

## Effects of Pharmaceuticals on Aquatic Invertebrates. Part I. The Antiepileptic Drug Carbamazepine

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**Abstract.** The effects of the antiepileptic drug carbamazepine (CBZ) were studied in three freshwater invertebrate species representing different taxonomic groups, life histories, and habitats in aquatic ecosystems. The oligochaete *Lumbriculus variegatus* was exposed by way of CBZ-spiked sediments at nominal concentrations between 0.625 and 10 mg/kg dry weight (dw) for 28 days. At the end of the test, reproduction and biomass were monitored as end points. The nonbiting midge *Chironomus riparius* was exposed to CBZ in a series of tests at nominal CBZ concentrations in sediment ranging from 0.16 to 100 mg/kg dw at 20°C and 23°C. Emergence and gender ratio were monitored at the end of the test. The freshwater snail *Potamopyrgus antipodarum* as the third test species was used in a chronic reproduction test for 28 days at aqueous CBZ concentrations from 0.4 to 250 mg/L. Whereas for the oligochaete and the snail no effects were observed, *C. riparius* exhibited a significant and concentration-dependent decrease of emergence in all test series. No observed effect concentrations and 10% effect concentrations were in the range of 33 to 140 and 70 to 210 µg/kg dw, respectively, based on measured CBZ concentrations in sediments. These low values indicate that CBZ may pose a potential threat for the survival of *C. riparius* and probably also for other aquatic insect populations in the field.

only few chronic data for other pharmaceuticals are available (e.g., Landsky and Halling-Sørensen 1997; Brooks *et al.* 2003). Although concentrations of pharmaceuticals in the aquatic environment are generally in the lower nanogram-per-liter and microgram-per-liter range, these compounds exhibit a high biologic activity, often associated with a high stability, such that concerns have been raised regarding their potential impact on aquatic wildlife even at the low reported environmental concentrations. The case of ethinylestradiol provides an example that wildlife fish populations may be affected by pharmaceuticals even in the sub-ng/L range (Young *et al.* 2002). Most investigations in this field have been dedicated to the effects of pharmaceuticals on aquatic vertebrates, namely fish, in aqueous-exposure experiments. The potential impact on invertebrates is by far less regarded as is the role of sediment binding, despite the fact that pharmaceuticals are often at least moderately lipophilic so that they might pose a potential risk especially for sediment organisms and the benthic community. This was the rationale for the current study to focus on representatives from different aquatic invertebrate taxa including sediment-dwelling species. This publication is the first in a series that investigates effects of human pharmaceuticals on freshwater invertebrates in the laboratory.

Sediments can serve as a sink for chemicals that have a tendency to adsorb to particulate matter (Fiedler and Rösler 1993), but they also act as a reservoir from which chemicals can be remobilized by resuspension or desorption. The bioavailability of the mainly sediment-sorbed chemicals to biota is important because the sediment reservoir may constitute a primary source of contamination for benthic organisms. To characterize the toxic potential of sediments, mostly arthropods (*Chironomus tentans*, *C. riparius*, *Hexagenia limbata*, *Polypedilum nubifer*, *Hyalella azteca*) and annelids (*Limnodrilus hoffmeisteri*, *Lumbriculus variegatus*) are used (Henry *et al.* 1986; Phipps *et al.* 1993; Egeler *et al.* 1997, 1999; Ristola *et al.* 1999; Brooks *et al.* 2003).

In our study, the nonbiting midge *C. riparius* (Arthropoda: Insecta: Diptera) and the endobenthic freshwater oligochaete *L. variegatus* (Annelida: Clitellata: Oligochaeta) served as test organisms. Both species have been widely used as standard organisms in bioaccumulation and sediment studies (Phipps *et*

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Recent reports have shown that pharmaceuticals do occur not only in effluents of sewage treatment works but also in receiving surface waters (Ternes 1998; Sacher *et al.* 1998; Drewes *et al.* 2002; Heberer *et al.* 2002; Metcalfe *et al.* 2003a). The list of pharmaceuticals detected in aquatic ecosystems is steadily increasing; however, our knowledge regarding their potential effects on aquatic wildlife is rather incomplete. Some ecotoxicity studies were performed for the synthetic steroid ethinylestradiol, used in oral contraceptives (Schweinfurt *et al.* 1996; Belfroid and Leonards 1996), but

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*al.* 1993; Leppänen and Kukkonen 1998; Groenendijk *et al.* 1999; Oetken *et al.* 2004) as well as in artificial indoor streams (Brust *et al.* 2001). They feed on detrital organic matter and represent, together with the mudsnail *Potamopyrgus antipodarum* (Mollusca: Gastropoda: Prosobranchia), key phyla for the structure and function of aquatic ecosystems. We used *P. antipodarum* in aqueous-exposure experiments for this study. Mudsnails have been employed earlier in studies focused on chemically induced endocrine disruption and proved to be a sensitive bioindicator for the identification of compounds with either androgenic (Schulte-Oehlmann 1997; Duft *et al.* 2003a) or estrogenic activities (Schulte-Oehlmann *et al.* 2001; Duft *et al.* 2003b).

