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Comparison of cholin- and carboxylesterase enzyme inhibition and visible effects in the zebra fish embryo bioassay under short-term paraoxon-methyl exposure

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Abstract
The acute zebra fish embryo test (*Danio rerio* Hamilton–Buchanan, 1822) is an accepted bioassay to assess the toxicity of waste water that may be used for the replacement of testing with adult fish. It is also suggested for chemical hazard characterization and assessment, although only a few groups of substances have yet been studied. Specifically acting substances such as neurotoxic insecticides pose a potentially hazard for non-target fish. To establish whether the proposed zebra fish embryo test protocol and the inhibition of cholinesterases (acetylcholinesterase EC 3.1.1.7, propionylcholinesterase EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) enzymes can be used in a similar fashion for hazard characterization and risk assessment of chemicals and environmental samples, two types of experiments were conducted. Visual effects of exposure to the organophosphate metabolite paraoxon-methyl after 24 and 48 h in the zebra fish embryo test system were analysed with the use of an inverse microscope (rate of mortality, developmental disturbances, heart rate and others). The inhibition to cholinesterases and carboxylesterase was also measured. Enzyme inhibition as a biomarker of exposure was about 70 times more sensitive than the effects in the zebra fish embryo test with an IC_{50} below 1.2 μmol compared with an EC_{50} of 91 μmol. The dose–response relationships showed different curve characteristics with a linear increase of enzyme inhibition compared with a sigmoidal curve for the overt effects. Significant overt effects could only be seen at concentrations at which already 80% of the activities of the different esterases were inhibited.

Keywords: *Cholinesterase, carboxylesterase, zebra fish embryo test, insecticide, biomarker, paraoxon-methyl*

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Introduction

The zebra fish (*Danio rerio* Hamilton–Buchanan, 1822) spawns eggs where the chorion stays transparent after fertilization. The rapid development of the embryo in the chorion can easily be followed, hatching occurring usually after 72 h post-fertilization (hpf) and mutants can be bred to study gene function. These are only some of several reasons why the zebra fish is one of the model organisms for developmental biology (Kimmel et al. 1995). Because different toxicity studies with

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waste water showed that the effects seen in zebra fish embryos are comparable with the effects in adult fish (Lange et al. 1995, Nagel 2002), the zebra fish embryo is a proposed test system for the replacement of acute tests with adult fish in ecotoxicological risk assessment. They may serve as a substitute for the acute fish test with the goldfish (*Carassius auratus*), the guppy (*Poecilia reticulata*) and the adult zebra fish or the fathead minnow (*Pimephales promelas*). For reasons of animal protection, the acute fish test for assessing the toxicity of waste water was abolished in Germany in 2004 in favour of an acute fish embryo test using a standardized protocol with zebra fish embryos (Anon. 2001) and 48 h of exposure. In order to establish whether the zebra fish embryo bioassay might also be used as an alternative not only in the toxicity assessment of waste water but also of hazard identification of chemicals or environmental samples, the investigation presented here included tests with embryos up to 48 hpf.

Insecticides of the organophosphorous type are used in agriculture. Since an insecticide such as parathion-methyl or its metabolites can harm non-target fish via run-off events or accidental spill a risk assessment for this type of chemical is compulsory. In addition, low-cost methods are needed which can be used to identify different substance classes with differing mode of actions (genotoxic, neurotoxic, teratogenic, etc.) in complex environmental samples. Parathion-methyl is — beside different degradation processes — metabolically activated to paraoxon-methyl. The mode of action of paraoxon-methyl is the acute inhibition of cholinesterase enzymes (acetylcholinesterase, AChE, EC 3.1.1.7) via phosphorylating the active site in the AChE protein. As shown by Beauvais et al. (2000, 2001), these cholinesterase inhibitors may have severe ecological impact because of their behavioural effects in older fish. Additionally, the exposure of early developmental stages of the zebra fish embryo to organophosphates might be followed by unspecific impairments as severe disturbance of somitogenesis was seen earlier (Hanneman 1992). Therefore, an acute sublethal effect in the early development — such as the inhibition of enzymes — might finally add up to effects in older stages. This could be even more prominent if repair mechanisms are not fully developed in the young animals. So with the above knowledge sublethal biomarkers such as enzyme inhibition in fish embryos could be useful to extrapolate to possible long term risks i.e. using it as a 'biomarker of chronic effect'.

