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Ecotoxicity of carbamazepine and its UV photolysis transformation products

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HIGHLIGHTS

- Carbamazepine is highly recalcitrant to standard wastewater treatment practices.
- This study investigated the ecotoxicity of carbamazepine UV-photolysis degradation products.
- Carbamazepine was less toxic to three standard test organisms than the tested degradation products.
- Recalcitrant mixture toxicity of the UV-treated solution was still observed at the end of treatment.
- UV-photolysis of carbamazepine may form toxicologically relevant 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 ultraviolet (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, and all three ecotoxicity endpoints were still inhibited >60% relative to control populations upon dosing with 90 + min UV-treated carbamazepine solution. 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 may 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.

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1. 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,b). 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. Heberer et al., 2004; Stackelberg et al., 2004, 2007; Togola and Budzinski, 2008). It is also noted to be highly resistant to biodegradation and thus highly recalcitrant under standard biological wastewater

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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 (Agüera 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 et al., 2009), acridine and 9(10H)-acridone. Kosjek et al. (2009) have also identified several other compounds which may form via UV photolysis of carbamazepine, however authentic standard compounds are not commercially available for these other degradation products. Thus, in addition to single compound testing of carbamazepine, acridine and acridone with 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 by 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 (including, but not limited to, acridine and acridone).

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 and is thus ideal for situations such as this one in which standard compounds are not available for all relevant transformation products. In 'effect driven' assessments, compounds are prioritised for transformation product isolation, purification, and further study if the reaction mixture increases in toxicity in parallel with decreasing parent compound concentration.

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 one of the most important long-term process affecting persistence and toxicity in the environment (Doll and Frimmel, 2003; Agüera et al., 2005).

2. 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 (New Jersey, USA). Acridine (97%, CAS 260-94-6) and 9(10H)-acridone (99%, CAS 578-95-0) were obtained from Sigma-Aldrich (St. Louis, USA). Stock solutions were freshly made prior to use and the volumetric flasks were covered in aluminium foil to prevent photodegradation.

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 h. 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. The limits of detection (LOD) were 2.3 μg L^{-1} for acridine, 14 ng L^{-1} for acridone and 11 ng L^{-1} for carbamazepine

2.3. Ecotoxicity testing

Three internationally standardised aquatic ecotoxicity tests (Table 1) were used to investigate the ecotoxicity of carbamazepine, acridine, and acridone. The same three assays were also used to monitor changes in ecotoxicity during UV-treatment of the 6 mg L^{-1} carbamazepine solution. 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).

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 min exposure to the test solution. Algal biomasses were determined by 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

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 ± 0.2	7.8 ± 0.2	8 ± 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	$5–10 \cdot 10^3$ 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	48 h	48 h
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)

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. 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 each testing period to check compliance with standard test conditions. Quality control tests with potassium dichromate were also performed ($K_2Cr_2O_7$ EC_{50} 0.8–1.4 mg L^{-1}). All ecotoxicity results are reported in terms of the toxic response relative to a measured control population. To be considered as valid results, population growth of algae control treatments must be exponential and immobilisation of *Daphnia* control populations must be <10%. In addition to standard control samples, DMSO/methanol solvent controls were also included. The test results for solvent controls did not differ significantly from ordinary controls and were used to represent the control population for statistical analyses).

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 the 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 min 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 min 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 min 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 °C 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 as in Broos et al. (2007). 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™ 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.

3. Results

3.1. Ecotoxicity of carbamazepine, acridine and acridone

Acute toxicity data for the individual compounds, expressed either as median effective concentrations (EC_{10}/EC_{50}) 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 acridine or acridone. Furthermore, of the two transformation products tested, acridone was consistently less toxic than acridine across all three assays. Table 3 presents a comprehensive

Table 2
Ecotoxicity testing results for carbamazepine, acridine and acridone (all values in mg L^{-1}). Results are based on measured solution concentrations. For tests where full response curves were obtained n = 39. For tests where full response curves could not be obtained additional NOEC/LOEC tests were run. NOEC/LOEC tests comprised 6 replicates for each concentration tested).

Ecotoxicity assay	Carbamazepine	Acridine	Acridone
Bacteria, *Vibrio fischeri* (5, 15, 30 min); inhibition of bioluminescence	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); inhibition of growth	NOEC = 27.4 LOEC = 40	EC_{10} = 0.11 EC_{50} = 0.61	NOEC = 0.69 LOEC = 1.09
Cladoceran, *Daphnia magna* (48 h); immobilisation	NOEC = 30	EC_{10} = 0.39 EC_{50} = 0.71	EC_{10} = 0.82 EC_{50} = 1.49