We report here on the effects of the antiepileptic drug carbamazepine (CBZ). These investigations are part of a larger project assessing the environmental impact of a range of pharmaceuticals. CBZ is prescribed at an amount of 78 t in Germany (Schwabe and Paffrath 2003). Recent studies have revealed the low biodegradability of the drug in sewage treatment (Ternes 1998; Clara *et al.* 2002, 2004; Heberer 2002), and the persistence of CBZ has also been confirmed by field studies (Lam *et al.* 2004). Hence, the drug has been detected in surface waters (Metcalf *et al.* 2003b; Scharf *et al.* 2004; Tixier *et al.* 2003; Wiegand *et al.* 2004). Heberer *et al.* (2002) found concentrations up to 1.1 µg/L in surface water samples in Berlin, Germany, whereas Sacher *et al.* (1998) and Ternes (1998) reported for the River Rhine a maximum CBZ concentration of 2.1 µg/L and a 90th percentile of 0.82 µg/L, respectively. Because of the properties of the compound (log  $K_{OW}$  = 2.45 and log  $K_{OC}$  = 3.59 as estimated by EPI-Suite Version 3.11 [United States Environmental Protection Agency 2000]), CBZ can be expected to adsorb to sediments. This assumption was confirmed in a study performed by Löffler *et al.* (2004). One hundred days after spiking CBZ by way of the water phase, the investigators found 40% of the compound in the sediment. Monitoring data on residues and distribution in sediments are not available with the exception of a study that analyzed CBZ in river sediments, the mean level of which was 4.16 µg/kg in 32 of 44 sediment samples (Furlong *et al.* 2004). Despite its wide occurrence in aquatic ecosystems, a recent literature review resulted in rare findings regarding the effects of CBZ on different organisms (Pflüger *et al.* 2000; Andreozzi *et al.* 2002; Cleuvers 2002; Ferrari *et al.* 2003, 2004). Most of these studies were acute-toxicity tests dealing with an exposure by way of water phase. Hence, data on chronic effects of CBZ, particularly on sediment organisms, are still lacking.

## Materials and Methods

### Test Substance

Carbamazepine [5H-dibenz[b,f]azepine-5-carboxamide; CAS No 298-46-4] was obtained from SigmaAldrich (Taufkirchen, Germany). Relevant physical and chemical properties are as follows: chemical purity >99%; water solubility 17.7 mg/L at 25°C; log  $K_{OW}$  2.45; and vapor pressure 1.84E-007 mm Hg (25°C). CBZ is licensed for use in newly diagnosed epileptic patients and in those whose epilepsy is uncontrolled by or who are unable to tolerate their current anticon-

vulsant therapy. CBZ is also used in the treatment of alcoholism (Sternebring *et al.* 1992) and management of opiate in opiate withdrawal (Montgomery *et al.* 2000). Bertschy *et al.* (1997) described the antidepressant efficacy for CBZ.

### Chemical Analysis

Samples (sediment and overlaying water) from the tests with *C. riparius* (series I through VI) were taken at day 0 measuring CBZ in 1 replicate/concentration. Up to 50 g sediment was successively extracted with organic solvents in an ultrasonic bath (45 ml methanol-ethyl acetate mixture, 50:50 v:v, followed by 3 × 45 ml ethyl acetate). The slurries of the solvent-sediment mixtures were ultrasonicated for 15 minutes and centrifuged for 7 minutes at 4,075 [xg], and the supernatant solvent phases filtered, combined, and evaporated in a gentle stream of nitrogen. Sediment extracts were dissolved in 3 ml methanol and diluted with 500 ml groundwater, which is known to be free of anthropogenic organic contamination (Löffler 2003).

