

From: Chemosphere [chem-eo@elsevier.com]
Sent: 26 February 2007 14:39
To: Armin Sturm
Subject: CHEM9049R1 - Editor decision - accepted

Dear Dr. Sturm,

I am pleased to inform you that the manuscript "Inhibition of rainbow trout acetylcholinesterase by aqueous and suspended particle-associated organophosphorous insecticides" (Dr. Armin Sturm) has now been accepted by the editor for publication.

Your manuscript will soon be passed to the production department for further handling. Then you will receive further notice.

Thank you for considering our journal for the publication of your research.

Kind regards,
For the Editor,

Mia Schouten, Journal Manager
Chemosphere

Manuscript submission to: Chemosphere

Title: Inhibition of rainbow trout acetylcholinesterase by aqueous and
suspended particle-associated organophosphorous insecticides

Corresponding author: Dr. Armin Sturm

Institute of Aquaculture

University of Stirling

Stirling, FK9 4LA

United Kingdom

armin.sturm@stir.ac.uk

Fax ++44 1786 472 133

25 Pages

4 Figures

2 Tables

38 quoted references

Word counts: total: 5733

title: 12

abstract: 261

1 INHIBITION OF RAINBOW TROUT ACETYLCHOLINESTERASE
2 BY AQUEOUS AND SUSPENDED PARTICLE-ASSOCIATED
3 ORGANOPHOSPHOROUS INSECTICIDES
4

5 ARMIN STURM^{1*}, TANJA S. RADAU², TORSTEN HAHN³, RALF SCHULZ⁴
6

7 ¹Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

8 ²Department of Zoology, University of Stellenbosch, Matieland 7602, South Africa

9 ³³Department of Chemical Risk Assessment, Fraunhofer Institute of Toxicology and
10 Experimental Medicine, 30625 Hannover, Germany

11 ⁴Institute for Environmental Sciences, University of Koblenz-Landau, 76829 Landau,
12 Germany,
13

14
15 *Corresponding author:

16 Armin.Sturm@stir.ac.uk

17 Fax ++44 1786 472 133

ABSTRACT

Spraydrift and edge-of-field runoff are important routes of pesticide entry into streams. Pesticide contamination originating from spraydrift usually resides in the water phase, while pesticides in contaminated runoff are to a large extent associated with suspended particles (SPs). The effects of two organophosphorous insecticides (OPs), chlorpyrifos (CPF) and azinphos-methyl (AZP), on acetylcholinesterase (AChE) activity in rainbow trout were compared between two exposure scenarios, simulating spraydrift- and runoff-borne contamination events in the Lourens River (LR), Western Cape, South Africa. NOECs of brain AChE inhibition, determined after 1 h of exposure followed by 24 h of recovery, were $0.33 \mu\text{g l}^{-1}$ for aqueous CPF, 200 mg kg^{-1} for SP-associated CPF and 20 mg kg^{-1} for SP-associated AZP (at 0.5 g l^{-1} SP). The highest aqueous AZP concentration tested ($3.3 \mu\text{g l}^{-1}$) was without significant effects. Previously reported peak levels of aqueous CPF in the LR ($\sim 0.2 \mu\text{g l}^{-1}$) are close to its NOEC (this study), suggesting a significant toxicological risk to fish in the LR. By contrast, reported levels of SP-associated OPs in the LR are 20- to 200-fold lower than their NOECs (this study). In a comparative in situ study, trout were exposed for seven days at agricultural (LR2, LR3) and upstream reference (LR1) sites. No runoff occurred during the study. Brain AChE was significantly inhibited at LR3. However, OP levels at LR3 (CPF $0.01 \mu\text{g l}^{-1}$; AZP $0.14 \mu\text{g l}^{-1}$) were minor compared to concentrations having effects in the laboratory (see above). Additionally, muscle AChE activity was significantly higher in caged trout from LR1 than in animals maintained in laboratory tanks.