Commonly, cholinesterase and recently also carboxylesterase inhibition are used as biomarkers for organophosphate exposure (Maxwell 2001). As fish/fish larvae or embryos might be harmed at runoff events, we used the enzyme inhibition assay of unhatched zebra fish embryos to test it for the possible use as a biomarker. Küster (2005) showed that the esterase enzyme activities increase with age of the zebra fish embryo. As quantification of enzyme activities was more difficult to detect at early developmental stages, embryos of the age 48 hpf were used.

As insecticides may also show lethal, sublethal and teratogenic effects, the analysis was not focused on enzyme activity measurements only. The four main lethal endpoints (coagulation, detachment of tail, development of somites and heart beat) as defined in the standard protocol and additional sublethal and teratogenic endpoints as proposed in the guideline of the Organization for Economic Co-operation and Development (OECD) (Anon. 2004) and as described in detail in Nagel (2002) were included in the final evaluation.

Therefore in this work a combination of the organismic zebra fish embryo test with the suborganismic enzyme assay was used to analyse possible correlations between these two sets of observation parameters and to assess the usefulness of the assay system for neurotoxic hazard identification in environmental samples.

Materials and methods

Chemicals

The esterase substrates acetylthiocholine-iodide (ASCh), CAS RN 1866-15-5, acetyl-ß-(methylthio)choline-iodide (AMSCh), CAS RN 1866-17-7, propionylthiocholine-iodide (ProSCh), CAS RN 1866-73-5, *S*-phenylthioacetate (PSA), CAS RN 934-87-2 were purchased from ICN Biomedicals (Eschwege, Germany). Triton X-100 and sodiumdihydrogenphosphate-monohydrate were obtained from Merck (Darmstadt, Germany). The chromoagent DTNB (5,5-dithio bis-2-nitrobenzoate) CAS RN 69-78-3 was purchased from Serva (Heidelberg, Germany). DC Protein Assay was purchased from BioRad (Munich, Germany) while the bovine serum albumin (BSA) was obtained from Serva (Heidelberg, Germany). The parathion-methyl metabolite paraoxon-methyl (CAS RN 950-35-6) was a certified pure compound (98% purity) from the company Dr. Ehrenstorfer (Augsburg, Germany). All other chemicals were at least *pro analysi* quality.

Fish culture and embryo collection

Culture and embryo collection were done as described in Küster (2005). In short, adult fish originally obtained from West Aquarium (Bad Harzburg, Germany) and in culture for several years, kept in glass aquaria (120 litres) with activated carbon filtered and equilibrated tap water at $27 \pm 2^{\circ}$C with a density of 5 fish l^{-1} and a female to male ratio of 2:1 (Westerfield 2000). The light/dark regimen was set to 12/12 h. The fish were fed twice daily with commercial dry food (Tetramin, Tetra, Melle, Germany) and once per day with *Artemia* sp. *nauplii* (Sanders, Ogden, UT, USA). The zebrafish are photoperiodic in their breeding, and produce embryos every morning, shortly after sunrise (Westerfield 2000). Mating and spawning were therefore induced by the onset of light and by the addition of synthetic spawning substrate. Eggs were collected using a grid-covered dish and successively cleaned with aerated ISO standard dilution water (ISO-water) as specified in Anon (2004). The developmental stages were identified according to Kimmel et al. (1995).

Fish embryo test and eco-toxicological endpoints (LC/EC_{50} determination)

Briefly, the fish eggs were collected and washed twice with ISO-water and exposed to paraoxon-methyl without regard of fertilization. The exposure commenced within 20 min after completed spawning of the adult fish. Unfertilized eggs were differentiated from fertilized eggs after 1 h (in the four- to eight-cell stage) and excluded from the test. Stock solution of the highest test concentration of paraoxon-methyl in ISO-water was freshly prepared the afternoon before the test and stored at 4°C until initiation of the bioassay the next morning. The other test solutions were prepared by sequential dilution at the morning of the test. Preceding reverse phase high-performance liquid chromatography (HPLC) analysis of the highest test concentration (paraoxon-methyl

