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## Chapter 16

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# Effects of Pharmaceuticals on Aquatic Invertebrates – The Example of Carbamazepine and Clofibric Acid

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## 16.1 Introduction

Pharmaceuticals and their metabolites are widely distributed in the aquatic environment (Kümpel et al. 2001, Tixier 2003). Concerns about potential ecological effects have been raised because these compounds are usually highly active, with the example of ethinylestradiol and other estrogens demonstrating that such compounds may cause effects already at concentrations between 1 and 10 ng l<sup>-1</sup> (Routledge et al. 1998). Though many data about the occurrence have been collected, not much is known in fact about possible impact on aquatic wildlife.

Actually, mostly acute tests are used to evaluate the risk of pharmaceuticals for the environment. Effects occurring in these tests are often obtained at non-environmentally relevant concentrations. Potential risks are assessed by calculating with risk factors ranging up to 25,000 (Hanisch et al. 2002). Furthermore, these tests do only account for the aquatic phase.

The approach of the present study is to investigate whether exposure via sediment shows any effects of pharmaceuticals which were not revealed by aqueous exposure. Sediments can serve as a sink for xenobiotics that are lipophilic (Prorsi and Müller 1987; Fiedler and Rösler 1993). In the same way, they act as a reservoir from which sediment-bound chemicals can be remobilized when the sediment is churned up (Kram et al. 1989). Their role in binding and releasing of potentially harmful substances in the environment is actually underestimated as most studies focus on water exposure. Especially as pharmaceuticals are mostly complex molecules with several functional groups (see Chap. 2), a negative log  $P_{ow}$  is not a good measure for the distribution of the compound between water and sediment. For the current approach, to assess the effects of representatives from different classes of pharmaceuticals, carbamazepine and clofibric acid were chosen as test compounds (Fig. 16.1).

Carbamazepine is used widely as an antiepileptic agent for newly diagnosed cases of epilepsy and for patients who cannot tolerate their current therapy. It is also used in depression treatment (Kudoh et al. 1998), in opiate and alcohol withdrawal management (Bertschy et al. 1997; Sternebring et al. 1992). It is prescribed in amounts of about 80 tons per year in Germany (Schwabe and Paffrath 2003).

Clofibric acid is the active metabolite of the lipid lowering compounds clofibrate, etofibrate and etofyllinclofibrate (Mutschler 1991). Though the use of these lipid regulators decreases, clofibric acid is still detected in the environment (Hanisch et al. 2002).

Carbamazepine and clofibric acid have been detected in many environmental samples. The concentrations measured are quite high, reaching up to 2.1 µg l<sup>-1</sup> for carbamazepine in rivers and streams (Sacher et al. 1998, Ferrari et al. 2003). These values

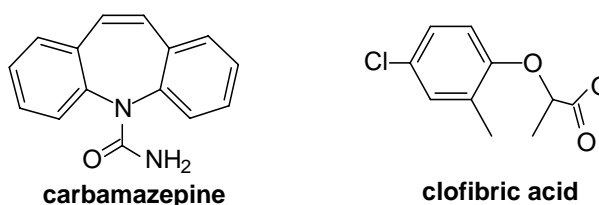
occurred 1996 in the river Rhine due to the discharge of untreated industrial wastewater. As the discharger has improved his wastewater treatment, the values are lower now with maximum concentrations of  $0.22 \mu\text{g l}^{-1}$  in 2000 (RIWA 2002). Ternes (1998) detected 90-percentiles of  $3.7 \mu\text{g l}^{-1}$  in sewage effluents and  $0.82 \mu\text{g l}^{-1}$  in rivers and streams for carbamazepine as well as 90-percentiles of  $0.72 \mu\text{g l}^{-1}$  in sewage effluents and  $0.21 \mu\text{g l}^{-1}$  in rivers and streams for clofibric acid.

Acute aqueous exposure tests with daphnids and algae did not indicate any risk for carbamazepine and clofibric acid at environmentally relevant concentrations. Clofibric acid showed a reproductive toxicity on daphnids with a LOEC (lowest observed effect concentration) of  $10 \mu\text{g l}^{-1}$ , which is rather close to measured concentrations. Hanisch et al. (2002) considered this substance as a possible risk.

The objective of the present study was therefore not only to perform additional acute toxicity tests with both compounds at environmentally relevant concentrations, but also to determine whether exposure in chronic or life cycle tests would reveal any effects. Because several studies have shown that pharmaceuticals can also bind to sediments (Drewes et al. 2002; Heberer et al. 2002) and the physicochemical properties of both compounds, particularly the  $\log P_{\text{OW}}$  (Table 16.1), indicate that carbamazepine and clofibric acid could bind to the sediment, this route of exposure was also considered in the current approach.

