Toxicity of Organophosphorus Insecticides in the Zebra Mussel, *Dreissena polymorpha* P.

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Abstract. The 96-h toxicity of four organophosphates (thiometon, disulfoton, malathion, and demeton-S-methyl, the oxygen analogue of thiometon) in the freshwater bivalve mollusc *Dreissena polymorpha* was tested using different nominal concentrations ranging between 6 and 50 mg/L. No mortalities were observed in mussels exposed to malathion and demeton-S-methyl (26 mg/L and 6 mg/L, respectively), and at the lowest concentrations of thiometon and disulfoton (6 and 10 mg/L, respectively). At higher thiometon and disulfoton concentrations, mortalities occurred. At the highest concentrations of 50 mg thiometon/L and 30 mg disulfoton/L, mussel mortalities of 88 and 93%, respectively, were determined. Organophosphate concentrations of up to a factor 10 times higher than in the ambient water were found in exposed mussels, irrespective of whether they were alive or dead. The search for organophosphate metabolites via GC/MS analysis of mussel tissue extracts was negative, suggesting lacking or low oxidative activation of the insecticides used. The mollusc is highly resistant to toxic effects of organophosphate insecticides and their biological active oxygen analogues.

On November 1, 1986, a pesticide storing facility at Sandoz in Basel (Switzerland) caught fire. In order to extinguish the fire, water was pumped at high volumes from the nearby river Rhine. This water found its way back into the river Rhine due to overflowing sewage canals (Capel *et al.* 1988; Güttinger and Stumm 1992). Along with the fire-extinguishing water, pesticides stored in the affected facility were swept into the river. Among these pesticides were organophosphate insecticides, mainly thiometon and disulfoton, mercurial-based fungicides, and herbicides. Consequently, the river Rhine ecosystem

faced high water concentrations of these pesticides. Measured peak concentrations close to the source of contamination were 500 µg/L and 600 µg/L for thiometon and disulfoton, respectively. These pesticide concentrations severely affected insect, crustacean, and fish populations of the Rhine ecosystem. In particular, eels (*Anguilla anguilla* L.) appeared to be highly susceptible to organophosphate intoxication, as fish of this species were found dead up to 400 km downstream of the source of contamination (Güttinger and Stumm 1992).

Based on the chemical characteristics of these compounds, it was assumed they would undergo quick hydrolysis and biodegradation, and would not remain in the Rhine sediments (EAWAG 1987). However, up to 3 1/2 months following the incident, copious concentrations of unhydrolyzed parent compound of the major contaminants, thiometon and disulfoton, were found in the Rhine river sediment near the site of original contamination (430 ppm and 3,000 ppm, respectively, Institute of Toxicology, unpublished data).

In addition to the initial fish deaths, unusually high numbers of dead diving ducks, namely tufted duck (*Aythya fuligula*) and pochard (*Aythya ferina*), were found around the site of maximal water and sediment contamination up to four months after the incident. As zebra mussels (*Dreissena polymorpha* P.) represent the predominant part of the diet of these birds (Suter 1982), mussels from this part of the Rhine were inspected for viability and organophosphate contamination. Surprisingly, they presented no obvious signs of intoxication despite the high organophosphate concentrations determined in the surrounding sediment. Furthermore, unexpected high tissue concentrations of unhydrolyzed thiometon (up to 20 mg/kg) and disulfoton (up to 300 mg/kg) were found in the *Dreissena polymorpha* analyzed (Institute of Toxicology, unpublished data). Based on the high transformation rate of organophosphates in vertebrates, it had been assumed that accumulation of organophosphorus pesticides by aquatic organisms was not likely to occur (De Bruijn and Hermens 1991). The causal relationship between the observed death of diving ducks and the fire incident demonstrates that this is not true for *Dreissena*. Because zebra mussels represent an important component of the aquatic food chain, it was important to investigate the basis for the accumulation of highly