Keywords: biomonitoring fish biomarker spraydrift runoff

INTRODUCTION

Organophosphorous insecticides (OPs) are widely used in agriculture and are characterised by a low persistence under most environmental conditions (Hill, 1995). However, due to their high toxicity, OPs can impose hazard on nontarget species. The toxic action of OPs is based on the inhibition of the enzyme acetylcholinesterase (AChE), which hydrolyses the neurotransmitter acetylcholine in cholinergic synapses of the central and peripheral nervous system. Inhibition of AChE leads to the accumulation of acetylcholine in the synapses, resulting in excessive stimulation of postsynaptic cholinergic receptors (Pope, 1999). OPs inhibit AChE by an irreversible mechanism, so that recovery of enzyme activity after exposure is slow, relying on de novo synthesis of AChE (Main, 1964; Ferrari et al., 2004). Insecticidal carbamates, a second group of anti-AChE insecticides, inhibit AChE by a mechanism similar to that of OPs (Wilson et al. 1960); however, carbamylated AChE can slowly recover (Wilson et al. 1960) while phosphorylated AChE cannot. The measurement of the AChE activities in fish has been suggested as a diagnostic biomarker, with decreased activities indicating water contamination by OP and/or carbamate insecticides (Weiss and Gakstatter, 1964; Coppage and Braidech, 1976; Zinkl et al., 1987).

Freshwater systems in agricultural areas may receive temporary pesticide contamination after pesticide applications to crops. Important routes for the non-point pollution of freshwater systems by pesticides are spraydrift and edge-of-field runoff (Williams et al., 1995; Kreuger, 1998). These entry routes are associated with quite distinct exposure scenarios for aquatic nontarget species. Contamination through spraydrift generally leads to an input of pesticides in the aqueous phase (Schulz et al.,

67 2001a), while runoff-borne pesticides are usually to a large extent associated with
68 suspended particles (Liess et al., 1999).

69 In the Western Cape, South Africa, orchards and vineyards form important
70 agricultural crops. The main pesticide application period (October to February) is
71 characterised by frequent rainfall events, raising the possibility of water
72 contamination through runoff. The entry and occurrence of pesticides in the Lourens
73 River (LR) catchment, Western Cape, has been intensely studied since 1998.
74 Contamination of the mainstream originates from inputs into tributaries, mainly via
75 surface runoff (Schulz et al., 2001b; Dabrowski et al., 2002), but also to a lesser
76 extent by spraydrift (Schulz et al., 2001a).

77 Pesticides, including OPs, have been reported to reach high transient
78 concentrations in the LR during the peak of contamination events. Measured
79 concentrations (n=3) of azinphosmethyl (AZP) and total endosulfan in sediment and
80 water during three runoff events in 1998 and 1999 averaged at $502 \mu\text{g kg}^{-1}$ ($0.67 \mu\text{g l}^{-1}$) for AZP and $4,167 \mu\text{g kg}^{-1}$ ($1.63 \mu\text{g l}^{-1}$) for total endosulfan (Schulz, 2001b). Peak
81 concentrations of various particle-associated pesticides measured in runoff during a
82 rainstorm include $1,247 \mu\text{g kg}^{-1}$ AZP, $924 \mu\text{g kg}^{-1}$ chlorpyrifos (CPF) and $12,082 \mu\text{g kg}^{-1}$ total endosulfan (Schulz, 2001a). Even higher concentrations were measured in a
83 puddle on an orchard plot in which runoff conglomerated before flowing into a
84 tributary of the LR ($17,831 \mu\text{g kg}^{-1}$ AZP, Schulz, 2001a).

87 During recent decades a shift to lower water quality in Western Cape rivers has
88 been attributed to intensified agriculture, erosion problems and loss of indigenous
89 vegetation (Tharme J., Personal communication). The few studies which have
90 assessed the effects of pesticides on nontarget species in Western Cape rivers, have
91 focussed on invertebrates (Schulz, 2001; Schulz et al., 2001a; Schulz, 2003; Thiere

and Schulz, 2004). However, information on the potential effects of pesticides on fish is of particular importance, as fish kills have been reported from the LR (Krause V., Personal communication).