The non-biting midge *Chironomus riparius* and the oligochaete *Lumbriculus variegatus* were the two test species used for the aqueous exposure acute toxicity tests and also for the chronic sediment assays. Both test organisms have been widely used in sediment toxicity tests (OECD 2001, Phipps et al 1993, West et al 1998). Chironomids play an important role in the food web, serving as food for many fishes and other aquatic organisms. They are frequently the most abundant insect species in freshwater ecosystems (15,000 species are estimated worldwide, 1,000 species in Europe) and

**Fig. 16.1.** Chemical structure of the test compounds



**Table 16.1.** Physicochemical characteristics of carbamazepine and clofibric acid (Syracuse Physprop Database 2003)

	Molecular weight	Solubility in water ( $\text{mg l}^{-1}$ )	Melting point ( $^{\circ}\text{C}$ )	Log $P_{\text{OW}}$	Vapor pressure (mm Hg (25 $^{\circ}\text{C}$ ))	Henry's Law constant ( $\text{atm}^{-1} \text{m}^3 \text{mole}^{-1}$ )
Carbamazepine	236.28	17.7	190.2	2.45	1.84E-007	1.08E-010
Clofibric acid	214.65	583	118-119	2.57	0.000113	2.19E-008

their larvae serve as food for many aquatic organisms. Therefore, it is important to monitor effects on these midges as adverse effects on chironomids may lead to a food shortage in aquatic ecosystems. This could possibly endanger wildlife of rivers and lakes. Aquatic oligochaetes also serve as food for predators in aquatic ecosystems, particularly fish. They are possibly affected by sediment-bound pollutants as they ingest the complete sediment while feeding. Therefore, it is of interest whether these organisms are affected by pharmaceuticals.

As acute test in the present study, a 24 h-mortality assay using first instars of *C. riparius* and a 96 h-mortality test with adults of *L. variegatus* was applied. For the 28 d sediment assays *C. riparius* was used in a life-cycle test and *L. variegatus* in a chronic reproduction test. In addition to these two sediment tests, the toxicity via aqueous exposure was assessed in a further chronic reproduction test with the freshwater mud snail *Potamopyrgus antipodarum*. This plant- and detritus-feeding species has been introduced in aquatic ecotoxicology while performing studies on endocrine disruption as effects are easy to determine by counting the embryos that the snails carry in their brood pouches. (Schulte-Oehlmann 1997; Duft et al. 2003).

## 16.2 Materials and Methods

Carbamazepine was purchased from Sigma-Aldrich, Taufkirchen, Germany and clofibric acid from Acros Organics, Geel, Belgium. All test organisms (*Chironomus riparius*, *Lumbriculus variegatus* and *Potamopyrgus antipodarum*) were bred from our own laboratory culture.

The acute tests were performed using 96-well-microtiter plates. Eight worms or eight larvae (1st instars), respectively, were used per treatment, one per well. The acute toxicity of carbamazepine was assessed at the following concentrations: 0.5, 10, 200, and 4 000  $\mu\text{g l}^{-1}$ . For clofibric acid, the following concentrations were used: 0.05, 1, 20, and 400  $\mu\text{g l}^{-1}$ . The first concentration of each compound reflects the median environmental concentration. It was raised by a factor of 20 in each of the following treatments. If an effect had been detected at these concentrations, the compound would, calculating with an adequate assessment factor, probably pose a risk to the test organism. The acute toxicity on *L. variegatus* was determined during 96 h. For *C. riparius*, the testing time had to be reduced to 24 h because newly hatched larvae starve after this time. Lethal endpoints were lysis and lack of blood circulation for *L. variegatus* and lysis, immobility and lack of reaction for *C. riparius*.

For the chronic sediment tests, artificial sediment was used according to OECD (2001). Kaolin was admittedly not added. The sediment consists mainly of quarry sand. The particle sizes were as follows: 90-125  $\mu\text{m}$ : 1%, 125-180  $\mu\text{m}$ : 57%, 250-355  $\mu\text{m}$ : 14% and 355-500  $\mu\text{m}$ : 1%. Additionally, 1.6% by weight of ground leaves of alder were added to the sediment for the *L. variegatus* assay and 1% of a mixture of ground alder- and stinging-nettle-leaves for the *C. riparius* assay. Stinging-nettle and alder leaves were used instead of *Sphagnum* moss peat. As the aim was to create a sediment similar to that found in real rivers, plants that grow on the banks should serve as carbon source. For the same reason, no kaolin was used. The content of fine particles would have been too high, limiting the bioavailability of the test compound.

Due to this addition of organic carbon, feeding was not necessary during the test.

