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Organophosphates in the Zebra Mussel *Dreissena polymorpha:* Subacute Exposure, Body Burdens, and Organ Concentrations

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Abstract. Subacute exposures (10 d) of the freshwater mollusc *Dreissena polymorpha* to disulfoton (10 mg/L), thiometon (6 mg/L), and its activated oxygen analogue demeton-*S*-methyl (6 mg/L) corroborate earlier findings of organophosphate resistance and accumulation in the organism. Mortality occurred not before the ninth day of exposure. Mortality was induced at high ambient water concentrations and must be due to unknown specific organophosphate effects. Body burdens reached saturation levels within one week being around 40 mg/kg wet weight for thiometon and 60 mg/kg for disulfoton. Mussels dying during the tests showed lower tissue concentrations. Elimination of accumulated organophosphates was so low in the mussel, that an efficient metabolism of these compounds in the mussel was unlikely. Different organs of *Dreissena* previously acutely exposed (96 h) to the organophosphate thiometon (6, 12, 25, 50 mg/L) were analyzed for their thiometon content. Thiometon could be found in all organs, but were highest in the anterior part of the viscera (230 mg/kg), where it was accumulated either in the digestive gland and/or in the gonadal tissue.

After a fire at pesticide storage facilities at Basel (Switzerland) in 1986, several tons of organophosphates, mainly thiometon and disulfoton, were swept accidentally into the river Rhine. High water concentrations of thiometon and disulfoton were measured close to the source of contamination (Güttinger and Stumm 1992, Table 1). Organophosphates are cholinesterase inhibitors and are used worldwide as insecticides. The Rhine biota was heavily damaged for several hundred kilometers downstream (Capel *et al.* 1988). Based on the fact that thiometon and disulfoton underly quick hydrolysis and biodegradation in water (Wanner *et al.* 1989), it was assumed that only small amounts of active thiometon and disulfoton would remain in the Rhine sediments and that these substances would therefore not accumulate in exposed animals. Three and a half months after the incident, however, unexpectedly high concentrations of thiometon and disulfoton were measured in the freshwater bivalve *Dreissena polymorpha.* Although no health effects seemed to occur in the mussels, mortality of diving ducks feeding on the molluscs was observed. The causal relationship between the observed high mortality of diving ducks in early 1987 and the spill could be established (Institute of Toxicology, unpublished data). The unexpected still high pollution of the Rhine sediment may have been responsible for the elevated tissue concentrations in the mussels (Table 1). Additionally, the limited ability of molluscs to metabolize xenobiotic compounds (Becker van Slooten and Tarradellas 1994; Solé *et al.* 1994) may have contributed to the observed organophosphate accumulation in *Dreissena.*

The tolerance of zebra mussels to high organophosphate concentrations in ambient water and tissue observed after the incident at Basel was confirmed by acute laboratory experiments (Dauberschmidt *et al.* 1996). The exposure of *Dreissena* to tenfold the concentration of thiometon found in the Rhine water immediately following the chemical spill (6 mg/L) induced no mortality within 96 h. At 50-mg thiometon per liter, 88% mortality was observed within 96 h.

The following study was performed in order to understand more about organophosphate resistance and accumulation in *Dreissena polymorpha* observed under natural as well as acute laboratory conditions. For determining timepoint and organophosphate body burdens of the steady-state exposures longer than 96 h were performed. Putative xenobiotic bioconcentration mechanisms in mussels were investigated by analyzing different organs (instead of whole mussels) of acutely exposed (96 h) *Dreissena* for their thiometon content.

Materials and Methods

Chemicals

Thiometon (*O*,*O*-dimethyl-*S*-[2-ethylmercaptoethyl] -phosphorodithioate, purity 53.1%), disulfoton (*O*,*O*-diethyl-*S*-[2-ethylmercaptoethyl]-phosphorodithioate, 92.3%), and ShellSol® were donated by Sandoz Agro, Basel, Switzerland. ShellSol® is used as a solvent for organophosphates in the formulation of the end product, it consists of approximately 80 to 85% aromatic and 15 to 20% aliphatic hydrocarbons. This

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Table 1. Different thiometon and disulfoton concentrations measured in Rhine water and sediment, in tissues (per kg wet weight) of *Dreissena* and dead predatory diving ducks (*Aythya fuligula*) collected close to the incident site