Table 3
Comprehensive listing of all published aquatic organism acute (short-term) toxicity data for carbamazepine, acridine, and acridone. EC_{50}/LC_{50} values relate either to solution concentrations or porewater concentrations as relevant to the particular test. All values 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*); 5/15/30 min; bioluminescence ([a]Kim et al., 2007; [b]Ferrari et al., 2003; [c]Jos et al., 2003)	52.2[a]; >81[b]; 64–79[c]		
Algae (*Chlorella vulgaris*); 48 h; growth inhibition (Jos et al., 2003)	37		
Cladoceran (*Daphnia magna*); 48 h; immobilisation ([a]Kim et al., 2007; [b]Ferrari et al., 2003; [c]Jos et al., 2003; [d]Cleuvers, 2002); (*Ceriodaphnia dubia*); 48 h; immobilisation ([e]Ferrari et al., 2003)	>100[a]; >13.8[b]; 98[c]; >100[d]; 77.7[e]		
Cnidarian (*Hydra attenuata*); 96 h; tulip phase morphology; (Quinn et al., 2008)	29.4		
Bivalve, Zebra mussel (*Dreissena polymorpha*); 96 h; cell cytotoxicity; haemocytes, gill and digestive gland cells; (Parolini et al., 2011)	5.1–6.8		
Algae spp. (multiple species tested); 96 h growth inhibition ([a]Dijkman et al., 1997); 24 h growth inhibition [b]Eisentraeger et al., 2008)		0.08–0.79[a]; 2.1[b]	
Cladoceran (*Daphnia spp.*); 24/48 h; immobilisation ([a]Southworth et al., 1978; [b]Eisentraeger et al., 2008; [c]Parkhurst et al., 1981)		1.71[a]; 4.6[b]; 2.3[c]	
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*); 96 h (Bleeker et al., 1999)		0.40	>4.8

list of published aquatic ecotoxicity test results for these three compounds. These values clearly support the results presented above, strongly suggesting that UV photolysis of carbamazepine can produce degradation products of greater toxicity that the parent compound itself.

3.2. Carbamazepine UV-treatment experiment

Experiments to investigate the effects of UV-treatment of 6 mg L^{-1} carbamazepine solution were carried out over a 120 min period and samples were removed periodically over the course of these experiments for chemical analysis and ecotoxicity testing. Chemical quantification analyses were performed on duplicate samples taken during one of the 6 mg L^{-1} carbamazepine UV-treatment tests, whilst ecotoxicity endpoints were measured on samples from three replicate UV-treatment experiments (n = 9). The starting concentration of 6 mg L^{-1} carbamazepine is higher than the measured environmental concentrations (Lacey et al., 2012), but facilitated the collection of measureable toxicity data from beginning to end of the UV-treatment experiments.

Quantification results confirmed the ongoing degradation of carbamazepine throughout the 120 min treatment period, together with concurrent increases in acridine and acridone throughout the first 20 min of treatment (Fig. 1). These compounds, which were not present at Time 0, were quantifiable in solutions sampled from 2 to 90 min after the onset of UV-treatment, confirming the formation of these compounds as a function of the UV-photolysis of carbamazepine (see Kosjek et al., 2009). However, from 25 min onwards the measured concentrations of both acridine and acridone consistently decreased, indicating that the transformation rates of these compounds were greater than their production rates for the remainder of the experimental period. Both compounds were below the detection limits after 120 min of UV-treatment. The degradation of these compounds during the treatment period indicates that additional photolysis products were likely to have been produced during the remaining treatment period (see discussion below).

Although methanol, a potential radical scavenger, was used as the solvent 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 using the same benchtop UV-treatment system as the current experiment 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,

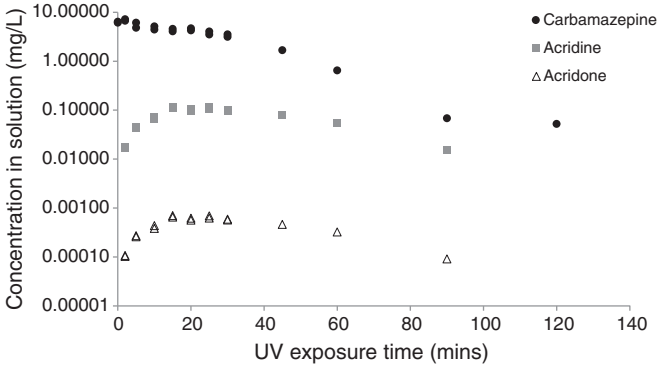

Fig. 1. Measured concentrations of carbamazepine, acridine, and acridone in samples removed periodically from a 6 mg L^{-1} carbamazepine UV-treatment experiment. Measurements were carried out in duplicate and both results are plotted.

the presence of methanol is not believed to have affected the chemical analysis results obtained.

The pH of the carbamazepine UV-treatment test solution decreased during treatment from ~pH 5.8 to pH 3.6. This decrease in pH is unlikely to have caused significant degradation of carbamazepine as the recovery of carbamazepine from a 1.5 M HCl solution after 48 h has been reported to be 97.3% (Rao and Belorkar, 2010). However, it was necessary to amend all samples to a standardised pH prior to ecotoxicity testing in order to prevent pH-induced toxic responses (i.e. hydrogen ion toxicity) from confounding the results. Samples were therefore amended with 0.1 M NaOH to obtain 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*). Fig. 2 summarises the results from the three ecotoxicity bioassays. The toxic response for all endpoints increased over time during the UV-treatment experiments in parallel with the formation of carbamazepine degradation products. These results indicate that the mixture of transformation products formed (including those which could not be quantified due to the lack of available standard compounds) was more toxic than the parent compound by itself.