The bulk sediment was spiked with the test chemicals to obtain the following concentrations: 0.625, 1.25, 2.5, 5 and 10 mg kg<sup>-1</sup> on a dry weight base for carbamazepine. For clofibrac acid, the following, dry-weight-based concentrations were used: 0.1, 0.3, 0.9, 2.7 and 8.1 mg kg<sup>-1</sup>. These concentrations were calculated according to the aqueous concentrations in the following manner: The maximum aqueous concentration value was multiplied by the product of the log P<sub>OW</sub> to estimate the sediment concentration. The aqueous concentrations used as the basis for the calculation of predicted sediment concentrations were 2.1 µg l<sup>-1</sup> for carbamazepine and 0.2 µg l<sup>-1</sup> for clofibrac acid according to Sacher et al. (1998). All concentrations provided here are nominal concentrations. Ethyl acetate was used as a solvent. After spiking of the bulk sediments with the dissolved test compounds, the solvent was evaporated to dryness and finally, water was added to the test vessels. For the *C. riparius* test 100 g sediment was used for each replicate and covered with 400 mL water, for the *L. variegatus* assay the respective amounts were 40 g sediment and 200 mL water. The vessels were aerated and the sediment was aged two weeks before test organisms were added to ensure equilibration of the test compound between water and sediment.

For the test with *L. variegatus*, it had to be ensured, that all worms were at the same developmental stage. Therefore, the worms were cut into half four weeks before the begin of the test. As *L. variegatus* reproduces asexually by morphallaxis, this procedure imitates the natural reproduction. Within four weeks, the headed half of the worm regenerates a new tail and the tail regenerates a new head (Brust et al. 2001). Only the posterior fragments were used for the test. When inserted to the test vessels, the worms had just completed the regeneration of the head. Ten worms were added to every test vessel (day 0). For each test concentrations and the controls four replicates were considered. Effects on *L. variegatus* were assessed by counting the worms at the end of the test (day 28) and by measuring the biomass of the worms.

For the chronic 28 d sediment toxicity test with *C. riparius*, which is often also referred to as a life cycle test, larvae were given into the test immediately after hatching as first instars. Per vessel, twenty larvae were added. For each test concentration and the controls four replicates were considered. Effects on *C. riparius* were determined by daily control of the emergence. The total emergence was documented. The life cycle test with *C. riparius* was conducted four times. The first series was a range-finder covering a larger range of carbamazepine concentrations (0.16, 0.8, 4, 20, and 100 mg kg<sup>-1</sup> dw) while for the remaining three series the above mentioned concentrations between 0.625 and 10 mg kg<sup>-1</sup> dw were used. The first three series were conducted at 20°C ± 1°C, the OECD-recommended temperature. Series IV was conducted at 23°C ± 1°C to assess whether a higher temperature had an additional influence.

Additionally to these two sediment assays an aqueous exposure reproduction test with the prosobranch snail *P. antipodarum* was applied. 80 snails were held in 1 L-Erlenmeyer flasks which were aerated via glass pipettes. Test organisms were fed twice a week and the test medium was also renewed twice a week. The concentrations of the test chemicals were 0.4, 2, 10, 50 and 250 µg l<sup>-1</sup> for carbamazepine and 0.04, 0.2, 1, 5 and 25 µg l<sup>-1</sup> for clofibrac acid. Ethanol was used as solvent. The concentrations were calculated in a way similar to those for the acute tests with the second lowest approximately reflecting the maximum aqueous concentration measured (Sacher et al. 1998; Ternes 1998). The concentration range in the reproduction tests were chosen lower than those in the acute tests because the objective of these tests was to detect ef-

fects at environmentally relevant concentrations. As the reproduction tests aim at effects occurring during chronic exposure, the effects on the endpoint reproduction could therefore be assessed in a lower concentration range.

To determine potential effects on reproduction of *P. antipodarum*, the embryos in the brood pouch were counted. Therefore, 20 snails were taken from the test vessels in weekly intervals over a period of 4 weeks (carbamazepine test) or 2, 4, and 8 weeks after the start of the experiment (clofibric acid test). They were narcotized in a 2.5% solution of  $MgCl_2$ , the shell was broken and the embryos in the brood pouch were counted (for details cf. Duft et al. 2003).

Statistical analysis was performed using Statistica 5.0 and SPSS 6.1 Software. For normality, data were analyzed using the Kolmogorov-Smirnov-Test. The homogeneity of variances was estimated with the Cochran-Test. NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values were calculated via an one-way-ANOVA followed by a Tukey-HSD-Test as post-hoc-comparison. In case of not meeting the requirement for parametric tests, data were analyzed using the Kruskal-Wallis-Test with the Nemenyi-Test as post-hoc comparison. Probit transformation was used to determine  $EC_x$  and  $LC_x$ -values.

## 16.3 Results and Discussion

### 16.3.1 Acute Tests

Aqueous exposure of *Lumbriculus variegatus* and *Chironomus riparius* to carbamazepine and clofibric acid during the acute test did not result in an increased mortality for any of the considered concentrations in the tested range. Therefore, the acute tests determined a  $LC_{50}$  of  $> 4 \text{ mg l}^{-1}$  for carbamazepine and of  $> 0.4 \text{ } \mu\text{g l}^{-1}$  for clofibric acid in both species (Table 16.2). No toxic effects occurred at these concentrations.