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Table 1. Amounts of organophosphates added once every 24 h for a total of 4 times, resulting average water concentrations, mortalities, and tissue concentrations in dead and surviving *Dreissena polymorpha* at the end of the 96 h experiments: Exposures to thiometon and disulfoton, the spilled chemicals at Sandoz, and exposures to malathion and demeton-S-methyl

Added amounts	Average water concentrations	96-h mortality	Tissue concentrations after 96 h exposure	
			Dead mussels	Live mussels
Thiometon				
6 mg/L	2.7 mg/L	0%	—	63 mg/kg
12 mg/L	4.6 mg/L	39%	59 mg/kg	50 mg/kg
16 mg/L	5.8 mg/L	44%	42 mg/kg	66 mg/kg
25 mg/L	9.0 mg/L	63%	44 mg/kg	83 mg/kg
50 mg/L	12.9 mg/L	88%	96 mg/kg	128 mg/kg
Disulfoton				
10 mg/L	1.8 mg/L	0%	—	30 mg/kg
15 mg/L	2.1 mg/L	13%	51 mg/kg	72 mg/kg
24 mg/L	2.2 mg/L	31%	55 mg/kg	73 mg/kg
26 mg/L	2.3 mg/L	80%	73 mg/kg	113 mg/kg
30 mg/L	3.7 mg/L	93%	83 mg/kg	106 mg/kg
Malathion				
10 mg/L	2.7 mg/L	0%	—	2 mg/kg
26 mg/L	4.7 mg/L	0%	—	9 mg/kg
Demeton-S-methyl				
6 mg/L	4.0 mg/L	0%	—	13 mg/kg
Control				
0 mg/L	0.0 mg/L	0%		

toxic compounds in this mollusc which may lead to a potential hazard for higher trophic levels.

The goal of this study was to demonstrate the high resistance of *Dreissena* towards organophosphates in laboratory experiments, to see whether mussels retain organophosphates without showing signs of intoxication, to determine metabolites of thiometon and disulfoton, and to try to elucidate the basis of the resistance observed in the field.

Materials and Methods

Exposure of *Dreissena polymorpha* in the Laboratory

Zebra mussels (*Dreissena polymorpha*) were collected at an average depth of 5 m from Lake Zurich by scuba diving. Thereafter, they were kept in a flow-through aquarium with dechlorinated tap water (average hardness 4.8 meq/L) for a few weeks to several months. The water temperature ranged between 12° and 18°C. *Dreissena* were fed twice a week with fresh plankton during the summer months and with commercially available, dried *Spirulina platensis* and *Chlorella pyrenoidosa* cultures during winter months as described by Nichols (1992).

In the first series of experiments, *Dreissena* were exposed to high thiometon and disulfoton concentrations, the two organophosphates mainly spilled at the Sandoz incident (Table 1). Mussels collected in July were exposed during the months of August to December. The experimental set-up allowed the investigators to run a thiometon, a disulfoton, and a control exposure simultaneously (3 aquaria). Three days before the 96 h exposure experiments were started, 40 mussels (valve length 1.6–2.3 cm) were placed into each glass aquaria (7.5 L dechlorinated tap water continuously recirculated). The mussels were acclimatized slowly to the test water temperature of $18 \pm 1^\circ\text{C}$ and pH 8.5. During this acclimatization phase, as well as during the experiments, the mussels were not fed. The tests were carried out by adding the respective amount of the different organophosphates to the aquaria once every 24 h during a total period of 96 h (Table 1). Evaporation of water and/or test compound was prevented by covering the aquaria.

Dead mussels were removed daily, and stored at -20°C until further processing. The shell-closing reflex served as a parameter for mussel viability. Mussels lacking this reflex were considered dead. For comparison to a different organophosphate, *Dreissena* were exposed to malathion in two additional experiments. The amounts of malathion added corresponded to the 10 and 26 mg/L/d disulfoton experiment. Finally, *Dreissena* were exposed to the potent oxygen analogue of thiometon, demeton-S-methyl, at the lowest concentration used for thiometon (Table 1).