● Rhine water, November 1, 1986[a]				
Thiometon	0.5 mg/L			
Disulfoton	0.6 mg/L			
● Rhine sediment [mg/kg], February 19, 1987[b]				
Sampling relative to the outflow	100 m above	100 m beneath	200 m beneath	300 m beneath
Thiometon	—	430	30	900
Disulfoton	—	3000	500	2000
● *Dreissena* [mg/kg], February 19, 1987[b]				
Sampling relative to the outflow	100 m above	100 m beneath	200 m beneath	300 m beneath
Thiometon	n.d.	16	0.5	n.d.
Disulfoton	n.d.	283	21	1.5
● Tufted duck, content of stomach, February 2, 1987[b]				
Disulfoton	1–4 mg/kg			
● *Dreissena*, after 96-h acute exposure (to 6-mg thiometon/L or 10-mg disulfoton/L)[c]				
Thiometon	63 mg/kg			
Disulfoton	30 mg/kg			

[a] Güttinger and Stumm (1992)
[b] Institute of Toxicology, unpublished data
[c] Dauberschmidt *et al.* (1996)
n.d.: not detectable

was confirmed by gas chromatography/mass spectrometry (GC/MS). Demeton-*S*-methyl (the oxygen analogue of thiometon, purity 95.2%) was obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany. For direct comparison with thiometon, demeton-*S*-methyl was diluted with the solvent ShellSol to the same concentration (purity) as thiometon (53.1%). Analytical grade *n*-hexane, petroleum benzene, dichlormethane and sodium sulphate anhydrous (extra pure) used for organophosphate extraction were purchased from Merck ABS.

Animals

Zebra mussels (*Dreissena polymorpha* P.) were collected at an average depth of 5 m from Lake Zurich by scuba diving. Thereafter, they were kept in a flowthrough tank with dechlorinated tap water (average hardness 4.8 meq/L) for several weeks. The water temperature ranged between 12 and 18°C. Although starvation up to several weeks does not affect survival of *Dreissena,* mussels were fed twice a week with freshly caught plankton. During winter they were fed with commercially available, dried *Spirulina platensis* and *Chlorella pyrenoidosa* cultures as described by Nichols (1992).

Subacute Experiments

The protocol used for the subacute exposures of *Dreissena* was similar to the one of the acute exposures to organophosphates (Dauberschmidt *et al.* 1996a) with modifications of the exposure time and observation period. The experimental setup allowed the investigators to run two organophosphates and a control exposure simultaneously (3 aquaria). Three days before the 10-d experiments were started, 100 mussels (valve length 1.6–2.3 cm) were placed into the respective glass aquaria. All aquaria contained 7.5 L of dechlorinated tap water (18 ± 1°C), which was continuously recirculated. During the preceding three day acclimatisation phase, as well as during the experiments the mussels were not fed. The tests were started by adding 6 mg thiometon, 10 mg disulfoton or 6 mg demeton-*S*-methyl per liter to the respective aquaria once every 24 h during a total period of 10 d. Collection of subsamples of mussels (8) was started after 2 d of exposure. Mussels lacking the shell closing reflex were considered dead and were collected separately. Dead as well as living mussels were stored at −20°C until organophosphate extraction. The subsequent observation (depuration) period lasted 5 d. The final timepoint of determination the organophosphate tissue concentration of dead and surviving mussels was day 15 (Figure 3).

Organophosphate Extraction

The tissue extraction followed the protocol for determination of organophosphate pesticides published by the DFG (1982). The mussels were thawed, shucked and the mussel water was drained off and discarded. Pooled tissue (whole mussels or organs) of 2 to 10 mussels was ground with six times its wet weight of anhydrous sodium sulphate with a mortar until homogeneous. The homogenate was extracted under reflux three times for 10 min with 50-ml petroleum benzene. The petroleum benzene extracts were evaporated *in vacuo* to dryness and the residue was taken up in *n*-hexane (1 ml or 100 µl). No further clean-up steps were necessary as shown earlier (Dauberschmidt *et al.* 1996). Recoveries of 55 ± 6% and 86 ± 6% ($n = 3$) were found for thiometon and disulfoton, respectively.

From the subacute demeton-*S*-methyl experiment, seven water samples (10 ml) were taken at different timepoints within the first 3 d, and once daily on days 11 to 13. These samples were extracted three times with 10 ml of petroleum benzene. Petroleum benzene extracts were evaporated *in vacuo* to dryness. The residue was taken up in 1-ml *n*-hexane. Recovery was 53 ± 1% ($n = 2$).