Acute toxic effects in the tests with *L. variegatus* and *C. riparius* are far beyond environmental relevance. As still no toxic effects were observed in the tested concentration range, it can be stated that both compounds do not pose an acute hazard when occurring in the amounts normally measured in the environment. The lowest toxic concentrations measured in the past were  $85 \text{ mg l}^{-1}$  for carbamazepine and  $145 \text{ mg l}^{-1}$

**Table 16.2.** *Lumbriculus variegatus* and *Chironomus riparius*. Acute toxicity ( $LC_{50}$ ) of carbamazepine and clofibric acid

Species	Exposure time [h]	$LC_{50}$ [ $\text{mg l}^{-1}$ ]	
		Carbamazepine	Clofibric acid
<i>L. variegatus</i>	96	$> 4$	$> 0.4$
<i>C. riparius</i>	24	$> 4$	$> 0.4$

for clofibrilic acid (Cleuvers 2002). These values are  $EC_{50}$  values in an algae test. As long as there are only few ecotoxicological data for the compounds, environmental risks have to be calculated with high assessment factors. Although it is clear that no acute toxic effects can be expected at environmentally relevant concentrations, based on the currently available data, further research is needed for a more appropriate evaluation of the effects of both pharmaceuticals.

### 16.3.2

#### Life-Cycle-Test and Reproduction Tests

##### 16.3.2.1

##### **Carbamazepine**

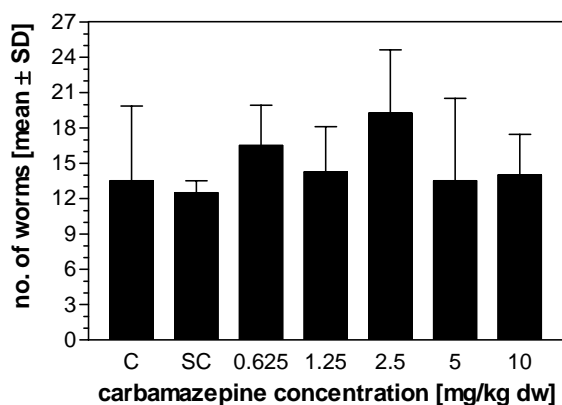
Concentrations for the sediment tests with *Lumbriculus variegatus* and *Chironomus riparius* were calculated according to the EU Technical Guidance Document (EU 1996). The test concentrations had to be calculated from water concentrations by using  $\log P_{OW}$  (Sacher et al. 1998) as analytical data on residues in sediments are still missing.

The sediment was spiked by preparing a stock solution of carbamazepine. By solving the required amount of stock solution in 33 ml ethyl acetate and soaking the sediment with this solution, the test compound was distributed evenly. It can be assumed that the whole sediment was spiked.

*Lumbriculus variegatus*. As demonstrated in Fig. 16.2, the numbers of *L. variegatus* were comparable between all treatment groups, irrespective of the applied carbamazepine concentration with no statistically significant differences ( $p > 0.05$ , one-way-ANOVA). This indicates that the vegetative mode of reproduction is not affected in this annelid in the tested concentration range. Likewise the biomass of all worms in the different samples did not show any significant difference when compared to the controls. Consequently, there is no evidence for a potential hazard originating from this antiepileptic drug to *L. variegatus* in the tested concentration range.

Carbamazepine did not show negative effects on *L. variegatus*, although it has to be taken into account that the reproduction factors of 1.4 up to 2 in the carbamazepine test

**Fig. 16.2.** *Lumbriculus variegatus*. Chronic 28 d sediment toxicity test. Test substance: carbamazepine. Mean number of individuals at the end of the test ( $\pm SD$ ). C, control; SC, solvent control

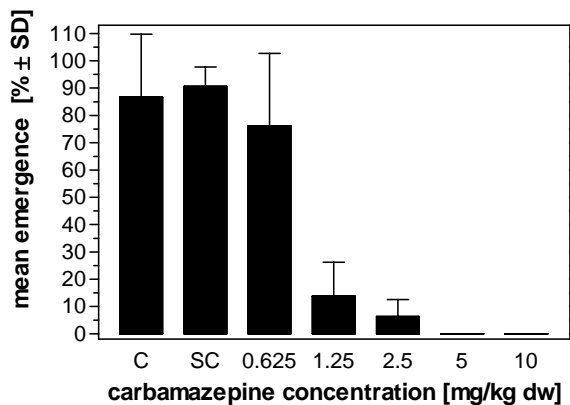


are generally lower than in other studies exhibiting an increase of the number of worms in the controls by a factor of up to 4 (Brust et al. 2001; Oetken et al. 2001). If carbamazepine had a negative effect on the reproduction of *L. variegatus*, the control treatments should show a better reproduction than the spiked treatments.