dissolved in water) revealed a loss of 7% after storage for one month at 4°C (data not shown). Castillo et al. (1997) calculated a half-life for paraoxon-ethyl of 4–5 days in river and estuarine water (being more stable than the parent compound parathion-ethyl with a half-life of 3–4 days in the respective water types). Therefore, possible loss of paraoxon-methyl in the fish embryo assay was estimated to be about 25% (a linear decrease over time was assumed) in the 48-h testing time. This hypothesis is supported by studies of Sakellarides et al. (2003) who quantified the photodegradation kinetics of different pesticides including parathion-methyl and its metabolites in different types of water. The group calculated half-lives of parathion-methyl from 25- to 35-day in lake water and distilled water, respectively. But the data also revealed a steep decrease of parathion-methyl in the first 3–5 days of about 30% being in line with the assumed 25% loss in the assay system used here. The embryos were exposed under static conditions to the toxicant for 48 h at a temperature of 27.5 ± 1°C under 12/12-h light/dark (30 µmol photons m^{-2} s^{-1}) exposure. The concentrations of test solutions were calculated initially in mg l^{-1}. For better comparison with literature data all mg l^{-1} concentrations were converted to molar concentrations. Concentration range was from 2.5 and 50 mg l^{-1} (0.01 −0.2 mM).

The fish embryo test was carried out in 24-well plastic multiplates (Techno Plastic Products, Trasadingen, Switzerland) and as recommended in the German standard protocol (Anon. 2001) and the OECD draft guideline for testing of chemicals (Anon. 2004).

The embryos were transferred to the multiplates. In each well one single embryo was incubated in 2 ml of medium. On the same multiplate two concentrations (ten embryos/concentration) were tested simultaneously. The remaining four wells in each multiplate were filled with one embryo each, serving as an internal control for the specific multiplate. One separate multiplate served as external control. It was filled with 20 embryos in ISO-water only. The well plates were covered with self-adhesive plastic film and stored in a translucent plastic bag to reduce evaporation. Seven different concentrations of the insecticide metabolite were tested in triplicate.

Determination of the developmental stages was accomplished according to Kimmel et al. (1995) using a 50 × magnification with an inverse microscope (Olympus IX70-S8F, Hamburg, Germany) after 24 and 48 h. Endpoints used were derived from the German (DIN) standard protocol (Anon. 2001) and the OECD draft guideline for testing of chemicals (Anon. 2004). The endpoints in these two protocols are subdivided in three groups of endpoints (lethal, sublethal and teratogenic). Defined are four lethal endpoints which are coagulation of eggs, non-development of somites, non-detachment of the tail and no presence of heartbeat. If these effects are observed, the embryos will not survive the next hours or days and are therefore defined as dead. In contrast, the sublethal and teratogenic endpoints of the extended test in the same protocols include effects that might give extra information about (sublethal or teratogenic) mode of action of the tested samples. These include eighteen other endpoints from which the following seven sublethal endpoints were used in the interpretation of test results: incomplete gastrulation, eye formation, spontaneous movement, blood circulation, heart rate, pigmentation and oedema. In addition, two teratogenic endpoints (malformation of tail and general growth retardation) were used (Schulte & Nagel 1994, Ensenbach 1998, Nagel 2002). The effect endpoints of each 'endpoint group', i.e. lethal, sublethal and teratogenic, were analysed with the conception of possible differences in sensitivity of the specific endpoints to

paraoxon-methyl. As no differences could be seen at all endpoints except the heart rate, the heart rate was analysed singly while the other endpoints were weighted equally and then added up to the final percentage effect compared with the controls. So, if an embryo of the same test group showed either retardation of development (seen most often), low pigmentation (seen rarely) or malformations (seen very rarely) or other effects, these effects were treated and counted the same. This means if (in a group of 20 embryos), three embryos showed growth retardation, and two other showed low pigmentation and one embryo had an oedema this was calculated as 30% effect.

Exposure experiments for enzyme analysis

On the basis of data from Küster (2005) where a single concentration of 0.4 μM paraoxon-methyl was tested, different lower and higher concentrations were used in the exposure experiments for refinement of these data. The concentrations of test solutions e.g. the concentrations were initially calculated in mg l^{-1}. For better comparison with literature data all mg l^{-1} concentrations were converted to molar concentrations.

Embryos from three independent spawning events were used for the paraoxon-methyl experiments. Tests were carried out with a minimum of 20 fish embryos per replicate. Four replicates per concentration (30 μg l^{-1} to 20 mg l^{-1}, i.e. 0.12 – 80.92 μmol) were used (with a density of one embryo/2 ml) to evaluate the inhibition by paraoxon-methyl. Three independent exposure experiments were done. After 48 hpf the surviving embryos from each treatment and concentration were rinsed thoroughly in clean ISO-water (to lower the amount of possibly adhered paraoxon-methyl to the egg chorion), introduced into 2 ml microcentrifuge tubes and snap frozen in liquid nitrogen. The animals were successively stored at −20°C pending analysis. The period of storage never exceeded 4 weeks.