The aim of this study was to compare the effects of aqueous and particle-associated OPs on rainbow trout, using inhibition of AChE activity as the endpoint. AChE inhibition represents a well-accepted biomarker of exposure to anti-AChE insecticides and is mechanistically related to their toxicity. The two OPs selected for this study, azinphosmethyl (AZP) and chlorpyrifos (CPF), are among the most commonly used insecticides in agricultural sites within the LR catchment area, and the only anti-AChE insecticides used. The per annum use of AZP in orchards in the Western Cape, South Africa, has been estimated at 52,000 kg active ingredient (Schulz et al., 2002). Quantitative data on CPF use in the Western Cape are not available; however, CPF has been estimated to be among the most intensively used OPs in agriculture (Larson et al., 1997). Results obtained in indoor microcosms were compared to in situ exposures of rainbow trout at three sites in the LR showing different degrees of OP contamination.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide and 5,5-dithiobis(2-nitrobenzoic acid) were obtained from Sigma-Aldrich Chemical (Deisenhofen, Germany). All substances were of 95 to 99% purity. Azinphos-methyl (O,O-dimethyl S-(4-oxo-1,2,3-benzotriazin-3(4H)-yl)-methyl phosphorodithioate; Azin 200SC[®], Sanachem, Durban, South Africa) and chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothioate; Dursban[®], AgrEvo, Halfway House, South Africa) were obtained from the Lourensford Estate, Western Cape, South Africa.

Study area and study sites

The Lourens River (LR) rises at an altitude of 1080 m in a naturally vegetated fynbos area and flows in a southwesterly direction for 20 km before discharging into False Bay at The Strand (S34°06'; E18°48'), South Africa. The middle reaches of the catchment region are characterised by intensive orchard and vineyard farming. The LR has a total catchment area of 92 km² and receives a mean annual rainfall of 915 mm. About 87% of its mean annual discharge of 35 x 10⁶ m³ occurs during the autumn, winter and early spring months between April and October. The main soil type is silty loam and the slopes in the catchment vary between < 2% and < 8% (Schulz et al., 2001b). Consequently, field-runoff following rainfall constitutes an important route of pesticide entry into the river (Dabrowski et al., 2002).

Three LR sites were selected within a 6 km distance for measurements of physical and chemical water parameters, aqueous-phase and particle-associated pesticide levels, and field studies with rainbow trout. The reference site LR1 was

located upstream of agriculturally used areas and previously shown to lack pesticide contamination (Schulz, 2001a). Sites LR2 and LR3 were located within areas of intense agriculture and have been found to show elevated levels of sediments and nutrients, as well as contamination by pesticides of agricultural origin (Thiere and Schulz, 2004).

Microcosm studies

Indoor microcosm experiments were carried out to assess the effects of aqueous- and particle-associated OPs, simulating the conditions of spraydrift and runoff events, respectively. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local hatchery and allowed to acclimate to laboratory conditions for one week. In experiments, trout (body length 10.9 ± 1.6 cm, body weight 14.6 ± 6.1 g) were randomly assigned to treatments (10-13 individuals). Fish were exposed for one hour to azinphos-methyl (AZP) or chlorpyrifos (CPF) in stream microcosms (length = 1.5 m, width = 0.21 m, height = 0.15 m). After exposure, fish were transferred into microcosms with clean water and allowed to recover for 24 h before sampling. The microcosms contained 30 l river water from LR1. In the recirculating systems, a paddle wheel generated a water current (~ 0.11 m s⁻¹). pH, temperature, and oxygen were in the range of 7.1-7.2, 17.5-18.5 °C, and 8.5-8.7 mg l⁻¹, respectively. Nitrite and ammonium levels never exceeded 0.05 mg l⁻¹. Levels of toxic metals (aluminium, copper, zinc, mercury and lead) and pesticides (CPF, AZP, fenvalerate, deltamethrin, prothiofos and endosulfan) in the testing water were below detection limits (metals, 0.005-0.25 mg l⁻¹; pesticides, 0.01 µg l⁻¹).