Chemicals

The test chemicals used in the experiments were thiometon, disulfoton, demeton-S-methyl, and malathion. Thiometon (*O,O*-dimethyl-S-[2-ethylmercaptoethyl]-phosphorodithioate, purity 53.1%), the solvent ShellSol®, and disulfoton (*O,O*-diethyl-S-[2-ethylmercaptoethyl]-phosphorodithioate, 92.3%) were a gift from Sandoz Agro, Basel, Switzerland. Demeton-S-methyl (the oxygen analogue of thiometon, purity 95.2%) and malathion (98.5%) and the reference compound disulfoton sulfoxide (10 ng/μl in toluene) were obtained from Dr. Ehrenstorfer GmbH, Augsburg, FRG. For direct comparison with thiometon, demeton-S-methyl was diluted with the solvent ShellSol to the same concentration as thiometon (53.1%). To obtain the sulfone/sulfoxide analogues, parent thiometon and disulfoton were oxidized with potassium permanganate as described in Wisson *et al.* (1976).

Analytical grade *n*-hexane, petroleum benzene, dichloromethane, and sodium sulphate anhydrous (extra pure) were purchased from Merck ABS. Florisil (PR®, 60–100 mesh) was obtained from Fluka.

Water Analysis

Water samples (10 ml) were taken 10 min after addition of the organophosphate and 24 h thereafter to monitor organophosphate concentrations. These samples were extracted three times with 10 ml of petroleum benzene. Petroleum benzene extracts were evaporated *in vacuo* to dryness. The residue was then taken up in 1 ml *n*-hexane.

Percent recovery of the organophosphates from water samples were determined by adding 100 μ l of the respective standard (100 ppm) to 10 ml tap water. These spiked samples were extracted using the method described above. A recovery of $68 \pm 13\%$ ($n = 3$), $92 \pm 6\%$ ($n = 3$), $53 \pm 1\%$ ($n = 2$), and $89 \pm 4\%$ ($n = 2$) was determined for thiometon, disulfoton, demeton-S-methyl, and malathion, respectively.

The average water concentration during the experiment was calculated by dividing the area under the curve by the exposure time.

Extraction of Organophosphates in Mussel Tissue

The mussels were thawed, shucked, and the excess water drained off before the tissue was weighed and dissected. The ensuing tissue extraction followed the protocol for determination of organophosphate pesticides published by the DFG (1982). Pooled tissue of 5 mussels was ground with six times its 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. The residue was taken up in 1 ml *n*-hexane.

For the tissue extracts of mussels exposed to thiometon and disulfoton, a clean-up procedure was introduced prior to GC analysis. A column was filled with 5.0 g Florisil® (deactivated with 20% water) swelled in petroleum benzene. The mussel extract was placed onto the column and eluted with 60 ml of petroleum benzene/dichloromethane mixture (8:2, v/v). The eluate was concentrated with a rotary evaporator and dissolved in 1 ml *n*-hexane. Since experience had shown that it was possible to achieve reproducible and reliable analytical results with direct injection of the residues dissolved in *n*-hexane, the clean-up step was left out for the extracts of demeton-S-methyl and malathion-exposed mussels.

The reliability of the tissue extraction and clean-up method was tested by determining the recovery of the organophosphate compound. This was done by adding a known amount of the respective organophosphate standard to untreated mussel tissue and by running these spiked samples through the extraction and clean-up method as described above. The recovery was expressed as % of the added amount. A recovery of $55 \pm 6\%$ ($n = 3$) and $86 \pm 6\%$ ($n = 3$) was determined for thiometon and disulfoton, respectively, while recoveries of $92 \pm 4\%$ ($n = 2$) and $100 \pm 7\%$ ($n = 2$) were determined for demeton-S-methyl and malathion.