Biochemical analyses

The analysis of the enzyme activities of cholinesterase (AChE), methylcholinesterase (MChE), propionylcholinesterase (ProChE) and carboxylesterase (CaE) were done as described in Küster (2005). Briefly, at least 20 snap frozen embryos were thawed and homogenized on ice in 0.4 ml ice-cold phosphate buffer (pH 7.5, 0.1 M $NaH_2PO_4 \times H_2O$ and 0.1% v/v Triton X-100). Homogenization was done for 20 s using a metal-bladed homogenizer (Ultra Turrax, IKA Labortechnik, Staufen, Germany) at speed 4–5. The homogenates were centrifuged at 4°C for 15 min at 10 000*g*. Supernatants were removed and used either directly for enzyme analysis or they were stored at −20°C until analysis. Storage of the samples never exceeded 4 weeks. The preceding experiments showed no decrease of enzyme activity under the above conditions (Küster 2005). Enzyme assays were carried out in quadruplicate per sample at 22°C. The assays followed the method described by Ellman et al. (1961) adapted to microtitre plates. Kinetics (mOD min^{-1}) were recorded at 412 nm for 10 min (SpectraMax 250 Photometer, Molecular Devices, Sunnyvale, CA, USA). The microtitre wells were successively filled with 0.05/0.01 ml supernatant (or buffer in controls), 0.05/0.09 ml homogenization phosphate buffer (pH 7.5) in ChE- or CaE-assays, respectively, and 0.1 ml DTNB (final test concentration of 0.3 mM). The solution was incubated for 10 min at room temperature to correct for non-specific

spontaneous hydrolysis of the DTNB chromoagent. The reaction was started with the addition of 0.1 ml substrate (final concentration of 0.45 mM for the different types of Cholinesterases and 0.776 mM for CaE). The determination of carboxylesterase enzyme activity was carried out according to Basack et al. (1998) using DTNB as the chromogenic reagent too and *S*-phenylthioacetate (PSA) as substrate.

The specific enzyme activity is expressed as units (U) per mg of protein, with 1 U defined as the amount that hydrolysed 1 µmol of substrate per min. Protein concentration of the samples was determined in quadruplicate at 750 nm using a commercial kit (DC Protein Assay, BioRad, Munich, Germany) based on the Lowry assay (Lowry et al. 1951). A total of 5 µl of supernatant was sufficient for the protein determination with maximal protein concentrations of 10 mg l^{-1} with 20 embryos (48 hpf). Bovine serum albumin, fraction V was used as the standard protein. A non-linear standard curve using the quadratic formula:

$$y = a + bx + cx^2$$

was generated to correct for the Folin reagent reaction (Peterson 1979, 1983). Each calibration curve was generated with five different protein dilutions. The percentage of AChE inhibition was derived by expressing the activity levels of exposed animals as the percentage of the activity in controls.

Results

Acute toxicity of paraoxon-methyl to the zebra fish embryo

The data of the effects of paraoxon-methyl after 24 and 48 h are shown in Figures 1 and 2. Depending on the endpoints used, different dose–response relationships could be seen. Using the four endpoints defined by the standard German DIN protocol for the testing of chemicals (coagulation of eggs, development of somites, detachment of the tail, development of heartbeat/circulation), only an incomplete dose–response

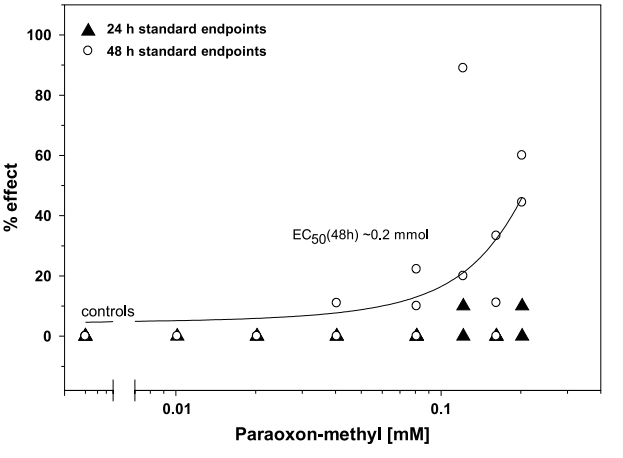

Figure 1. Dose–response relationship between paraoxon-methyl (concentration of 10–200 µmol) and visible effects in the zebra fish embryos (after 24 and 48 h) using the endpoints: coagulation of eggs, development of somites, detachment of tail and development of heartbeat. $n = 3$ with ten embryos per replicate. Curve parameters with $f = a/(1 + \exp(-(x - x_0)/b))$ with $x_0 = EC_{50}$; 48-h exposure, where $a = 100$, $b = 0.0698$ and $x_0 = 0.2133$.

relationship with a maximum effect of 45% could be detected after 24- and 48-h incubation with the two highest concentrations used (Figure 1). The other (sublethal or teratogenic) endpoints — described in the Materials and methods section — did not show any effects with one exception: the two highest concentrations stopped or retarded the development of the zebra fish embryos between the 10th and 18th somite stage.