For exposures to aqueous OPs, stock solutions were prepared in distilled water and appropriate amounts added to microcosms. For exposures to suspended particle

(SP)-associated OPs, stock solutions of SP-OP were prepared that were 500-fold concentrated with respect to both OP and SP. To 20 g oven-dried silt-loam sediment (grain size < 20 μm , total organic carbon = 15.4 %) from a pristine tributary of the LR, the appropriate amount of pesticide was added in 10 ml of acetone. After evaporation of the solvent, 80 ml of dilution water were added and serial dilutions using 250 g l^{-1} of dry sediment as the dilution medium. The obtained SP-OP stock solutions were aerated for 36 h at 17°C in the dark, and then added to microcosms, to give the desired nominal OP concentration and a final SP concentration of 0.5 g l^{-1} which is characteristic for conditions during runoff-borne contamination events in the LR (Schulz, 2001a). Control treatments consisted of untreated trout (exposure water only) and uncontaminated SP (treated with acetone only).

In situ exposures

Groups of ten trout were exposed in 20 l plastic buckets with large openings covered with 5-mm stainless steel mesh to allow water flow (0.15 m s^{-1}) through the bucket. Exposure took place for 7 days (7/2/2002 to 14/2/2002).

Sampling and water parameters for in situ experiments

Selected physical and chemical parameters were recorded using electronic meters from WTW (Weilheim, Germany) and Lange (Düsseldorf, Germany) between 4.12.2001 and 6.3.2002, either weekly (discharge, total suspended solids (TSS), conductivity, oxygen, temperature and pH) or at 2-week intervals (concentrations of orthophosphate, nitrate, nitrite and ammonium). Suspended sediment samplers used to obtain SP samples (Liess et al., 1996) have been described elsewhere. Previous field observations have shown that during the application season spraying of orchards in

the LR catchment occurs almost every workday of the week (Schulz, 2001b). To estimate potential water pollution by spraydrift, pesticide concentrations in water were measured from composite samples taken at each site over a period of 5 h on a workday, as described earlier (Schulz et al., 2001a).

Pesticide analysis

Sample extraction and GC analysis was carried out as described in detail elsewhere (Schulz et al., 2001a). Detection limits were $0.01 \mu\text{g l}^{-1}$ and $0.1 \mu\text{g kg}^{-1}$ dry mass for water and suspended sediments, respectively, and spiked recovery efficiencies were between 79 and 106%.

Biochemical analyses

Prior to tissue sampling, fishes were stunned by a blow to the head and killed by cervical dislocation. The whole brain and approximately 150 mg of skeletal white muscle tissue were sampled immediately and stored at -20°C in 1 ml buffer (potassium phosphate, 0.1 mol l^{-1} , pH 7.4). For analyses, samples were thawed and homogenized on ice using a Polytron homogeniser. The supernatant obtained after centrifugation ($10,000 \text{ g}$, 4°C , 15 minutes), containing the low-salt soluble fraction of cholinesterases (Massoulié and Bon, 1982), was diluted 1:20 in buffer and used in enzymatic and protein measurements. AChE activity was determined at a temperature of 25°C by the method of Ellman et al. (1961) as adapted for 96-well microplates (Sturm et al., 1999a). Cholinesterase activity in brain and white muscle of rainbow trout consisted exclusively of AChE (EC 3.1.1.7, nomenclature of the International Union of Biochemistry and Molecular Biology), as apparent from its lack of activity on the selective butyrylcholinesterase (EC 3.1.1.8) substrate, butyrylthiocholine

iodide (data not shown). Protein concentrations were determined by the Coomassie blue method using a commercial kit (Carl Roth, Karlsruhe, Germany) with bovine serum albumin as the standard.

Data analysis

Bartlett's test indicated that the variances in some subsets of the data were non-homogenous. Therefore, the non-parametric Kruskal-Wallis test was used to compare AChE activities among treatments or sites. To obtain lowest observed effect concentrations (LOECs) and no observed effect concentrations (NOECs), Dunn's multiple comparison test was used (Zar, 1996). Differences were considered significant if the probability value (P) was < 0.05 .

