge treatment or advanced oxidation vs. sunlight induced photodegradation). It is unrealistic to expect that all relevant compounds can be tested using conventional toxicity testing of individual compounds at multiple trophic levels, especially as the majority of these substances may not be commercially available. Identification, synthesis and isolation of such compounds is extremely time consuming, making experiments such as this, where mixture toxicity of the degradation products together as a whole is assessed, particularly useful for identifying cases where degradation pathway toxicity may be an issue. Further information on effect-driven approaches for assessing the toxicological relevance of transformation products can be found in Escher and Fenner's critical review (2011).

## 4. CONCLUSION

The results of this study indicate that degradation products considerably more toxic than carbamazepine itself are likely to be produced as a result of UV treatment of wastewater effluents and/or photo-induced degradation of carbamazepine in natural waters. These findings are clearly relevant to ongoing debates regarding the potential environmental effects of pharmaceutically-derived compounds in the environment, highlighting the need to consider mixture toxicity and the formation and persistence of toxicologically relevant degradation products when assessing environmental risk.

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# SUPPLEMENTARY MATERIAL

Table S1: Published acute toxicity testing for carbamazepine, acridine, and acridone. The table includes a range of different aquatic species as well as results for benthic invertebrates. EC50/LC50 values relate either to solution concentrations or porewater concentrations as relevant to the particular test. All vales are given in mg L$^{-1}$.

| Organism, test duration, and ecotoxicological endpoint | Carbamazepine EC$_{50}$/LC$_{50}$ | Acridine EC$_{50}$/LC$_{50}$ | Acridone EC$_{50}$/LC$_{50}$ |
|---|---|---|---|
| Bacteria (Vibrio fischeri); 15 min; bioluminescence (Kim et al., 2007) | 52.2 | | |
| Bacteria (*Vibrio fischeri*); 30 min; bioluminescence (Ferrari et al., 2003) | > 81 | | |
| Bacteria (*Vibrio fischeri*); 5, 15, 30 min; bioluminescence (Jos et al., 2003) | 64 - 79 | | |
| Algae (*Chlorella vulgaris*); 48 h; growth inhibition (Jos et al., 2003) | 37 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Kim et al., 2007) | >100 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Ferrari et al., 2003) | > 13.8 | | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Jos et al., 2003) | 98 | | |
| Cladoceran (*Daphnia spp.*); 48 h; immobilisation (Cleuvers, 2002) | > 100 | | |
| Cladoceran (*Ceriodaphnia dubia*); 48 h; immobilisation (Ferrari et al., 2003) | 77.7 | | |
| Cnidarian (*Hydra attenuata*); 96 h; tulip phase morphology; (Quinn et al., 2008) | 29.4 | | |
| Bivalve, Zebra mussel (*Dreissena polymorpha*); 96 h; cell cytoxicity; haemocytes, gill and digestive gland cells; (Parolini et al., 2011) | 5.1 – 6.8 | | |
| Algae (*Desmodesmus subspicatus*); 24 h; growth inhibition (Eisentraeger et al., 2008) | | 2.1 | |
| Algae spp. (7 species tested); 96 h; growth inhibition (Dijkman et al., 1997) | | 0.08 – 0.79 | |
| Cladoceran (*Daphnia magna*); 48 h; immobilisation (Eisentraeger et al., 2008) | | 4.6 | |
| Cladoceran (*Daphnia pulex*); 48 h; mortality (first instar) (Parkhurst et al., 1981) | | 2.3 | |
| Cladoceran (*Daphnia pulex*); 28 d; reproduction (adult) (Parkhurst et al., 1981) | | 0.8 | |

| | | | |
|---|---|---|---|
| Cladoceran (*Daphnia pulex*); 24 h; immobilisation (adult) (Southworth et al., 1978) | | 1.71 | |
| Benthic invertebrate, midge (*Chironomus riparius*); 96 h; survival (first instar larvae) (Bleeker et al., 1998) | | 0.07 | |
| Bivalve, Zebra mussel (*Dreissena polymorpha*); 48 h; filtration rate (Kraak et al., 1997) | | 0.96 | |
| Calonoid copepod (*Diaptomus clavipes*); 14 h; mortality (first naupliar) (Cooney and Gehrs, 1984) | | 1.55 | |
| Benthic invertebrate, midge (*Chironomus riparius*); 28 d; survival (emergence) (Paumen et al., 2008) | | 0.37 | 1.02 |
| Benthic invertebrate, midge (*Chironomus riparius*); 96h; survival (first instar larvae Bleeker et al (1999). | | 0.40 | > 4.8 |
| Benthic oligochaete (*Chironomus riparius*); 28 d; mortality (Paumen et al., 2009) | | 0.25 | 0.75 |
| Benthic oligochaete (*Lumbriculus variegatus*); 28 d; reproduction (Paumen et al., 2009) | | 0.23 | 0.1 |