After a 48-h test duration, an EC_{50} of about 202 μmol could only be extrapolated because concentrations that cause effects of 100% were not reached and high variability of responses occurred with the used test concentration of 202 μmol. Higher concentrations were not tested for practical reasons. Reanalysis of the data by using sublethal and teratogenic endpoints (Anon. 2004) led to a left shift of the dose–response curve (Figure 2). Fish embryos showed a retardation/disturbance of development including a missing detachment of the tail and the underdevelopment of eyes, otic vesicles and somites. The left shift of the curve is indicated by an EC_{50} of 137 μmol after a 24-h test duration and a further decrease to 91 μmol after 48 h of exposure. From the several sublethal and teratogenic effects observed, one endpoint (embryo heart rates) was simplest to analyse. The heart rate was not much more responsive than the other endpoints, but it was analysed most easily. The shift of the concentration–effect curve to lower concentrations was seen at concentrations of 40 μM (Figure 3), which is about a factor of 2 less than the EC_{50} concentration calculated using the extended endpoints (Figure 2).

In vivo inhibition of the cholin- and carboxylesterases

All cholinesterase isozymes and the carboxylesterase analysed showed concentration-dependent effects with increasing concentrations of paraoxon-methyl (Figure 4) (for comparison, the fitted curve of Figure 2 — with the data from the 48-h acute fish embryo test — was included in Figure 4). Cholin- and acetylcholinesterases activities (using either ASCh or AMSCh substrates) were significantly decreased by

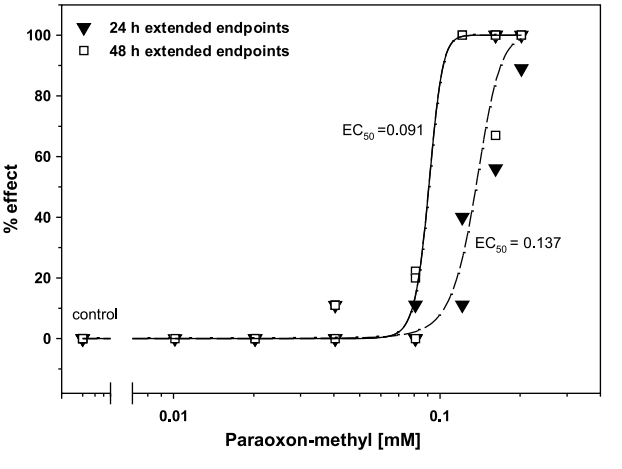

Figure 2. Dose–response relationship as shown in Figure 1 using additional and extended endpoints instead. $n=3$ with ten embryos per replicate. Curve parameters with $f=a/(1+\exp(-(x-x_0)/b))$ with $x_0=EC_{50}$; 24-h exposure, where $a=100$, $b=0.0149$ and $x_0=0.137$, 48-h exposure: $a=100$, $b=0.0055$ and $x_0=0.091$.

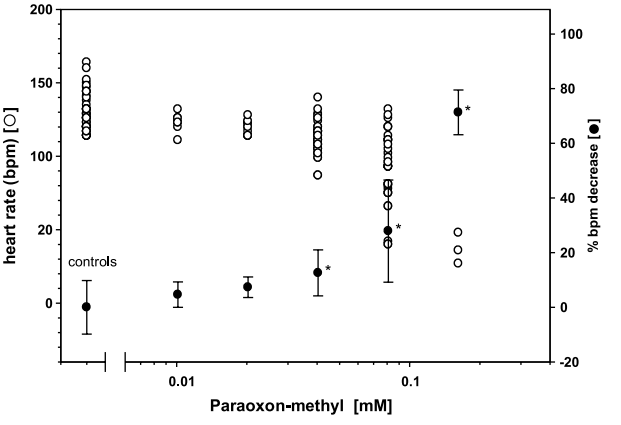

Figure 3. Relationship between increasing paraoxon-methyl concentrations and heart rate in zebra fish embryos (48 h post-fertilization) displayed as beats per min, i.e. bpm (¡) and as percentage bpm decrease (l) compared with controls (mean ± SD). *Significantly different from control with $p < 0.001$ (one-way ANOVA with Tukey–Kramer multiple comparison post-test).