Gas Chromatography

The analysis of both the water and tissue 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 $\times$ 0.30 mm glass capillary columns coated with 0.25 μ m PS086 (polysiloxane containing 86% methyl and 14% phenyl) in the case of thiometon and disulfoton, and 0.15 μ m OV-31-OH (polysiloxane containing 83% methyl and 17% 3-cyanopropyl) in the case of demeton-S-methyl and malathion. 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 μ l of the sample dissolved in *n*-hexane was injected at a split ratio of 1:10. For thiometon and disulfoton samples, the temperature was programmed to increase from 150°C (hold time 2 min) up to 240°C at a rate of 10°C/min. For the analysis of demeton-S-methyl and malathion, the temperature was held at 200°C and 230°C, respectively (isothermal analysis). For quantification, reference compounds dissolved in *n*-hexane were employed. The NPD signals were recorded and integrated using 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.

Fig. 1. Organophosphate concentrations (mg/L) in the glass aquaria without zebra mussels following a single addition of 10 mg/L thiometon $\square$, disulfoton Δ , malathion $\diamond$, and demeton-S-methyl $\circ$, respectively (t_0 = first measurement, 10 min after addition)

Metabolites

In order to answer the question whether organophosphates are activated either to more toxic metabolites, as demonstrated in mammals (Gallo and Lawryk 1991), or to non-toxic metabolites, tissue samples of exposed mussels were analyzed for metabolites. To ensure extraction of the slightly more polar metabolites, acetonitrile was used instead of petroleum benzene. The aliquots of extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) without previous purification. The analysis was made with a Finnigan 4500 mass spectrometer. Separation was performed under the same conditions as the other thiometon and disulfoton samples. The temperature was programmed to increase from 60°C (hold time 1 min) up to 200°C at a rate of 20°C/min, and then up to 280°C at a rate of 5°C/min.

Results

The stability of the organophosphate compounds in water without zebra mussels was monitored for 96 h after one single addition of 10 mg/L of the respective compounds. Concentrations decreased rapidly for thiometon and especially for disulfoton and malathion, whereas demeton-S-methyl, the oxygen analogue of thiometon, seemed to be more stable in water (Figure 1). Figure 2 depicts the time-courses of thiometon and disulfoton following addition of the respective compound every 24 h to the experimental units containing zebra mussels.

Control zebra mussels remained in a clustered array following introduction into the glass aquaria, maintained a quick valve closing reflex throughout the experimental period, produced byssus threads for attachment to the glass, and filtered actively. In contrast, zebra mussels exposed to thiometon and disulfoton at concentrations which induced mortality filtered only sporadically or remained closed. Inexistent or decreased production of byssus threads was observed and in addition, depending on the concentration level used, byssus threads were rejected. This resulted in a single organism array of the normally clustered zebra mussels. Following exposure to high concentrations, mussels presented with widely opened valves and demonstrated a delayed closing reflex and enhanced mucus production.

Fig. 2. Organophosphate concentrations in water (■) and tissue of dead (+) and live (●) *Dreissena polymorpha* exposed to 50 mg thiometon and, respectively, 30 mg disulfoton/L/d for 96 h (single analysis of water or pooled tissue of 5 mussels)

Mortality increased with exposure concentration (Table 1) and exposure time. The higher the concentration, the earlier the appearance of dead mussels. At similar water concentrations, thiometon induced higher mortality than disulfoton. No mortality could be observed in the experiment with the oxygen analogue of thiometon, demeton-S-methyl. Malathion induced no mortality, even at water concentrations higher than lethal disulfoton concentrations.

Zebra mussel tissue concentrations increased with exposure time. A plateau concentration was not reached within the 96 h of exposure (Figure 2). The highest tissue concentrations were always found in mussels still alive after 96 h of exposure, i.e., 128 mg thiometon and 106 mg disulfoton/kg tissue. Lower concentrations were found in mussels dying before the end of the 96 h experiment. Exposure to malathion or demeton-S-methyl resulted in much lower tissue concentrations than those found for similar ambient water concentrations of thiometon and disulfoton (Table 1).

In the GC/MS analysis, no signals of the typical fragment ions of thiometon and disulfoton metabolites (sulfoxides, sulfones, and oxygen analogues) known from the literature (Yashiki *et al.* 1990) were observed (detection limit 0.5 ng). The concentration-ratio of the parent compound to the potentially existing metabolites was greater than 25.