Solid-phase extraction (SPE) was performed for the enrichment of water samples (Ternes 2001; Ternes *et al.* 2001) and served also as a cleanup step for aqueous sediment extracts (Löffler and Ternes 2003). Aqueous samples were filled up to 500 ml with groundwater and were adjusted, if necessary, to a neutral pH (7.0 to 7.5) with H<sub>2</sub>SO<sub>4</sub> (3.5 mol/l), and 10,11-dihydrocarbamazepine (Alltech, USA) was used as a surrogate standard. The glass cartridges were manually packed with 500 mg RP-C<sub>18</sub>sec material (ICT, Bad Homburg, Germany) and conditioned before sample extraction with 6 ml n-hexane, 2 ml acetone, 10 ml methanol, and 10 ml groundwater. Aqueous samples were passed through the SPE cartridges at a flow rate of 20 ml/min. Afterward, cartridges were dried with nitrogen for 1 hour and then eluted with 4 × 1 ml methanol. The extracts were evaporated to dryness in a gentle nitrogen stream, and the residue was finally dissolved in 50 µl methanol and 450 µl phosphate buffer (20 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>, pH 7).

Samples were separated on an Agilent 1100 high-pressure liquid chromatograph system at 25°C after filtration using a 100 × 4.6-mm Chromolith Performance RP-18ec 100 column (Merck, Darmstadt, Germany). Eluent A was prepared by adding 100 ml acetonitrile to 900 ml water containing 5 mmol/L ammonium acetate (pH 5.7), and eluent B consisted of 40% eluent A and 60% acetonitrile. A flow rate of 1 ml/min was chosen for the binary gradient. The eluent composition was 100% of eluent A held for 2 minutes, a linear gradient to 100% eluent B within 8 minutes, linear gradient back to 100% A within 0.5 minute, and 100% eluent A for 6.5 minutes. The injection volume was always 10 µl. CBZ was analyzed with an API 4000 triple-stage quadrupole mass spectrometer (MS) (Sciex, Concord, Canada) using electrospray ionization in the positive-ion mode at 750°C and an ionization potential of 5500 V. Detailed information on the MS parameters is given in Table 1.

Recoveries of CBZ in sediment and water samples were calculated relative to a nonenriched standard and ranged between 102% and 125%. For quantification of the water and sediment samples, calibration series of spiked groundwater, including a blank and a recovery sample, was used. The limit of quantification was 2 ng/L for water samples and 0.5 ng/g for sediment samples; signal-to-noise ratios were always >10.

### Test Organisms

The oligochaete *L. variegatus* has been widely used in sediment toxicity tests (Phipps *et al.* 1993; Leppänen and Kukkonen 1998; West and Ankley 1998; Egeler *et al.* 1997, 1999) and is recommended by the American Society for Testing and Materials (1995) as a standard

**Table 1.** LC-MS/MS conditions

Compound	MRM-transition (m/z)	Declustering potential (V)	Collision energy (eV)	Dwell time (ms)
Carbamazepine	237 > 194	71	27	75
	237 > 179	71	49	75
10, 11-Dihydrocarbamazepine	239 > 194	66	31	75
	236 > 180	66	55	75

LC = Liquid chromatography.

MS = Mass spectrometry.

organism to be used in bioaccumulation and sediment studies. *L. variegatus* was purchased from BIO-International (NJ Horn, The Netherlands). The oligochaetes were kept in continuously aerated tap water in 5-L glass aquaria at 20°C ± 1°C under a 16 hours light–8 hours dark photoperiod. For the breeding culture, an artificial sediment with the following particle sizes was used: 90 to 125 µm = 1%, 125 to 180 µm = 27%, 180 to 250 µm = 57%, 250 to 355 µm = 14%, and 355 to 500 µm = 1%. Worms were fed once a week with TetraMin fish food *ad libitum*.

*L. variegatus* is known to have remarkable regeneration capabilities. Phipps *et al.* (1993) determined a population-doubling rate of approximately 10 to 14 days at 20°C. The reproduction of this species follows mainly asexual propagations by self-fragmentation, termed “morphallaxis” (Brinkhurst and Jamieson 1971). According to Brust *et al.* (2001), this process starts with the loss of a posterior fragment by a darkly pigmented donor worm. This posterior fragment regenerates a new head within 10 to 14 days, whereas the donor worm generates a new tail. Regenerated parts are clearly recognizable because the new segments are unpigmented. Organisms from the stock culture were cut in the middle of their body to use worms of the same developmental and physiologic status. After 14 days, the posterior fragments were grown to complete worms, which then were used in the experiment.