paraoxon-methyl at all concentrations tested. Significant inhibition was already accomplished at the lowest test concentration used (0.12 μmol). This was followed by an even higher inhibition with increasing paraoxon-methyl concentrations. The pseudocholinesterases (using ProSCh as substrate) were inhibited in a similar pattern. A total of 98% inhibition was reached at the highest test concentration of 80.9 μmol. At 20.2 and 40.4 μmol the inhibition of these pseudocholinesterases reached a maximum of 90 and 95%, respectively. Below these concentrations the inhibition was 70% (4.1 and 2.8 μmol), 50–60% (1.2 μmol) and 40% (0.41 μmol) compared with

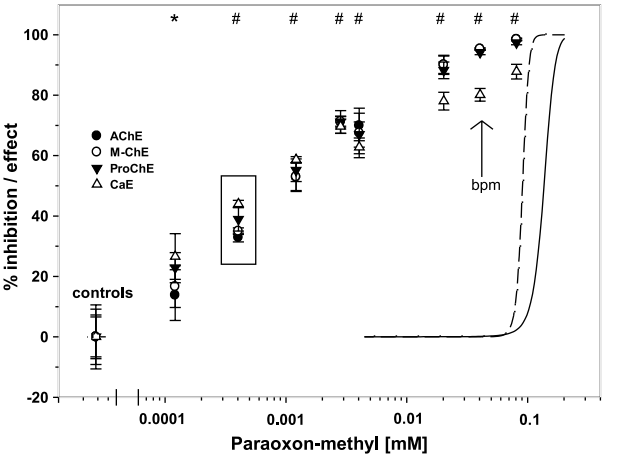

Figure 4. Dose–response relationship between paraoxon-methyl and the inhibition of cholin- and carboxylesterases in supernatants of whole embryo homogenates (48 h post-fertilization). $n = 4$ with 20 embryos per replicate. Data are means ± SD. AChE, acetylcholinesterase; MChE, methylcholinesterase; ProChE, propionylcholinesterase; CaE, carboxylesterase. Significantly different from control with $^{*}p < 0.05$ and $^{\#}p < 0.001$ (one-way ANOVA with Tukey–Kramer multiple comparison post-test). The dataset in the rectangle (0.0004 mM) is taken from Küster (2005). Curve plots are taken from Figure 3. An arrow with beats per min indicates the concentration at which a significant decrease in heart rate was observed.

the respective controls. The IC_{50} can be estimated to be around 0.81 μmol for both the acetylcholinesterase and pseudocholinesterases (ProSCh as substrate).

The inhibition of carboxylesterase was in general similar to the inhibitions of the cholinesterases. Slight differences between the rates of inhibition were seen at the lowest three and the highest test concentration of 80.9 μmol. With 80.9 μmol paraoxon-methyl the inhibition of carboxylesterase did not increase above 88%. At the lowest three test concentrations (0.12–1.2 μmol) the inhibition of carboxylesterase was slightly but not significantly higher, while the inhibition at the highest concentration was about 10–15% lower than inhibition of cholinesterases.

Overall, the results shown in Figure 4 also illustrate differences in the concentration–effect relationships for the enzyme and the other (visible) endpoints. The response of the fish embryos to increasing doses of paraoxon-methyl clearly follows a sigmoidal curve progression (lethal, sublethal and teratogenic endpoints) with a steep slope. In contrast, the enzyme data show (1) a very different, i.e. much more shallow slope, and (2) measurable effects on the enzymes at much lower test concentrations than seen with the whole embryos.

Discussion

Fish embryotoxic potential of paraoxon-methyl

Toxicity data about the parathion-methyl metabolite paraoxon-methyl, which was used here, are very scarce. The only data could be found in the database of the Pesticide Action Network North America (PAN) (http://www.pesticideinfo.org/ January 2005), which lists four studies including two fish toxicity tests. This database retrieves databank information from the World Health Organization (WHO), the European Union (EU), the International Agency for Research on Cancer (IARC), the US Environmental Protection Agency (EPA) and other different US organizations such as the National Toxicology Program (NTP), the National Institutes of Health (NIH) and the State of California.