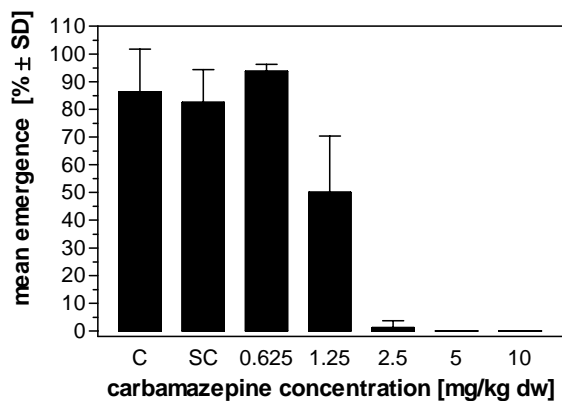
This was not the case. No effect on biomass was observed. A possible carbamazepine effect could have been the inhibition of food uptake due to repellent effects or due to a general reduction of activity for example. Based on these results, there is no evidence that *L. variegatus* might be affected by carbamazepine.

*Chironomus riparius*. The validity criteria according to the OECD guideline 218 (OECD 2001) were fulfilled: Emergence started at day 15, the mortality in the controls did not exceed 30% (Figs. 16.3 and 16.4). Oxygen content, pH and water temperature also stayed in the prescribed range. Figure 16.3 demonstrates that carbamazepine leads to a significant reduction of the emergence of *C. riparius*. At a sediment concentration of 1.25 mg kg<sup>-1</sup> dw, the emergence is significantly reduced compared to the solvent control, followed by an inhibition of more than 90% at 2.5 mg kg<sup>-1</sup> dw and a total inhibition at higher treatments ( $p < 0.05$ , one-way-ANOVA). Thus, the NOEC was calculated to 0.625 mg kg<sup>-1</sup> dw, the LOEC to 1.25 mg kg<sup>-1</sup> dw. At the highest carbamazepine

**Fig. 16.3.** *Chironomus riparius*. Chronic 28 d sediment toxicity test at 20°C (test series III). Test substance: carbamazepine. Mean emergence (%;  $\pm SD$ ). C, control; SC, solvent control



**Fig. 16.4.** *Chironomus riparius*. Chronic 28 d sediment toxicity test at 23°C (test series IV). Test substance: carbamazepine. Mean emergence (%;  $\pm SD$ ). C, control; SC, solvent control



pine concentrations the development was arrested in the fourth larval stage, disabling the formation of the pupae. Even in those cases, where the pupae were formed, imagines did not hatch. At all concentrations higher than  $0.625 \text{ mg kg}^{-1} \text{ dw}$ , the larvae were observed lying on the surface of the sediment and wincing in a convulsive manner. This observation indicates that carbamazepine may interact with a specific metabolic process that is required for further development from the fourth larval stage to the pupa and the imago. Carbamazepine could inhibit this process so that the pupa cannot be formed anymore. Alternatively, carbamazepine may act as an endocrine disruptor, probably interfering with synthesis, bioavailability or breakdown of juvenile hormones or ecdysteroids or by binding to their receptors.

The results of the four repeats of the life cycle test with *C. riparius* and carbamazepine are summarized in Table 16.3.

Obviously, carbamazepine inhibited the emergence in all series. The NOEC was calculated to  $0.8 \text{ mg kg}^{-1} \text{ dw}$  and the LOEC to  $4.0 \text{ mg kg}^{-1} \text{ dw}$  in series I and to  $0.625 \text{ mg kg}^{-1} \text{ dw}$  and  $1.25 \text{ mg kg}^{-1} \text{ dw}$ , respectively for series II to III. These results were confirmed in an assay that was conducted at  $23^\circ\text{C}$  (series IV, Fig. 16.4).

As already described, the compound acts in a very specialized manner indicating that a specific mode of action in *C. riparius* can be expected. The result shows that pharmaceuticals produced for human use can also affect invertebrates. Because in mammalian species carbamazepine acts in a receptor-mediated manner by inhibiting neurotransmitters (Ambrosio et al. 2002), it is possible that it can also bind to a receptor in chironomids and thus interfere with the activity of hormones.

*Potamopyrgus antipodarum*. Reproduction in *P. antipodarum* was not affected by carbamazepine. Fig. 16.5 indicates that the mean values for total embryo numbers do not differ significantly from the solvent control. The slight increase during the course of the experiment can be explained by the marked seasonality in the reproductive cycle of *P. antipodarum* (Schulte-Oehlmann, 1997). The maximum embryo numbers in the brood pouch can be found from March to August with a consequent drop to the lowest embryo numbers in November and December. The current experiment started in late winter during a phase of the reproductive cycle when embryo numbers generally In-

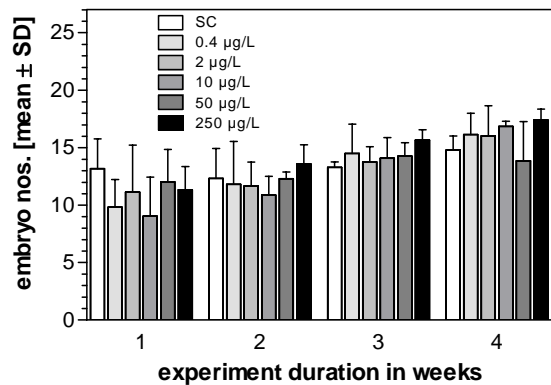
**Table 16.3.** *Chironomus riparius*. Results of the four 28 d sediment toxicity test series. Mean emergence (%;  $\pm SD$ ) after an exposure to different nominal carbamazepine concentrations