Table S2. Mean immobility of *Daphnia magna* exposed to solutions sampled from the 6 mg L$^{-1}$ carbamazepine UV-treatment experiments. Standard errors are given in brackets.

| Treatment Time (min) | Mean immobility after 24 h exposure (%) | Mean immobility after 48 h exposure (%) |
|---|---|---|
| 0 | 0 (0.0) | 0 (0.0) |
| 2 | 0 (0.0) | 0 (0.0) |
| 5 | 0 (0.0) | 0 (0.0) |
| 10 | 0 (0.0) | 0 (0.0) |
| 15 | 0 (0.0) | 0 (0.0) |
| 20 | 0 (0.0) | 0 (0.0) |
| 25 | 0 (0.0) | 0 (0.0) |
| 30 | 0 (0.0) | 0 (0.0) |
| 45 | 0 (0.0) | 7 (0.7) |
| 60 | 11 (0.9) | 17 (1.6) |
| 90 | 17 (1.2) | 87 (1.1) |
| 120 | 49 (1.4) | 100 (0.0) |

Figure S1. Mean inhibition in bioluminescence of *Vibrio fischeri* exposed to solutions sampled from triplicate runs of the 6 mg L$^{-1}$ carbamazepine UV-treatment experiment. Bioluminescence was measured after 5, 15 and 30 minutes exposure to the sample solutions. Error bars represent the standard error of the mean.
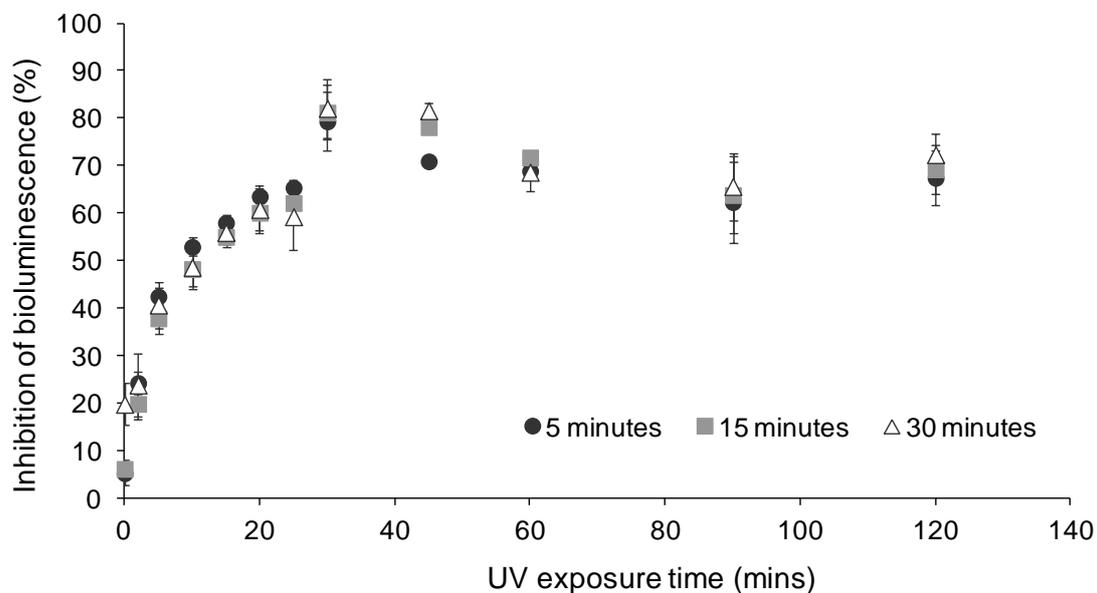


Figure S2. Mean inhibition in growth rate of *Pseudokirchneriella subcapitata* exposed to solutions sampled from the 6 mg L$^{-1}$ carbamazepine UV-treatment experiment. Cell density was measured after 0, 24 and 48 h exposure to the sample solutions and the mean of three replicate samples is presented for each exposure time. Error bars represent the standard error of the mean.