Gas Chromatography

The analysis of the tissue and water samples was carried out with a Carlo Erba HRGC gas chromatograph (GC) equipped with a split/

splitless injector and a nitrogen phosphorus detector (NPD). Separation was performed on 20 m × 0.30 mm glass capillary column coated with 0.15 µm OV-31-OH (polysiloxane containing 83% methyl and 17% 3-cyanopropyl). The carrier gas was hydrogen at a pressure of 40 kPa (linear velocity 0.5 ms^{-1}). The temperature of the injector and detector was 280°C. One microliter of the sample dissolved in *n*-hexane was injected at a split ratio of 1:10. The temperature was held at 200°C (isothermal analysis). For quantification, reference compounds dissolved in *n*-hexane were employed. The NPD signals were recorded and integrated with a Spectra-Physics SP 4290 integrator. Concentrations of the respective organophosphates were determined by integrating the area of the organophosphate specific peak and using a standard curve. The detection limit was at 0.5 ng.

Organ Concentrations (Animals, Dissection, Lipid Determination) and Histology

For determination of the thiometon contents of the different organs, mussels of earlier acute exposures (Dauberschmidt *et al.* 1996) were taken. The 96-h exposures had been performed similar to the subacute experiments. Briefly, varying amounts of thiometon (6, 12, 25, or 50 mg/L) had been added to the aquaria once every 24 h for a total duration of 96 h. Surviving mussels had been stored at −20°C. The mussels were thawed and dissected in shells, mantle, gills, foot, byssal threads with gland, muscles (adductor and byssal retractor muscles), and visceral mass dissected at the pedal gape in an anterior part and in a smaller posterior part. The anterior part contained most of the gonadal tissue and the digestive gland (Jenzer-Hofer 1981; Morton 1992). The tissues were weighed separately (wet weight). Thiometon extraction followed as described above.

Lipid contents of organs were determined by soxhlet extraction with dichlormethane, as described in Booij and van den Berg (1994). Fifty mussels were dissected and the different organs were pooled. The tissue was ground with six times its wet weight of anhydrous sodium sulphate in a mortar until homogeneous. The homogenate was poured into a glass column and extracted with approximately six times its volume dichlormethane. Dichlormethane was evaporated *in vacuo* to dryness. The lipid residues were weighed.

For the histological analysis, the shells of whole mussels were pierced. The mussels were then fixed in 4% neutral buffered formalin. Sections of 3 µm were stained with hematoxylin and eosin.

Results and Discussion

Thiometon concentrations found in different organs of *Dreissena* are shown in Figure 1. Highest burdens were found in the anterior viscera, especially of mussels exposed to 25 mg/L/d. Because of the lipophilicity of the organophosphorus compound, one might expect bioconcentration to occur by direct absorption in lipids. The fat contents (g/100 g wet weight) of the posterior viscera, the anterior viscera, the gills, the foot, the mantle, and the muscles were 1.77, 1.74, 0.88, 0.92, 0.87, and 0.49%, respectively. Comparison between the thiometon content of the different organs and their lipid content showed that mechanisms other than lipophilicity are needed to explain the very high concentrations found in the anterior viscera, especially of mussels exposed to 12 and 25 mg thiometon per liter daily. In spite of similar fat content in the whole viscera, thiometon concentrations in the posterior part were much lower than in the anterior one (Figure 1).

Because most of the gonadal tissue is located in the anterior viscera, the high thiometon concentration may indicate a

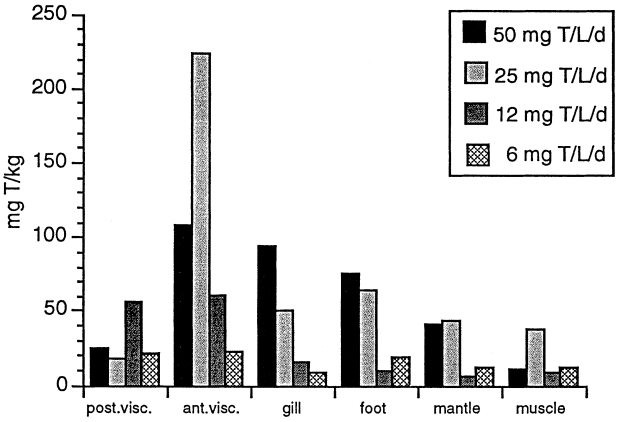

Fig. 1. Thiometon concentrations (T) per kg wet weight in organs of *Dreissena* exposed to 6-, 12-, 25-, and 50-mg thiometon per liter (added once every day for a total period of 4 d; pooled tissue of single analysis)

preferential accumulation in the gonads. A sequestration in lipids of the gonadal tissue, according to the hypothesis of Tanacredel and Cardenas (1991), would also explain the high resistance to effects of organophosphates observed in *Dreissena* (Dauberschmidt *et al.* 1996), since the effects of sequestered pesticides on essential biochemical processes would remain low (De Bruijn *et al.* 1991).