RESULTS

The effects of aqueous and SP-associated OPs on AChE activities in brain and white muscle were investigated in short-term (1 h) exposures of rainbow trout, followed by 24 h of recovery. Within the tested concentrations, there were no obvious signs of toxicity, except for a moderately impaired activity of trout in some of the highest concentrations. The treatment of trout with uncontaminated SP did not provoke a change in AChE activities when compared to dilution water controls (data not shown). In aqueous exposures, 61 to 95% of the nominal concentrations of the investigated pesticides were detected, while in particle-associated exposures actual levels were 45 to 68% of nominal levels (data not shown). Aqueous chlorpyrifos (CPF) caused significant decreases in rainbow trout brain AChE activities at nominal concentrations of $1 \mu\text{g l}^{-1}$ and $3.3 \mu\text{g l}^{-1}$, while no significant effects were observed with the highest aqueous azinphos-methyl (AZP) concentration tested, $3.3 \mu\text{g l}^{-1}$ (Fig.1). When OPs were tested in an SP-associated form at a SP concentration of 0.5 g l^{-1} , the lowest OP levels (expressed in mg kg^{-1} of SP) causing significant decreases in brain AChE were 660 mg kg^{-1} CPF and 66 mg kg^{-1} AZP (Fig. 2). Consequently, NOECs/LOECs obtained in this study were $0.33 \mu\text{g l}^{-1} / 1 \mu\text{g l}^{-1}$ for aqueous CPF, $200 \text{ mg kg}^{-1} / 660 \text{ mg kg}^{-1}$ for SP-associated CPF and $20 \text{ mg kg}^{-1} / 66 \text{ mg kg}^{-1}$ for SP-associated AZP. In general, the relative inhibition of AChE in white muscle was similar to that observed in brain (Figs. 1 and 2).

In a comparative in situ study, rainbow trout were exposed in cages at three LR sites for one week during the OP application period in February 2002. Some physical and chemical water quality parameters differed between agricultural sites LR2 and LR3 and the reference site LR1, notably total suspended solids, conductivity,

phosphate, nitrate, nitrite and total organic carbon (Table 1). An effect of these water quality parameters on AChE activity is unlikely, as these and other water quality parameters showed no correlation with cholinesterase activities in stickleback (Sturm et al., 1999b). Compared to LR1, brain AChE activities were significantly decreased by ~40% at LR3, but remained unchanged at LR2 (Fig. 3). To assess the general contamination status of the sites, SP was obtained from suspended sediment samplers installed in the streams (Liess et al., 1996). SP samples from the two week-interval surrounding the week of in situ-exposures showed detectable levels of CPF and AZP at both agricultural sites LR2 and LR3, but lacked OPs at the reference site LR1 (Table 2). This confirms the assumed contamination status of the sites; however the absence of significant rain events in the week of in situ exposures suggested that the SP in the samplers originated from runoff events that took place prior to the in situ exposures. To obtain an estimate of potential contamination from spraydrift, composite water samples were taken over a period of 5 h during one workday of the week of the in situ exposure (Table 2). Samples from the reference site LR1 lacked detectable residues of the OPs analysed, while low amounts of OPs were present in samples from both agricultural sites LR2 and LR3, with AZP being the major contaminant (Table 2).

During the study, we observed a noteworthy variability of muscle, but not brain, AChE activities between trout kept in laboratory aquaria and in situ exposed fish (Fig. 4). Muscle AChE activities of trout exposed at reference site LR1 were higher than those of most control groups of laboratory experiments (Fig. 4).