One study used young rainbow trout (10–15 g body weight) and tested the acute lethal toxicity (24 h) as the major endpoint (Leland 1968). The LC_{50} was calculated to be 0.34 μmol. A second study tested the toxicity by injecting paraoxon-methyl into pumkinseed sunfish (*Lepomis gibbosus*). Lethal toxicity was reached with doses of 32.4–80.9 μmol kg^{-1} body weight (Benke et al. 1973).

Although the amount of paraoxon-methyl in the zebra fish embryos was not quantified, it may be assumed that the concentration used in the sunfish tests (measured in μmol kg^{-1}; Benke et al. 1973) is somewhat comparable with the concentrations used in the zebra fish embryos (measured in $\mu mol \cdot l^{-1}$). As paraoxon-methyl has a log K_{OW} of 1.3, the substance is assumed to be not exactly equally distributed between the embryo and the surrounding water phase, but might be moderately enriched by a factor of 20. The volume to volume (v/v) ratio between the embryos and the test water is calculated to be 1/11 000 (egg diameter of 0.7 mm with a volume of 0.18 mm^3, i.e. 3.6 mm^3 for 20 eggs in a test volume of 40 ml). The concentration in the embryos might be a little higher. But the duration of exposure was 48 h only, so time might have been too short to reach a maximal concentration of paraoxon-methyl in the embryos. and as the embryos are rich in yolk, some of the paraoxon-methyl is possibly enriched in the yolk also and therefore building up in the embryo later when the yolk is used up. Altogether, literature data showed that

the acute toxicity of parathion-methyl/ethyl and paraoxon-methyl in different larvae and adult fish is in the low μM range while toxic effects in embryos can be seen at high μM range only.

Inhibition of esterases by paraoxon-methyl

In terms of sensitivity, the used zebra fish embryo enzymes are as responsive to organophosphates as the enzymes of different adult fish. The inhibition of enzymes in this work was measurable at concentrations above 121 nmol. Acute toxicity tests with 343 nmol parathion (presumably parathion-ethyl) decreased the AChE enzyme activity in the brain of goldfish to 40% of the control activities after 24-h exposure (Weiss 1959).

Ludke et al. (1975) and Moulton et al. (1996) postulate that a 20–30% reduction of AChE activity is a valid biomarker for organophosphate exposure in birds and mussels, respectively. As a significant inhibition of the enzymes was measurable in the zebra fish embryos at concentrations at which no visible effects could be seen, the esterases analysed here seem to be a useful biomarker of sublethal exposure.

Striking is the fact that visible effects in the zebra fish embryo after exposure to paraoxon-methyl are correlating with a 80–100% inhibition of the cholin- and carboxylesterases. This is in agreement with findings of Habig et al. (1986), Detra & Collins (1991), Carr et al. (1997) and Sturm & Hansen (1999) and who found lethal or close to lethal effects of anti-ChEs after a 70–100% inhibition of ChEs in different species such as *Daphnia*, chironomids, crabs and fish.

CaE was tested as a possible additional biomarker of exposure. CaE might serve as a secondary target sequestering the organophophates before ChE is inhibited (Garcia et al. 2003) and thereby being an important pathway of detoxification (Abbas & Hayton 1997). Küster (2005) showed that enzyme activities of CaE were much higher than the ChE enzyme activities at a very early developmental stage of zebra fish embryos. Therefore, it was possible to postulate that CaE might already serve as a stoichiometric buffer system at this early developmental stage. But the results from the paraoxon-methyl tests with the older embryos (48 hpf) in this work do not support this interpretation. The enzyme units and the magnitude of inhibition of CaE and ChE were similar at this late (prehatching) embryonic stage. Only a tendency towards higher inhibition could be seen at the three lowest test concentrations. This is in contrast to results achieved by Ozretić & Krajnović-Ozretić (1992) and Barata et al. (2004). They found an increased inhibition of CaE compared with ChE by organophosphates in the mussel *Mytilus galloprovincialis* and a twofold higher sensitivity of CaE than ChE to the organophosphate malathion in exposed *Daphnia magna*, respectively.