Imagines of the nonbiting midge *C. riparius* usually breed within 24 hours after emergence. Females extrude gelatinous egg clutches into water, and larvae hatch after 2 to 4 days. They go through four instar stages living in close contact with sediment, pupate, and emerge within 13 to 25 days at 20°C, essentially evaluating a complete life cycle in 1 month. The brood stock was received from Bayer AG Leverkusen in November 1999. Larvae were kept in a controlled climate room at 20 ± 1°C under a cycle of 16 hours light–8 hours dark in glass beakers containing 1 cm sandy sediment (preponderant particle size = 200 µm) and aerated tap water. The beakers stood in a flight cage (70 x 50 x 45 cm). The water was renewed once per week, and the larvae were fed daily with TetraMin (1 mg/larvae/d).

*P. antipodarum* is a small freshwater snail with shell heights reaching up to 6 mm. The mudsnail was introduced from New Zealand to Europe in the mid-19th century, most probably in ballast water of ships. Its current distribution range in Europe covers the entire continent including the British Isles (Hubendick 1950). Furthermore, *P. antipodarum* was introduced to North America and can now be found in the Great Lakes (Strayer 1999). They inhabit the solid substrate of aquatic sediments, feeding on plants and detritus. In contrast to New Zealand populations, European populations consist almost exclusively of female individuals with a parthenogenetic and ovoviparous mode of reproduction. The anterior oviduct section is differentiated as a brood pouch containing young snails of different developmental stages (embryos without shell in the posterior and embryos with shell in the anterior part). Snails used in the experiments came from our own laboratory culture, which was built up with specimens collected from a small creek near Ibbenbüren, Germany. The breeding stock was located in a controlled climate room at 16°C ± 1°C under a cycle of 16 hours light–8 hours light in 10 L-

aquaria with reconstituted water (demineralized water, 0.5 g NaHCO<sub>3</sub>, 5 g CaCO<sub>3</sub>, 530 µS/cm TropicMarin mineral salt, pH 7.9). The organisms were fed daily with pulverized TetraPhyll.

### Acute-Toxicity Tests

The acute toxicity of CBZ for *L. variegatus* and *C. riparius* was determined by using 24-well multiplates as described by Brust *et al.* (2001). Organisms were exposed to CBZ by way of reconstituted water at concentrations of 0.5, 10, 200, and 4,000 µg/L with 20 replicate animals/treatment placed individually in each well (2 ml test solution). Neither test species was fed during the tests. The lethal end points for the 96-hour *L. variegatus* acute test were lysis and lack of blood circulation. The acute-toxicity test with first-instar larvae of *C. riparius* was conducted in the same way. Because first-instar larvae of *C. riparius* were considerably smaller compared with the oligochaetes, a 96-hour exposure could result in starving the larvae. Therefore, the experiment duration was limited to 24 hours. Lethal end points were immobility and/or lack of reaction to touching.

### Twenty-Eight-Day Sediment Tests with *C. riparius* and *L. variegatus*

The 28-day sediment tests were performed according to guideline 218 of the Organisation for Economic Co-operation and Development (OECD 2004). However, the sediment was made without kaolin, and other than the proposed carbon sources were used. Quartz sand for sediments was obtained from Quarzwerke Frechen (Germany). The grain size of test sediments was identical to that of the culturing substrate for *L. variegatus* previously described. Pulverized leaves of alder (*Alnus glutinosa*), 1.6% (dw), were added as carbon source to the sediments used with *L. variegatus*. In the experiment with *C. riparius*, 0.5% (dw) pulverized leaves each of stinging nettle (*Urtica dioica*) and alder (*Alnus glutinosa*) were used. Because of these carbon sources, it was not necessary to feed the animals during the experiments. The total content of organic carbon in the sediment per beaker was 1.36% for *L. variegatus* and 0.85% for *C. riparius*. The whole sediment was spiked with CBZ. Therefore, the test compound was solved in ethyl acetate to produce a concentrated stock solution. The treatments were spiked with CBZ, and the same volume of solvent was used for every treatment. The solvent control was spiked with an appropriate volume of solvent only. After the solvent had evaporated overnight, each beaker was filled with reconstituted water. Using this spiking method, CBZ was mixed homogeneously in the sediments. After spiking, the test system was incubated for 14 days under test conditions to ensure equilibration of the test substance between water and sediment. After this equilibration period, the exposure started with insertion of the test organisms (day 0). Experiments were terminated on day 28.