Besides the gonadal tissue, the digestive gland is also located in the anterior viscera. The digestive gland has shown to be a major site of uptake of organic xenobiotics (Livingstone *et al.* 1989; Suteau *et al.* 1987). After 2 weeks' exposure to the organophosphate endosulfan, clams and mussels (*Mytilus*) showed the highest concentration in the digestive gland, followed by gonads and other organs (Roberts 1975). As in *Dreissena*, the digestive gland and the gonadal tissue are intermingled in the viscera, consequently, separate dissection of these organs for organophosphate concentration measurements was not possible.

Besides the anterior viscera, the gill concentrations showed a strict water concentration-dependent increase (Figure 1). This was paralleled with a water-concentration–dependent increase in mortality in mussels (0, 39, 63, and 88% in the 6-, 12-, 25-, and 50-mg thiometon per liter groups (Dauberschmidt *et al.* 1996). These correlations suggest that mortality may depend at least partly on the organophosphate concentration in the gills. The gill concentrations resulting from redistribution from the digestive pathway and/or direct uptake in this organ (Loskill and Nagel 1991) may be lethal by some unknown toxic mechanisms, such as inhibition of the ciliate function by mucus production (Posselt and Bender 1971) or organophosphate-induced disintegration of the gill tissue (Villar *et al.* 1994). From the few histological slides examined in our studies, it was not possible to state whether observable gill damages originated from the organophosphate exposures itself, from tissue disintegration between death of the mussels and fixation.

Results of the subacute exposure tests (Figure 2) confirmed the high resistance of *Dreissena* to organophosphates found earlier in the acute experiments and in the Rhine river. Mortality occurred not before the ninth day of thiometon exposure. For the active thiometon oxygen analogue, demeton-*S*-methyl, mortality was observed only three days later. Because of different stability in water (Dauberschmidt *et al.* 1996) mortal-

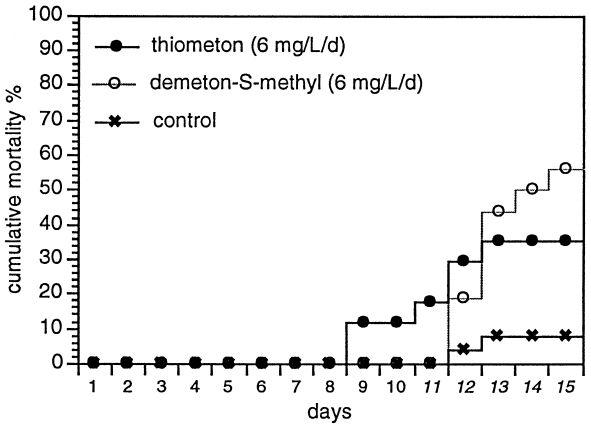

Fig. 2. Cumulative mortality of *Dreissena* at subacute exposures to organophosphates. Addition daily until day 10, no organophosphates on days 11 to 15 (*italic* numbers)

ity of the demeton-*S*-methyl exposure cannot directly be compared to the one of thiometon. Thiometon degrades very fast in the aquaria water and concentration level was below detection limit 24 h after the last addition of this compound. Demeton-*S*-methyl was more stable, only a part from the amount added daily was degraded within 24 h leading to a final water concentration of 17.6 mg/L at day 11. The results of the demeton-*S*-methyl exposure suggest that the repetitive dosing of demeton-*S*-methyl led to a slowly increasing, finally lethal water concentration. In the 96 h acute exposure experiments, the quickly hydrolyzed organophosphates thiometon and disulfoton were thought to be toxic to *Dreissena* due to the repeated high peak concentration (Dauberschmidt *et al.* 1996).

Maximal measured body burdens in the subacute exposure experiments were 41 mg thiometon/kg in living mussels, and 32 mg/kg in dead mussels (Figure 3). For disulfoton, the respective values were 67 mg/kg (alive) and 79 mg/kg (dead). Maximum thiometon body burdens had been slightly lower in mussels gathered in the Rhine river three and a half months after the chemical spill (16 mg/kg, Table 1), and slightly higher in mussels acutely exposed earlier (63 mg/kg, 96 h). Subacute disulfoton body burdens were in between the ones after acute exposure (30 mg/kg) and the ones found in Rhine mussels (283 mg/kg). The comparison suggests that the level of maximal organophosphate accumulation was reached—at least for thiometon—after one week of exposure. Body burdens were not primarily lethal as they were not higher in mussels dying from exposure than in living mussels (Figure 3). This was also observed in the earlier 96 h acute experiments (Dauberschmidt *et al.* 1996). The lower tissue concentrations in dead mussels may be explained by a different behavior of moribund mussels. Due to the strongly reduced filtration activity (shells mostly closed except when dying), the direct contact to the organophosphates was lower than in actively filtering mussels.