Temp.	Series	Control	SC	Mean emergence (%)				
Carbamazepine ( $\text{mg kg}^{-1} \text{ dw}$ )				0.16	0.8	4.0	20	100
20°C	I	88.0 ( $\pm 12.6$ )	92.0 ( $\pm 13.2$ )	88.8 ( $\pm 7.5$ )	91.3 ( $\pm 16.5$ )	47.5* ( $\pm 24.7$ )	0*	0*
Carbamazepine ( $\text{mg kg}^{-1} \text{ dw}$ )				0.625	1.25	2.5	5.0	10
	II	83.6 ( $\pm 17.6$ )	85.0 ( $\pm 7.07$ )	55.0* ( $\pm 10.8$ )	63.8* ( $\pm 8.54$ )	0*	0*	0*
	III	86.7 ( $\pm 23.1$ )	90.6 ( $\pm 7.12$ )	76.2 ( $\pm 26.5$ )	13.8* ( $\pm 12.5$ )	6.25* ( $\pm 6.29$ )	0*	0*
23°C	IV	86.0 ( $\pm 15.5$ )	82.5 ( $\pm 11.9$ )	93.8 ( $\pm 2.5$ )	50.0* ( $\pm 20.4$ )	1.25* ( $\pm 2.5$ )	0*	0*

14 days aged sediment was used. Data of the 4 replicates per treatment were pooled, 20 first instar larvae per replicate were used (one-way ANOVA; Tukey HSD test).

\* Significantly different from the solvent control (SC);  $p < 0.05$ .

**Fig. 16.5.** *Potamopyrgus antipodarum*. Chronic 28 d reproduction test. Test substance: carbamazepine. Mean numbers of embryos ( $\pm$ SD). SC, solvent control



crease. As in the *L. variegatus* assay, neither an acceleration nor an inhibition of the reproduction were observed.

Even if the snails would produce significantly more embryos under the influence of carbamazepine, as it has been shown for estrogenic compounds (Schulte-Oehlmann et al. 2001), this would also be an adverse effect. Like in almost all wildlife species, the energy budget of these snails is limited and thus the annual energetic contribution for reproduction. Consequently, an increase of the embryo numbers and of the reproductive effort at an unfavorable time of year, when young snails face lower survival rates, will reduce the overall reproductive success because less offspring can be produced during the main reproductive season.

Although *P. antipodarum* revealed as a very sensitive test species for endocrine active chemicals such as organotin compounds, alkylphenols and bisphenol A (Schulte-Oehlmann 1997; Schulte-Oehlmann et al. 2001; Duft et al. 2003), no effects on reproduction were observed for carbamazepine. Furthermore, the snails also did not show any change with respect to survival or behavior. Food uptake was visibly not inhibited. Despite its widespread presence in the aquatic environment, carbamazepine does not seem to pose a risk for aquatic mollusks, at least not for this species.

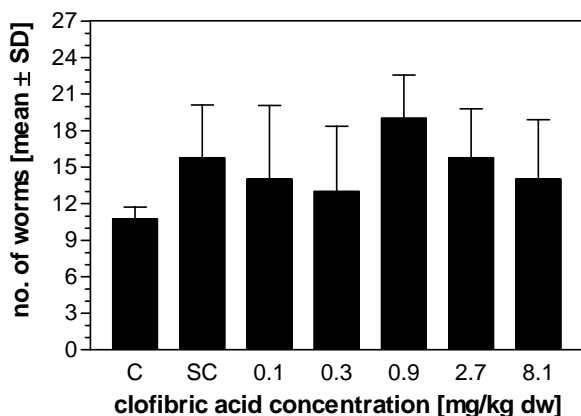
### 16.3.2.2

#### **Clofibric acid**

*Lumbriculus variegatus*. As it is shown in Fig. 16.6, no evidence was found that clofibric acid might affect vegetative reproduction in *L. variegatus*. The numbers of individuals in every treatment range from about 12 to 17 individuals, comparable to the results of the carbamazepine assay (Fig. 16.2). A statistical analysis revealed no significant differences between the groups exposed to clofibric acid and the solvent control ( $p > 0.05$ , one-way-ANOVA). Due to the fact that clofibric acid is a stereo isomer to the herbicide mecoprop (BLAC, 1998), it is interesting to examine whether this substance also causes toxic effects. Nevertheless, the results do not show such effects.