To evaluate reproducibility, the study with *C. riparius* was performed three times (series I through III) at  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$  under a cycle of 16 hours light–8 hours dark (light intensity 500 to 1000 lux). A further test (series IV) was conducted at  $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$  using the same light-to-dark cycle as in series I through III. The experiments were conducted in 600-ml glass beakers measuring 9 cm in diameter. The beakers contained 1 cm artificial sediment, corresponding to a total of 100 g dw, covered with 400 ml reconstituted water. Except for the organic carbon content, the same artificial sediment as in the tests with the oligochaete was used. The sediment was spiked with the following CBZ concentrations: 0.16, 0.8, 4, 20, and 100 mg/kg (series I) and 0.625, 1.25, 2.5, 5, and 10 mg/kg (series II through IV) expressed on a dw basis. The concentrations were referred to as treatments 0.16, 0.8, 4, 20, and 100 and 0.625, 1.25, 2.5, 5, and 10, respectively. All tests were run with four replicates including control and solvent control. In each beaker, a glass Pasteur pipette was fixed 2 to 3 cm above the sediment layer for gentle aeration. Twenty first-instar larvae were placed randomly into each test beaker. The emergence and the gender ratio were recorded daily, and the emerged adults were removed.

The test with *L. variegatus* was conducted in a controlled-climate room at  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$  under a cycle of 16 hours light to 8 hours dark (light intensity 500 to 1000 lux). For the experiment, 500-ml glass beakers measuring 8 cm in diameter and covered by a plastic screw cap were used. Each beaker was filled with 40 g dw artificial sediment and 200 ml reconstituted water. In this experiment, the same CBZ concentrations as in the experiment with *C. riparius* were chosen. Ten worms of the same developmental status were randomly placed into each test beaker. A glass Pasteur pipette was fixed with the plastic cap 0.5 cm above the sediment layer for gentle aeration. At the end of the test, the worms were removed from the sediment, and the number of worms and their biomass (dry weight) were recorded as end points.

#### Twenty-Eight-Day Water Test with *P. antipodarum*

The prosobranch snail *P. antipodarum* has successfully been used in a number of studies assessing the reproductive toxicity of freshwater sediments (Duft *et al.* 2002) and investigating the effects of endocrine-disrupting chemicals with either androgenic (Schulte-Oehlmann 1997; Duft *et al.* 2003a) or estrogenic activities (Schulte-Oehlmann *et al.* 2001; Duft *et al.* 2003b). In this study, the mudsnail was used to evaluate the reproductive toxicity of CBZ in an aqueous-exposure experiment.

Adults of *P. antipodarum* (shell height >3.7 mm) were exposed to CBZ by way of water at nominal concentration of 0.4, 2, 10, 50, and 250 mg/L. For each concentration, control, and solvent control (ethyl acetate), 3 replicates were used. The experiment was conducted in a 48-hour semistatic renewal system in 1-L Erlenmeyer flasks under constant conditions of  $16^{\circ}\text{C} \pm 1^{\circ}\text{C}$  under a cycle of 16 hours dark to 8 hours light. All snails were fed daily with TetraPhyll. At the beginning of the experiment (day 0), 80 snails/replicate were exposed to CBZ in the test vessels. The number of embryos (differentiated in individuals with and without shell) of 20 individuals/replicate in the brood pouch of each maternal snail was determined weekly. To count the embryos, the maternal snails were anesthetized in  $\text{MgCl}_2$  (2.5%), and the shell was broken to pieces (for details see Duft *et al.* 2003a, b).

#### Statistical Analysis

Statistical analysis was performed using Statistica 5.0 and SPSS 6.1 software. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined by analyses of variance (one-way) followed by Tukey Honestly Significant Difference test as a post-hoc comparison. LC(x) and EC(x)

**Table 2.** Twenty-eight-day-sediment toxicity test with *C. riparius* (series III)<sup>a</sup>

Nominal concentration (mg/kg dw)	Measured concentrations	
	Sediment (mg/kg dw)	Overlying water (mg/L)
Control	ND	ND
Solvent control	ND	ND
0.625	0.140	0.164
1.25	0.234	0.332
2.5	0.467	0.606
5	0.722	1.22
10	2.90	2.85

<sup>a</sup>Measured concentrations of carbamazepine on day 0 in sediment and overlying water.

ND = not detected (detection limit in water = 2 ng/L and in sediment = 2 µg/kg dw.  
dw = dry weight).

concentrations were calculated by probit transformation (Litchfield and Wilcoxon 1949). To compute differences in gender ratio, chi-squared analysis of the 2 x 2 table test was performed. The limits of significance ( $p$  value  $\leq 0.05$ ) were taken from a statistical benchmark (Sachs 1992).