In mammals, the mechanism of toxicity of organophosphates is a specific inhibition of acetylcholinesterase. In mussels, this inhibitory effect was not found (Dauberschmidt *et al.* 1997). Other mechanisms such as the discussed gill concentration must be at hand to explain the fact that, although *Dreissena* tolerates high organophosphate body burden and ambient water concentrations, mortality occurs at intense exposures.

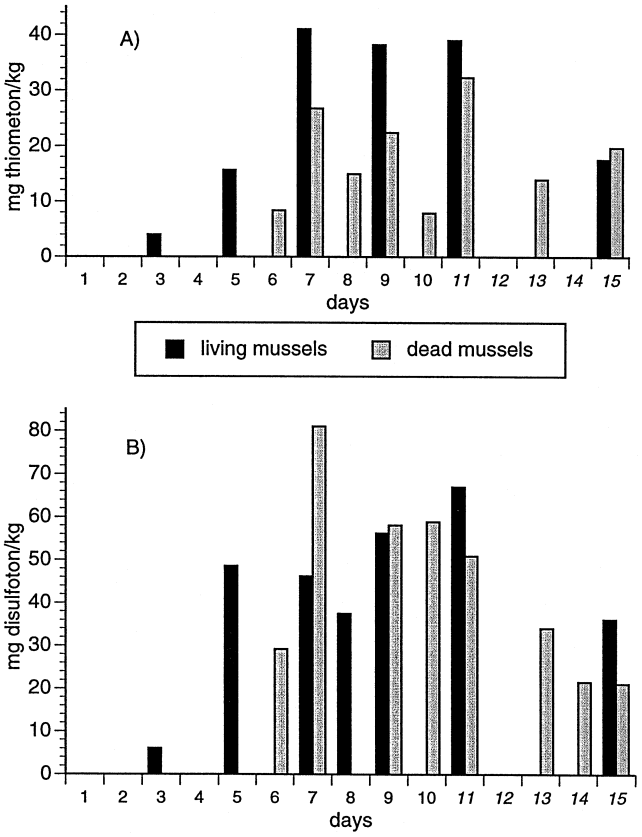

Fig. 3. Tissue concentrations per kg wet weight (pooled tissue of single analysis) of mussels exposed to thiometon (6 mg, A) and disulfoton (10 mg, B) added once every day until day 10. No organophosphates were added thereafter (days with *italic* numbers)

Twenty-four hours after the last addition of thiometon and disulfoton to the aquaria (day 10), the levels of these compounds were below detection limit (see above), allowing the mussels to eliminate accumulated organophosphates. The decrease of parent thiometon and disulfoton during this depuration period in mussel tissue was low (Figure 3) questioning an efficient metabolism of organophosphates in *Dreissena.* Limited ability of molluscs to metabolize xenobiotic compounds have been suggested earlier (Becker van Slooten and Tarradellas 1994; Solé *et al.* 1994). In vertebrates, organophosphates are readily metabolized (Hill 1988) and body burdens decrease rapidly when exposure ceases. For example, disulfoton decreased within hours to nondetectable values in man (Yashiki *et al.* 1990) and to 80% within 2 d in fish (Takase and Oyama 1985). Molluscs may nevertheless be able to metabolize xenobiotics actively. In freshwater pond snails body burdens of the organophosphate fenitrothion were almost completely eliminated 3 days after exposure. An active excretory oxidation was suggested as mechanism for accomplishing this (Takimoto *et al.* 1987). Additionally, there is evidence of active metabolism of organophosphates in *Dreissena* (Dauberschmidt *et al.* 1997b).

The results of the laboratory exposures and the ones found three and a half months after the spill at Basel (Table 1) showed that chemical characteristics such as quick hydrolysis in water and/or a high biotransformation rate in vertebrates did not prevent organophosphates from being accumulated in *Dreis-*

sena polymorpha. Residue levels in apparently healthy exposed mussels were higher than in moribund animals. The unexpected *in vivo* deposition of organophosphates in *Dreissena* posed a risk for the ecosystem through the food chain. Prey animals such as *Dreissena* may tolerate xenobiotic body burdens that are toxic to their warm-blooded predators (ducks).

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