DISCUSSION

In this study, exposure of trout to $1 \mu\text{g l}^{-1}$ of CPF resulted in the inhibition of brain AChE activity by about 40%, and while no significant effects were seen with AZP at the concentrations tested, there were indications of reductions of about 30% in AChE activity with $3.3 \mu\text{g l}^{-1}$ AZP. The inhibition of brain AChE following acute exposures to low concentrations of OPs in the $\mu\text{g l}^{-1}$ range has been reported for various fish species including salmonids. For instance, 96 h of exposure to $2.5 \mu\text{g l}^{-1}$ of CPF resulted in an inhibition of brain AChE by about 40% in steelhead trout, the anadromous form of *Oncorhynchus mykiss* (Sandahl and Jenkins, 2002). The 96 h LOEC of brain AChE inhibition by CPF in fathead minnow has been determined as $0.27 \mu\text{g l}^{-1}$ (Jarvinen et al., 1983). After AZP exposure, Van Dolah et al. (1997) reported 24 h EC50s of $1.1 \mu\text{g l}^{-1}$ in mummichog and $5.3 \mu\text{g l}^{-1}$ in red drum, and Ferrari et al. (2004) found a 96 h EC50 of $0.4 \mu\text{g l}^{-1}$ in rainbow trout. Considering that different factors may affect results, including the species and the age of test fish, as well as the duration and the temperature of exposures, this study's results on AChE inhibition following water-borne exposures in the absence of SP are in accordance with previous findings.

When associated with SP, 660 mg kg^{-1} CPF provoked an inhibition of AChE of about 60% at a SP concentration of 0.5 g l^{-1} in the exposure water. Comparing this with the aqueous CPF concentration causing a similar degree of AChE inhibition, $3.3 \mu\text{g l}^{-1}$, the association with SP decreased the toxicity of CPF roughly 100-fold. Because aqueous AZP did not show significant effects, an effect of SP cannot be ascertained with this OP. However, attenuating effects of SP, if any, on the toxicity of AZP must have been less than 10-fold, as follows from comparing the LOEC of SP-

associated AZP (66 mg kg^{-1}) to the highest tested aqueous concentration that was without effect ($3.3 \text{ } \mu\text{g l}^{-1}$). A more pronounced effect of SP on the toxicity of CPF compared to AZP was expected, as CPF is more hydrophobic than AZP (CPF $K_{oc} = 7000 - 25000 \text{ l kg}^{-1}$; AZP $K_{oc} = 1000 \text{ l kg}^{-1}$; <http://pmep.cce.cornell.edu/profiles/extoxnet/>) and thus would be more strongly adsorbed to SP.

To assess the risk of potentially hazardous compounds in the environment, toxic benchmark concentrations (usually NOECs, no observed effect concentrations) are compared to predicted or measured environmental levels of the compound. NOECs determined in this study were $0.33 \text{ } \mu\text{g l}^{-1}$ for aqueous CPF, 200 mg kg^{-1} for SP-associated CPF, and 20 mg kg^{-1} for SP-associated AZP. No aqueous NOEC can be given for AZP, because the $\sim 30\%$ decrease in AChE activity at the highest water-borne concentration tested ($3.3 \text{ } \mu\text{g l}^{-1}$) was not significant. However, an inhibition of AChE activities by more than 20% is often considered indicative of an exposure to anti-ChE agents (Ludke et al., 1975).

One hour average aqueous concentrations of CPF of 0.19 to $0.2 \text{ } \mu\text{g l}^{-1}$ have been reported in two earlier studies describing runoff events in the LR (Schulz et al., 2001a; Dabrowski et al. 2002). These levels are very close to the NOEC of $0.33 \text{ } \mu\text{g l}^{-1}$ (this study), suggesting that CPF can exert a significant risk on fish in the LR through a water-borne route. Concerning AZP, previously determined maximal aqueous concentrations in the LR are $1.5 \text{ } \mu\text{g l}^{-1}$ during a runoff event (Schulz et al., 2001a) and $1.7 \text{ } \mu\text{g l}^{-1}$ AZP (in a tributary to the LR) following contamination by spraydrift (Schulz et al. 2001a). Thus, the highest aqueous AZP concentrations reported in samples from the LR system are only about 2-fold lower than $3.3 \text{ } \mu\text{g l}^{-1}$, a concentration at which there were at least some indications that decreased AChE

activity might have been due to OP exposure. Regarding SP-associated OPs, a previous study reported one hour-average concentrations of AZP and CPF during a runoff event in the LR at about 1 mg kg^{-1} (Schulz, 2001a), i.e. 20-fold higher than the NOEC in the case of AZP and 200-fold lower than the NOEC in the case of CPF. This suggests that for CPF, and possibly also for AZP, contamination occurring in the LR in the SP-associated form is less toxicologically relevant than concurrent aqueous phase contamination.