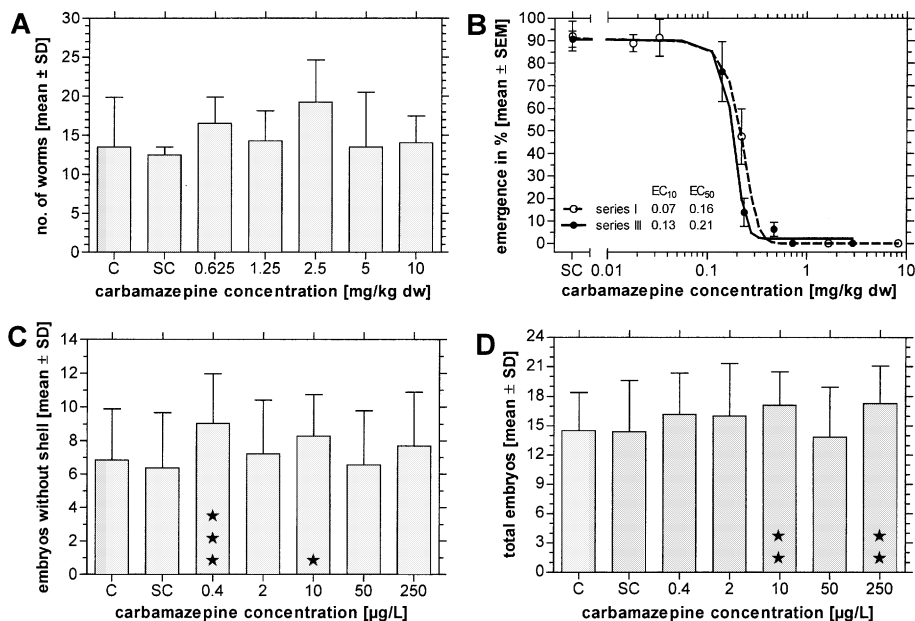
## Results and Discussion

### Acute-Toxicity Test

CBZ did not cause any acute toxic effect on the test organisms at the tested concentration range of up to 4 mg/L. This result is in line with literature data. Andreozzi *et al.* (2002) found under static conditions no inhibition of growth on the green algae *Selenastrum capricornutum* at a concentration range from 2.1 µg/L to 20 mg/L. Cleuvers (2002) estimated for the green algae *Desmodesmus subspicatus* a median effect concentration (EC<sub>50</sub>) of 85 mg/L for the same end point and for the water flea *Daphnia magna* an EC<sub>50</sub> of 157 mg/L. Ferrari *et al.* (2004) determined an LC<sub>50</sub> (48 hours) of 77.7 mg/L for the same species. The findings indicate that CBZ seems not to be acutely toxic at environmentally relevant concentrations. However, these data might be helpful in the interpretation of the results of the 28-day sediment toxicity tests.

### Chemical Analysis

Table 2 lists measured CBZ concentrations at day 0 in the sediment and in the overlying water for the test series III with *C. riparius* as an example. The chemical analysis of the other test series gave comparable results (data not shown). Although in the control and solvent control CBZ was not found, the concentrations in the treated sediments varied from 0.14 to 2.9 mg/kg CBZ, corresponding to 14% to 29% of nominal values. The analysis of sediment samples taken on days 0 and 28 showed no indication for a decrease in test compound concentrations during the experiment. At day 28, between 69% and 109% of the measured initial concentrations were still present in the sediments. Also, in the overlying water phase,



**Fig. 1.** Effects of carbamazepine exposure on *L. variegatus*, *C. riparius*, and *Potamopyrgus antipodarum* (C and D). (A) Number of *L. variegatus* worms at day 28 (SD; n = 4). (B) Concentration response curves for *C. riparius* emergence in test series I and III at day 0 (SD; n = 4). Number of *Potamopyrgus antipodarum* embryos without shell (C) and total number of *Potamopyrgus antipodarum* embryos (D) in the brood pouch of maternal snails at day 28 (n = 3). \**p* < 0.05. \*\**p* < 0.01. \*\*\**p* < 0.001. C = control; SC = solvent control.

there was no indication for a decrease in CBZ concentrations within the 28-day test period. These observations are supported by a report on the persistence of CBZ in sediment and water systems (Löffler *et al.* 2004). Therefore, biologic effects in the sediment tests are related to measured initial CBZ concentrations in sediment.