Discussion

A major problem in static aquatic toxicity tests is that, in some cases, the active ingredient rapidly disappears. Stable water

concentrations were not attained with any of the organophosphates tested in our studies. Similar observations were made by other authors (Miller *et al.* 1966; Takase and Oyama 1985; Wanner *et al.* 1989; Frank *et al.* 1991). The short half-lives in the water are most likely due to hydrolysis and adsorption to the glass walls of the aquaria, silicon adhesive (ASTM 1990), and to the pump. Takase and Oyama (1985) calculated for disulfoton a half-life in water of 16 h at 25°C and aeration, whereas thiometon and disulfoton degraded much more slowly when stirred at 10°C, with half-lives of 5 and 7 days, respectively (Wanner *et al.* 1989). This demonstrates that the disappearance of thiometon and disulfoton is dependent on recirculation, temperature, and aeration. Following oxidation, organophosphates appear to be more stable in water. This is demonstrated by the higher persistence of demeton-S-methyl in this study (Figure 1) as well as of the disulfoton oxygen analogue (Yashiki *et al.* 1990).

The low sensitivity of *Dreissena* observed in the field situation towards organophosphates could be confirmed with the first series of experiments. Exposure to concentrations 10-fold the one in the Rhine water immediately after the spill had no effects on the mussels within 96 h. Mortality occurred only at more than 20-fold the measured Rhine water concentrations (12 mg thiometon/L, 15 mg disulfoton/L, Table 1). For disulfoton, 96 h LC₅₀ are known also for other aquatic species, i.e., rainbow trout 1.85 mg/L, stonefly 5 µg/L (Mayer and Ellersieck 1986) and eel 37 µg/L (Arnold and Braunbeck 1992). However, it is difficult to compare the latter data with the ones obtained in the study presented as the methods of exposure, i.e., static (rainbow trout and stonefly data) vs. continuous flow-through (eel data) vs. recirculation and dose renewal (data from this study), are completely different. In order to roughly compare our results with literature data, the average measured concentrations of the experiments were used. Thus, an approximately average disulfoton water concentration of 2.2 mg/L induced 50% mortality in the zebra mussel within 96 h. When compared to the eel, *Dreissena polymorpha* is 60 times less susceptible to disulfoton than the eel. As only nominal concentrations were given in the static test for the determination of disulfoton toxicity in rainbow trout, the 96 h LC₅₀ of 1.85 mg/L reported by Mayer and Ellersieck (1986) is questionably high and can neither be compared directly to the eel nor to the data of this study.

Taking into account the tissue concentrations for thiometon and disulfoton (Table 1), the question arises whether the observed mortality in *Dreissena* was a result of the 96 h exposure to a mean concentration of organophosphates, or whether the mortality was induced by the repeated peak concentrations the mussels were exposed to every 24 h. Indeed, the minute differences in the average concentrations between the different exposures alone seem unlikely to have induced the observed differences in mortality (Table 1). The data suggest that the repeated concentration peaks (Figure 2) rather than the average effective water concentrations were responsible for the observed toxicity in the mussels. It is probable that *Dreissena* could survive even higher average concentrations than the ones used in the experiments presented here, if the mode of application would be continuous rather than intermittent (24 h intervals).

The higher resistance of *Dreissena* towards organophosphates compared to other animal species seems to be even more distinct in the case of malathion. Mammals and aquatic species demonstrate a higher sensitivity to malathion (e.g., 96 h LC₅₀ rainbow trout in static tests 4–200 µg/L; Mayer and Ellersieck 1986)

than to disulfoton and thiometon, whereas in our study the measured average water concentration of 4.7 mg/L produced no mortality in *Dreissena polymorpha*.