To compare results obtained in laboratory to the field situation, trout were exposed in cages at three sites in the LR during the OP application period. After one week of in situ exposure, brain AChE activities at one agricultural site, LR3, were significantly decreased compared to the reference site. In the week of in situ exposures, there was no runoff event, suggesting that OPs which have originated from spraydrift caused of the inhibition. The higher AChE inhibition at LR3 than LR2 seemed to correspond with OP levels in composite water samples taken over a period of 5 h ($0.04 \text{ } \mu\text{g l}^{-1}$ AZP at LR2; $0.15 \text{ } \mu\text{g l}^{-1}$ AZP at LR3). However, AZP concentrations in water samples from both sites were much lower than the highest concentration tested in the laboratory microcosms ($3.3 \text{ } \mu\text{g l}^{-1}$), at which only non-significant decreases in AChE were observed in this study. Thus, the levels of OP contamination detected in samples from LR3 are too low to explain the decrease of AChE activities at this site. This discrepancy suggests that the true OP exposure of trout at LR3 might have been higher than it appeared from the water concentrations measured in this study, which depicted the situation in the LR only at a certain resolution. For budget reasons, composite water samples could be analysed only for one day, and results are possibly not representative for the entire exposure interval. Moreover, taking into account that water contamination in a running water system is

transient, temporary concentrations might have exceeded the 5 h-average values. Finally, although all anti-ChE pesticides which to our knowledge are used in the investigation area were considered in the chemical analyses, the potential presence of further compounds cannot be excluded with absolute certainty. Thus the data from the in situ study suggest that OP water contamination by spraydrift has the potential to adversely affect fish in the LR, and further demonstrate the usefulness of the biomarker AChE in an exposure scenario where it is difficult to get a representative picture of contamination with conventional chemical analytics, at least with an economically realistic effort.

The comparison of laboratory exposure effects with those measured after in situ caging also indicated issues associated with the definition of reference values, a central problem in biomarker studies. Because of the possibility of undetected background pollution, it can be problematic to rely on reference sites as the sole point of comparison. Alternatively, reference values can be derived from animals kept under defined control conditions in the laboratory. In this study, muscle AChE activities were about 2-fold higher in trout exposed at the reference site LR1 than in animals kept in the laboratory. Similarly, in a previous study (Sturm et al. 1999b) muscle cholinesterase activities were up to 45% higher in stickleback from reference sites than in fish from the laboratory. The major natural variables known to affect AChE activities in fish are body size (Sturm et al., 1999b; Beauvais et al., 2002) and acclimation temperature (Baslow and Nigrelli, 1964). However, within the size range of trout used, there was no correlation between body length or body weight and AChE activities (data not shown). The temperature conditions in the laboratory and the reference site were virtually the same, with slightly higher temperatures at the agricultural sites. While AChE activities are not affected by gender in most fish

species (Gruber and Munn, 1998; Sturm et al., 1999b; Beauvais et al., 2002), sex differences in AChE levels have been reported occasionally in mature adults of certain taxa (Di Marzio et al., 1998). In this study, immature trout were used, so that an influence of the sex of the animals appeared unlikely.

In mammals, chronic increases in the levels of neuromuscular activity can cause significant increases in muscle AChE activity, while brain AChE activity remains unchanged (Sveistrup et al., 1995; Ryhanen et al., 1988). We therefore speculate that higher levels of physical exercise of caged compared to laboratory trout may have caused the increase in white muscle AChE activities in situ. Indeed, it has been suggested that a correlation exists between the general physical activity of fishes and their levels of skeletal muscle AChE, with higher AChE activities present in active than sluggish species (Baslow and Nigrelli, 1961). The easy accessibility of skeletal muscle compared to brain, particularly in small species, might be the reason why this tissue has been used in many biomarker studies with fish AChE. However, this study suggests that the background variability of AChE activities can be higher in muscle than in brain.