#### Twenty-Eight-Day- Sediment Toxicity Test with *L. variegatus*

On day 28, mean values of pH, dissolved oxygen, and conductivity in all controls and treatments in the water ( $\pm$  SD) were as follows: pH  $8.68 \pm 0.2$ , dissolved oxygen  $7.9 \pm 0.43$  mg/L, and conductivity  $871 \pm 94$   $\mu$ S/cm. As summarized in Figure 1A, the mean number of worms on day 28 ranged from 12.5 (solvent control) to 19.25 (treatment 2.5). Because the test started with 10 individuals/group on day 0, an evident reproduction occurred in the controls and in all treatments. However, the reproduction rate was low compared with that seen in other studies. Oetken *et al.* (2001) found within a comparable sediment toxicity test for the solvent control a reproduction factor of 3 for this species. In the present study, neither a significant increase nor decrease in the number of worms compared with the controls was observed. Furthermore, biomass per worm was also not affected by the test compound (data not shown). Hence, effects caused by the antiepileptic drug could be found neither on the reproduction nor on the biomass of the test organisms.

#### Twenty-Eight-Day Sediment-Toxicity Test with *C. riparius*

Emergence of imagines as an end point has been used in numerous studies with *Chironomus* spp. (Hatakeyama 1987; Timmermans 1991; Ristola *et al.* 1999). In test series I through

IV, all criteria of validity (OECD 2004) were fulfilled. On day 28, mean values ( $\pm$  SD) in the water of all controls and treatments were as follows: pH  $8.67 \pm 0.15$ , dissolved oxygen  $7.99 \pm 0.48$  mg/L, and conductivity  $1347 \pm 170$   $\mu$ S/cm.

In series I applying a concentration range of 0.16 to 100 mg/kg dw (nominal concentrations), the total emergence of *C. riparius* ranged from 47.5% in treatment 4 to 92% in the control (Table 3). In the two highest concentrations (20 and 100 mg/kg dw, respectively), no emergence was observed. A significant decrease in emergence was calculated for treatments 4, 20, and 100 (Table 3). Hence, for this end point NOEC and LOEC values of 0.8 mg/kg dw and 4 mg/kg dw, respectively, were determined based on nominal CBZ concentrations. Table 4 lists the NOEC, LOEC, EC<sub>10</sub>, and EC<sub>50</sub> values based on measured concentrations. The results for the four test series were consistent and indicated a negative impact of CBZ on emergence of midges at sediment concentrations >70  $\mu$ g/kg (EC<sub>10</sub> in series I). Specifically, we observed a blockade of pupation in CBZ-exposed *C. riparius* larvae such that they survived up to 4 weeks without undergoing pupation until they finally died. Figure 1B provides concentration response curves for CBZ in the *C. riparius* test using the examples of test series I and III. The suppression of pupation in *C. riparius* under CBZ exposure refers to a specific mode of action, most probably some interference with a physiologic pathway first activated in this life stage or to some modulation of endocrine functions. In this case, CBZ could not only pose a hazard for chironomids, it could also be dangerous to other aquatic insects. Clearly, this assumption needs further experimental validation.

In all experiments with *C. riparius*, the gender ratio (male-to-female) was between 0.65 (control in series III) and 4.0 (treatment 2.5 in series III), and there were no significant differences between the control and solvent control and the treatments (chi-squared analysis *p* < 0.05). McCahon and Pascoe (1991) and Hatakeyama (1987) described that heavy metals such as cadmium did not affect the gender ratio of

**Table 3.** Sediment-toxicity tests with CBZ using *C. riparius*<sup>a</sup>

Temperature	Series	Control	SC	Carbamazepine (mg/kg dw)					
				0.16	0.8	4.0	20	100	
20°C	I	88.0 (± 12.6)	92.0 (± 13.2)	88.8 (± 7.5)	91.3 (± 16.5)	47.5* (± 24.7)	0* (-)	0* (-)	
		Control	SC	Carbamazepine (mg/kg dw)					
	II	83.6 (± 17.6)	85.0 (± 7.07)	55.0* (± 10.8)	63.8* (± 8.54)	0* (-)	0* (-)	0* (-)	
		III	86.7 (± 23.1)	90.6 (± 7.12)	76.2 (± 26.5)	13.8* (± 12.5)	6.25* (± 6.29)	0* (-)	0* (-)
			Control	SC	Carbamazepine (mg/kg dw)				
			0.625	1.25	2.5	5.0	10		
23°C	IV	86.0 (± 15.5)	82.5 (± 11.9)	93.8 (± 2.5)	50.0* (± 20.4)	1.25* (± 2.5)	0* (-)	0* (-)	

<sup>a</sup>Mean emergence in patient (± SD,  $n = 4$ ) observed during four test series (I through IV) after 28-day exposure (nominal concentrations in mg/kg dw).