In mammals, thiophosphate insecticides are metabolically oxidized. Due to their better binding and thus stronger inhibition of cholinesterases, they then become 3- to 4-fold more toxic than the respective parent compound (Gallo and Lawryk 1991). The fact that thiometon and disulfoton metabolites (oxygen analogues, sulfoxides, sulfones) were not detected in the tissue of exposed mussels in this study indicates that organophosphates are not metabolized by *Dreissena* at all or at least very slowly. Indeed, lacking or slow metabolism of xenobiotics in general has been reported repeatedly in aquatic molluscs (Payne *et al.* 1987). However, the fact that no metabolites were detected is not a sure indication that they are not produced. In mammals, it has been shown that in plasma, active oxygen analogues are present at levels 30–100 times below parent compound concentrations (Willems *et al.* 1993). Because of the detection limit in this study of 0.5 ng, potentially existing metabolites would have been detected only if they were present at levels 25 times below parent compound concentrations.

Nevertheless, a possible missing oxidative metabolism in the mussels does not *a priori* explain the observed resistance of the zebra mussels toward these insecticides. *Dreissena* proved to be highly resistant not only to the parent compound thiometon, but also towards demeton-S-methyl. This might indicate that this acetylcholinesterase inhibitor, potent in mammals, either does not interact with the mussel cholinesterases, or that an interaction does not necessarily lead to severe toxic effects. Even though acetylcholine serves as a neurotransmitter in zebra mussels (Rosza 1984), the importance of acetylcholine as well as its esterases in neurotransmission processes in molluscs has not been clearly elucidated (Heyer *et al.* 1973; Mercer and McGregor 1982).

The biological effects of cholinesterase-inhibiting compounds in zebra mussels might well be due to effects other than inhibition of acetylcholinesterase. It can be hypothesized that less specific adducts with enzymes or proteins other than cholinesterase could occur. These adducts could be responsible for the observed accumulation and possibly also for the mortality at high doses. Specific as well as unspecific binding of organophosphates to proteins other than cholinesterases has been reported earlier. For instance, the enzyme neuropathy target esterase is inhibited by organophosphates due to phosphorylation of a hydroxyl residue of serine at the catalytic centre, similarly to the binding to serine of acetylcholine (Johnson 1975; Lotti 1992). There is evidence that organophosphates attack serine residue-containing active sites of other enzymes (Mahendru and Agarwal 1983) or receptors (Ward *et al.* 1993). Organophosphate binding to various enzymes or proteins in blood serum was also reported by Singh *et al.* (1985).

Organophosphorus pesticides were also shown to have strong inhibitory effects on aerobic metabolism and phosphatase activity in snail (Mahendru and Agarwal 1983). Abnormal head regeneration of planaria (Villar *et al.* 1994) and damaged membranes in mammals (Gallo and Lawryk 1991; Prozorovskii and Skopichev 1993) were shown as well.

It has been demonstrated that bioconcentration of organophosphates in mussels (Miller *et al.* 1966; McLeese *et al.* 1979; Ferrario *et al.* 1994) is compound-specific as in other aquatic animals such as amphibians (Hall and Kolbe 1980) and fish (De Bruijn and Hermens 1991). Relative to the effective water concentration, *Dreissena* showed in the present study a higher

accumulation of disulfoton than thiometon (Figure 2), which agrees with the three times higher octanol-water partition coefficient of disulfoton (EAWAG 1987). The lower tissue concentration found after demeton-S-methyl exposure could be explained for example with its more than 10 times higher water solubility than that of the parent compound, thiometon.

It could be shown that the highest body burdens were always measured in surviving mussels. As living mussels are active water filters, the total amount of filtered organophosphate-containing water therefore largely depends on the exposure time and the viability of the animal. Furthermore, the results indicate that the body burdens were not primarily lethal to the mollusc. This is corroborated by the 3-times higher disulfoton body burdens of the mussels collected in the Rhine river after the Sandoz incident (Introduction) than the surviving mussels after the acute experiments. The fact that the Rhine mussels had, contrary to the results of this study, much higher disulfoton than thiometon tissue concentrations can be explained by the much higher disulfoton concentrations still present in the Rhine sediments 3 1/2 months after the incident.

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