As already observed in the carbamazepine assay, the test compound did not affect the vegetative mode of reproduction in *L. variegatus* in any respect. Neither an acceleration nor an inhibition occurred. Therefore, it can be stated, that, according to these

**Fig. 16.6.** *Lumbricus variegatus*. Chronic 28 d sediment toxicity test. Test substance: clofibric acid. Mean number of individuals at the end of the test ( $\pm$ SD). C, control; SC, solvent control



data, clofibric acid should not be hazardous for *L. variegatus* at environmentally relevant concentrations. A statistically significant biomass reduction was found in the treatment group exposed to 2.7 mg kg<sup>-1</sup> when compared to the solvent control ( $p < 0.05$ , one-way-ANOVA). Because the highest treatment, spiked with 8.1 mg clofibric acid kg<sup>-1</sup>, did not show this effect, the reduction is possibly due to experimental conditions. An effect of clofibric acid is therefore also unlikely for this toxicological endpoint.

In spite of the fact that reproduction rates of *L. variegatus* are quite low again, almost identical with those found during the carbamazepine test (Fig. 16.2), clofibric acid cannot be considered as a threat for *L. variegatus* because the reproduction factors are low in every treatment. The same adverse effects could have been expected as those discussed for carbamazepine. As no effects on the reproduction are observed, the subsistence of *L. variegatus*-populations is not endangered by clofibric acid, at least according to present data. Biomass data admittedly recorded some significant reduction in biomass. As not many worms were present in all assays, no clear conclusion can be drawn out of this observation, even more if the reduction did not show any tendency that might be attributed to the concentrations of clofibric acid. The research on this compound has to be continued to collect more data and to come to more substantiated conclusions.

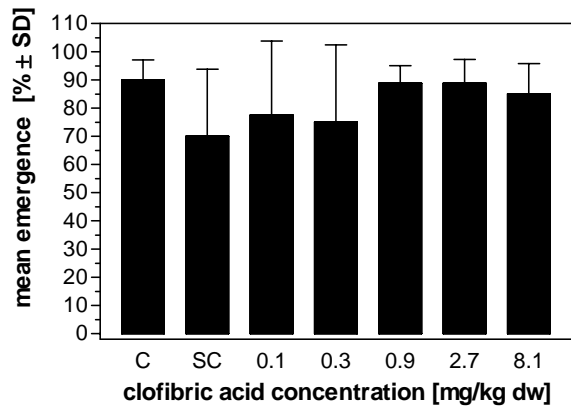
*Chironomus riparius*. The non-biting midge *C. riparius* was also not affected by clofibric acid. As it can be seen in Fig. 16.7, nearly every treatment ends up with a high emergence (for example 75% at 0.1 mg kg<sup>-1</sup> dw or 89% at 2.7 mg kg<sup>-1</sup> dw). Statistical analysis did not detect any significant difference between the groups ( $p > 0.05$ , ANOVA). Again, it is shown that, in spite of the stereo isomery to mecoprop, clofibric acid does not exert any toxic effect. The emergence rates in the spiked treatments do not differ from the solvent control. An effect as for carbamazepine was not observed. All larvae developed in a normal way without showing any inhibition in their development. The larvae did not avoid the sediment and behaved in a normal way. No wincing or any other signs of inconvenience were observed. Any shifts in the emergence that might be due to clofibric acid were not observed.

In contrast to carbamazepine, this substance can be considered as harmless for chironomids according to these data. As already discussed for *L. variegatus*, possible ef-

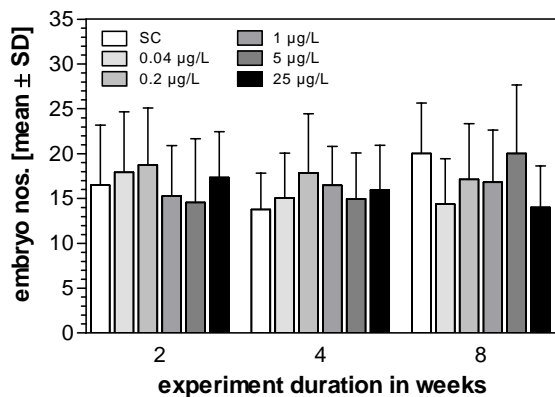
fects of clofibric acid are of interest because the compound is highly persistent and because it is, according to Hanisch et al. 2002, considered as environmentally relevant due to its acute toxicity on daphnids (NOEC: 0,01 mg l<sup>-1</sup>, Kopf 1995). *C. riparius* proved to be susceptible for adverse effects on pharmaceuticals in the carbamazepine assay. The complexity of the metabolism in chironomids, due to their holometaboly, offers many possibilities for xenobiotics to interfere with normal functions. Therefore, an effect similar to the one caused by carbamazepine is at least imaginable. Anyway, no effect occurred, neither on emergence nor on reproduction.