Peak toxicity to *V. fischeri* occurred around 30 min after the onset of UV-treatment when concentrations of acridine and acridone were also near their highest (0.12 and 0.0007 mg L^{-1} respectively). The inhibition of *V. fischeri* bioluminescence remained around 70% from 25 min to 120 min UV-treatment. As the acridine and acridone concentrations were below their respective detection limits by 120 min (Fig. 2) and concentrations of all individual compounds quantified were well below their EC50 values (Table 2), it appears that other transformation products (discussed below) must also have contributed to the ongoing toxic response evident in Fig. 2. This is also supported by the results of the *P. subcapitata* bioassay. In this case, growth inhibition reached 100% within 20 min of the onset of UV-treatment and stayed at 100% in all subsequent samples (i.e. up to 120 min UV-treatment). *D. magna* was less sensitive to the mixture toxicity of the UV-treated carbamazepine solution and only demonstrated a measurable toxic effect (i.e. *Daphnia* immobilisation) after 45 min of UV-treatment. However, in keeping with the other bioassays, the 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.

For clarity of presentation only the 15 min *V. fischeri*, 24 h *P. subcapitata*, and 48 h *D. magna* test results are shown in Fig. 2. However, it should be noted that the response data for the 5, 15, and 30 min *V. fischeri* exposure measurements were all very similar, both in terms of the overall response pattern and the measured inhibition values. The *D. magna* 24 h and 48 h measurements also compared favourably in terms of the pattern of response, and indicated a stronger toxic response after a longer exposure period (i.e. 48 h) as would typically be expected. Similarly, the 48 h *P. subcapitata* results showed greater growth inhibition in the 2, 5 and 10 min UV-treatments than was observed for the 24 h *P. subcapitata* measurement. The rest of the samples (20–120 min UV-treatment) showed 100% growth inhibition for both 24 h and 48 h measurements. The most interesting difference observed in the *P. subcapitata* tests was the apparent toxicity (~54% growth inhibition) in the 48 h exposure test of the Time 0 samples (data not shown). This response indicates that some photodegradation may have occurred in the Time 0 sample throughout the algae test itself. The growth lamp used for the algae tests (Philips TL-D 30 W/33-640) mainly emits light in the visible region (>400 nm) but also has a narrow emission peak at 365 nm. The *V. fisheri* test is much shorter (30 min) than the *P. subcapitata* test and does not involve significant illumination, whilst the *D. magna* test is carried out in the dark. Thus it is certainly plausible that the effects of in-test photodegradation would only be seen in the *P. subcapitata* results.

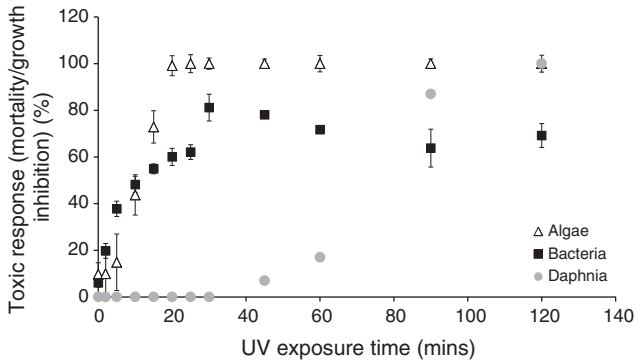

Fig. 2. Measured toxic response of *Vibrio fischeri* (inhibition in bioluminescence; 15 min), *Pseudokirchneriella subcapitata* (growth inhibition; 24 h), and *Daphnia magna* (immobilisation; 48 h) exposed to solutions sampled from triplicate runs of the 6 mg L^{-1} carbamazepine UV-treatment experiment. Error bars represent the standard error of the mean (n = 9).

4. 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 other published acute toxicity data for these compounds (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, Lürling 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 be 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 the 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 Total Organic Carbon 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. Acridine and acridone were positively confirmed by these researchers as carbamazepine UV photolysis degradation products through the use of commercially available authentic compound standards and multiple analytical techniques, whilst hydroxy-(9H,10H)-acridine-9-carbaldehyde, acridone-N-carbaldehyde, and 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione were tentatively identified using gas chromatography hyphenated with an ion trap mass spectrometer (GC–IT). 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 min 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 has been evaluated in previous studies (Hansen et al., 2007; Hansen and Andersen, 2012). 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 and Andersen, 2012), whereas some pharmaceuticals would require the equivalent of 20–40 min UV-treatment to be degraded by 90% (Hansen et al., 2007).

In this study, mixture toxicity was still high (>60% toxic response relative to control populations for all endpoints) at the end of the UV-treatment experiment (Fig. 2), even though the carbamazepine concentration had decreased to ~1% of the starting concentration 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., 2011). 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 by 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).

5. Conclusion

The results of this study indicate that degradation products considerably more toxic than carbamazepine itself may 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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