It must be added that the selection of *S*-phenylthioacetate (PSA) as the substrate for CaE can be criticized. PSA is also used as a substrate for the determination of paraoxonase/arylesterase (PON) activity in mammals. This PON was first characterized by its ability to hydrolyse organophosphates such as paraoxon (Aldridge 1953) or chlorpyrifos oxon. Therefore, PON is able to detoxify or decrease the toxic effects of organophosphates (Draganov & La Du 2004). Aldridge (1953) derived the hydrolysing esterases (PON) and the inhibited esterases (ChE and CaE) into the A-esterases and B-esterases, respectively. Therefore, the PSA might be a substrate not only for the CaE — as it was used by Basack et al. (1998) — but also for PON esterases. As we

used total homogenates of zebra fish embryos (with a possible mixture of the two enzymes PON and CaE), it might not be possible to differentiate sharply between the PON-hydrolysing activity and the CaE inhibition. Therefore, the inhibiting effects of the paraoxon detected might have been reduced by the PON-hydrolysing activity of the same paraoxon. In contrast, in the review by Draganov & La Du (2004) work by Li et al. (2000) is cited. They found that the hydrolysing efficiency of PON is dependent on the substrate and that the efficiency is actually low with paraoxon.

Comparison of enzyme inhibition and visible effects in the zebra fish embryo

The results shown in Figures 1–3 compared with the data shown in Figure 4 clearly show the difference between first signs of enzyme inhibition and observable effects. The first significant effects using sublethal and teratogenic endpoints in the fish embryo test can be analysed at about 0.08 mM paraoxon-methyl, while significant enzyme inhibition could already be seen at 0.12 μM (which is a factor of about 660). Including heart rate in the data interpretation, the sensitivity of the zebra fish embryo bioassay is raised by a factor of 2 with the first significant differences from control at a concentration of 0.04 mM.

Substance selectivity

The motivation of this work was not to develop another biomarker of insecticide exposure but to establish a tool to identify different modes of action (here neurotoxic mode of action) of substances in a complex contaminated environmental sample such as groundwater or with substances yet unknown for their neurotoxic potential. The identification of these specific modes of action with this biological detector then could help to identify the (ecotoxicological) relevant substances with chemical analytics such as HPLC or gas chromatography-mass spectrometry (GC-MS) in toxicity identification and evaluation (TIE) approaches (Brack 2003). As it was not clear from the literature at hand if the embryos of the zebra fish were able to metabolize parathion, paraoxon-methyl was preferred as a positive control to parathion-methyl although numerous data exist about the parent compound parathion-methyl (E601) and its effects to many different organisms (WHO 1992, Anon. 1997, 2005a). For metabolizing parathion into the bioactive form, the fish embryos need enzymes of the cytochrome P450 type that are part of the mixed-function oxidases (MFO) being responsible for metabolism. But the existence of one of the zebra fish cytochrome P450 (CYP1A1) proteins in the prehatched embryo is controversial. Mattingly & Toscano (2001) did find CYP1A1 mRNA in embryos before 24 hpf, but were unable to detect the corresponding protein before day 3 pf. Nevertheless, they were able to induce mRNA expression with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In contrast, Yamazaki et al. (2002) were able to prove CYP1A1 protein existence in prehatched embryos of zebra fish even without TCDD using Western blot analysis. Also, Anderson & Förlin (1992) showed that the amounts of cytochromes P450 increase very much after hatching in fish.

The third reason not to use parathion-methyl at first hand was that Van Leeuwen et al. (1985) showed that the lipids contained in the yolk of embryos might function as a temporary toxicant sink for lipophilic substances such as parathion-methyl, which has a $\log K_{OW}$ of 3.00–3.43 (Anon. 1997). Therefore, parathion might not provoke significant effects in the young embryo before the yolk is completely used up. In

contrast, the logK_{OW} of paraoxon-methyl is reported to be between 0.98 and 1.33 (Anon. 2005b).

These facts could be dealt with in different ways. For further clarification the fish embryo test and the respective enzyme assays should also be tested with other pesticides of different type and physico-chemical characteristics (high lipophilicity etc.). Alternatively a metabolizing activation step primary to the bioassay could be used that is similar to the S9 activation used in the umuDC (Oda et al. 1985) or Ames tests (Maron & Ames 1983). In this way, the suitability of the assay system could be verified or refuted for substances with very different toxic potential and properties and may be used for the overall hazard identification of potential neurotoxic substances.

Conclusions

Paraoxon-methyl shows a clear dose–response relationship in the acute (24- and 48-h) zebra fish embryo test with an EC_{50} of 136 and 91 μmol, respectively, when using sublethal endpoints in addition to standardized test protocols.

The IC_{50} of enzyme activity is two orders of magnitude lower than the EC_{50} (approximately 0.8 versus 91 μmol in the acute test) showing that the sublethal biomarker (esterase inhibition) is a much more sensitive endpoint than visible effects (including the decrease of heart rate).

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