*Potamopyrgus antipodarum*. The reproductive effort in the freshwater mud snail *P. antipodarum* during the clofibric acid aqueous exposure experiment, measured as the numbers of embryos, is summarized in Fig. 16.8. No statistically significant differences were found between the exposure groups and the solvent control ( $p > 0.05$ , one-way ANOVA). The mean embryo numbers ranged between 14 and 20 in all experimental groups during the test. These values are in good accordance with those of historical controls for *P. antipodarum* as provided by Schulte-Oehlmann (1997). During the test, some kind of biofouling with bacterial or fungal growth occurred in all treatments that

**Fig. 16.7.** *Chironomus riparius*. Chronic 28 d sediment toxicity test. Test substance: clofibric acid. Mean emergence (%;  $\pm SD$ ). C, control; SC, solvent control



**Fig. 16.8.** *Potamopyrgus antipodarum*. Chronic 28 d reproduction test. Test substance: clofibric acid. Mean numbers of embryos ( $\pm SD$ ). SC, solvent control



contained the solvent (ethanol). This biofilm formation continued although the test vessels were changed several times. Therefore, it cannot be excluded that the statistically insignificant differences in the number of embryos in Fig. 16.8 may also be due to the biofilm development on vessel surfaces. Probably, the bacteria and fungi used the solvent ethanol as a carbon source, because the biofilm development did not occur in the ethanol-free control. No dose response relationship could be observed.

Based on these results there is no indication that clofibric acid might affect reproduction in *P. antipodarum* or that it may pose a risk to the survival of freshwater mud snail populations in the field.

### 16.3.3

#### Sediments in further research on pharmaceuticals

The majority of environmental chemicals are tested with a rather limited range of ecotoxicological tests, especially with the so-called “aquatic trias”, consisting of tests with algae, daphnids and fish (Fent 1998). Often, only acute tests are performed, considering almost exclusively the aqueous exposure route (e.g. Hanisch et al. 2002). The results presented here show that the same test species may react in different ways when exposed once via water and once via sediments. Therefore, also sediments should be considered in ecotoxicological research on pharmaceuticals in the environment. Lipophilic pharmaceuticals may easily distribute to the sediment. They may pose no more a risk for completely pelagic organisms then, but may still affect sediment-dwelling organisms. Test systems like those conducted here should therefore be considered in future research.

All different tests applied in the current attempt have extensively been used in several studies before (e.g. Leppänen & Kukkonen 1998; Bleeker et al. 1999; Duft et al. 2003) or are already standardized, such as the *Chironomus riparius* and *Lumbriculus variegatus* tests (ASTM 1995; OECD, 2001). *L. variegatus* feeds by ingesting the whole sediment and is therefore theoretically susceptible for sediment-adsorbed xenobiotics, at least, if they are not bound by a covalent bond. *C. riparius* larvae, although not ingesting the sediment itself, are susceptible for substances that accumulate in sediments because they feed on material taken out from within the sediment or from beyond the sediment surface. In the same way, *Potamopyrgus antipodarum* is susceptible for sediment-bound residues. All three species can take up residues also via their integument.

With the three species presented here and several other standardized test organisms, a broad variety of methods is available not only for the testing of monosubstances or mixtures in the laboratory but also for biological effect monitoring purposes in the field. The testing of chemicals in the laboratory and a biomonitoring of pharmaceuticals and their metabolites should include sediment tests. The results presented here show that a compound cannot be considered as harmless if no acute toxicity is detected, especially if only aqueous exposure test were performed. Carbamazepine did not exert acute toxic effects on the first larval stage of *C. riparius*. The adverse effect did not show up before the fourth larval stage. This discrepancy shows that it is necessary to watch an organism's full life cycle for being sure that it is not affected by a xenobiotic like pharmaceuticals. Estrogens for example develop their feminizing effects at water concentrations in a range  $< 10 \text{ ng l}^{-1}$ , though no effects are revealed in acute toxicity tests (Routledge et al. 1998). Furthermore, the most appropriate way of

evaluating the toxicity of substances is to consider an environment that is as natural as possible for the test organism to be sure that potential effects are neither heightened nor masked by experimental conditions. Therefore, sediment tests should become an instrument in the routine testing and biological effect monitoring of chemicals.

As there may be very specific effects like the inhibition of development of *C. riparius* in the fourth larval stage found here, pharmaceuticals cannot be considered as harmless, based exclusively on results obtained with “aquatic trias” tests. Though all trophic levels are covered, these tests cannot display all possible effects nor do they consider the wide range of different systematic groups, especially within the invertebrates. Since it is known that many chemicals may for example act as endocrine disruptors, it is necessary to work with test organisms that can display such effects. Acute tests are generally not suited to detect such specified modes of action of test chemicals. The inhibition of the chironomid development obviously occurs shortly before pupating. This indicates that a very special process in the metabolism is affected which only occurs in this developmental stage. In addition, the organisms of the aquatic trias do not totally represent the whole spectrum of aquatic wildlife. Though they can give a first clue to the toxicity of certain substances, they do not allow for the statement that chemicals do not cause any risk for the biocenosis. The example of carbamazepine shows that very special effects can occur when substances with a very specialized mode of action are released. Thorough research on compounds like this is necessary to minimize risks for the environment and life cycle tests should be used in this research to make the best use of the methods that are available.

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