\*Significant to the solvent control;  $p < 0.005$ .

CBZ = carbamazepine.

SC = solvent control.

**Table 4.** Sediment-toxicity tests with CBZ using *C. riparius*<sup>a</sup>

Temperature	Series	Nominal concentration range (mg/kg dw)	NOEC	LOEC	EC <sub>50</sub> (95% confidence interval)	EC <sub>10</sub>
20°C	I	0.16–100	0.033	0.22	0.16 (0.13 – 0.19)	0.07
	II	0.625–10	< 0.14	0.14	0.19 (0.18 – 0.21)	0.14
	III	0.625–10	0.14	0.234	0.21 (0.19 – 0.23)	0.13
23°C	IV	0.625–10	0.14	0.234	0.28 (0.26 – 0.30)	0.21

<sup>a</sup>Effect concentrations for the end point total emergence referred to measured concentrations at day 0 (mg/kg dw).

CBZ = carbamazepine.

EC = median effect concentration.

LOEC = lowest observed effect concentration.

NOEC = no observed effect concentration.

chironomids. Also, for other insects there are no published reports on effects on gender ratio. After application of the insecticide fenvalerat on *Limnephilus lunatus* (Trichoptera), no significant differences in the gender ratio of the control compared with the treatment groups were observed (Schulz 1997).

The European Agency for the Evaluation of Medicinal Products (2003) proposed a scheme for an environmental risk assessment for pharmaceuticals based on a two-phase tiered assessment concept. Phase I consists of a crude initial predicted environmental concentration (PEC) assessment of the substance in surface water (PEC<sub>sw</sub>) based on the maximum daily dose of the active ingredient of the pharmaceutical. In phase II tier B, sediment tests are performed in case there is an extensive shift of the substance into the sediment. In this context, Löffler (2003) showed in a water–sediment study according to the OECD guideline 308 (OECD 2002) the high persistence as well as an “extensive shifting” of CBZ from the water phase into the sediment. Therefore, sediment toxicity assessment for CBZ is required (EMEA 2003). Based on the PEC<sub>sw</sub>, K<sub>OC</sub>, and the calculation provided by the European Union Technical Guidance Document (EU 2003), Liebig (2005) calculated a PEC<sub>sediment</sub> of 43.9

µg/kg dw for carbamazepine. Regarding the NOEC <0.14 mg/kg dw (series II) from our study and using an assessment factor of 50 (EU 2003), the PEC/(predicted no effect concentration) PNEC ratio is 15.7, indicating a risk for the compartment sediment.

#### Twenty-Eight-Day Water-Toxicity Test with *P. antipodarum*

Although at the beginning of the experiment the mean number of embryos (with and without a shell) in the brood pouch of each maternal snail was determined to be 13.7, the number of embryos decreased slightly after an exposure time of 1 week independent of the CBZ exposure. During the further course of the experiment, the number of embryos increased in the controls as well as in all treatment groups. At the end of the experiment (day 28), single treatments were characterized by a significantly higher number of embryos without shell (0.4 and 10 µg/L; Figure 1C) or the total number of embryos (10 and 250 µg/L; Figure 1D). However, these effects do not follow a monotonic or nonmonotonic (J-, U- or inverted U-shaped)

concentration response, so there is no evidence for an impact of the test compound on this parameter. In fact, the moderate increase in the number of embryos toward the end of the experiment reflects the seasonal changes of embryo numbers during the reproductive cycle of *P. antipodarum*. Schulte-Oehlmann (1997) found maximum embryo numbers in May and June and a minimum in November and December for this species. The present experiment was performed in early spring, and therefore seasonal effects may have caused the increase in the number of embryos.

## Conclusion

CBZ is characterized by low acute toxicity to aquatic organisms, particularly invertebrates. However, in chronic sediment-exposure experiments, we found a significant and specific effect: a blockade of pupation and emergence in the nonbiting midge *Chironomus riparius* with EC<sub>10</sub> values 70 to 210 µg/kg dw. Although there are currently no sediment concentrations for CBZ available in the literature, Furlong *et al.* (2004), reported CBZ at a mean level of 4.16 mg/kg from 32 of 44 sediment samples. Hence, it is likely that the drug may pose a risk for the survival of populations of benthic insects in the field.

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