Conclusions

- Comparatively high levels of suspended particulate-associated OPs ($\sim 1 \text{ mg l}^{-1}$), having been previously reported in the Lourens River (LR), were without effect on trout AChE activities in 1 h-exposures and probably pose no acute risk for fish.
- The measurement of brain AChE inhibition after the short-term (1 h) exposure of trout to different concentrations of water-borne CPF yielded a NOEC of CPF ($0.33 \text{ } \mu\text{g l}^{-1}$) that was close to previously reported CPF levels during

contamination events in the LR ($0.2 \mu\text{g l}^{-1}$). This suggests that temporary levels of water-borne OPs in the LR, as they occur as a result from losses during use of OPs in the catchment area, likely pose an ecotoxicological risk to fish.

- One-week in situ exposures of trout in the LR during the OP application period provoked AChE inhibition at one of two agricultural sites when compared to an upstream reference site. However, aqueous OP levels at the agricultural sites were too low to explain AChE inhibition. This discrepancy seems to reflect the difficulty in appropriately modelling the highly dynamic exposure conditions in streams. Because of their integrative nature, biomarkers such as AChE may be useful in scenarios where environmental concentrations are highly variable.
- Compared to AChE activities of trout maintained in the laboratory, muscle, but not brain, AChE activities in cage-exposed trout at the reference site were significantly increased. Caution should be applied in using muscle AChE in future studies with trout, and where possible, AChE activities should be measured in brain.

407 *Acknowledgement*—This study is part of the ENVIROMAP project and is funded by
408 the Volkswagen Stiftung, Hannover (I/76 177). Thanks are due to S. Bollmohr, G.
409 Thiere, J. M. Dabrowski, M. Gehlhar, S. Reinecke, A.J. Reinecke, P. Benecke, J.
410 Williams, S.K.C. Peall and M. Leaver.

REFERENCES

- Baslow, M.H., Nigrelli, R.F., 1961. Muscle acetylcholinesterase level as an index of general activity in fishes. *Copeia* 1, 8-11.
- Baslow, M.H., Nigrelli, R.F. 1964. The effect of thermal acclimation on brain cholinesterase activity of the killifish, *Fundulus heteroclitus*. *Zoologica* 49, 41-51.
- Beauvais, S.L., Cole, K.J., Atchinson, G.J., Coffey, M. 2002. Factors affecting brain cholinesterase activity in bluegill (*Lepomis macrochirus*). *Water Air Soil Poll.* 135, 249-264.
- Coppage, D.L., Braidech, T.E. 1976. River pollution by anticholinesterase agents. *Water Res.* 10, 19-24.
- Dabrowski, J.M., Peall, S.K.C., Reinecke, A.J., Liess, M., Schulz R., 2002. Runoff-related pesticide input into the Lourens river, South Africa: Basic data for exposure assessment and risk mitigation at the catchment scale. *Water Air Soil Poll.* 135, 265-283.
- Di Marzio, W.D., Alberdi, J.L., Saenz, M.E. Tortorelli, M.D. 1998. Effects of paraquat (Osaquat ® formulation) on survival and total cholinesterase activity in male and female adults of *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). *Environ. Toxic. Water* 13, 55-59.
- Ellman, G.L., Courtney, K.D., Andres, V.J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88-95.
- Ferrari, A., Anguiano, O.L., Soleño, J., Venturino, A., Pechen de D'Angelo, A.M., 2004. Different susceptibility of two aquatic vertebrates (*Oncorhynchus*

- 436 *mykiss* and *Bufo arenarum*) to azinphos methyl and carbaryl. Comp. Biochem.
 437 Phys. C 139, 239-243.
- 438 Gruber, S.J., Munn, M.D. 1998. Organophosphate and carbamate insecticides in
 439 agricultural waters and cholinesterase (ChE) inhibition in common carp
 440 (*Cyprinus carpio*). Arch. Environ. Con. Tox. 35, 391-396.
- 441 Hill, E.F., 1995. Organophosphorus and carbamate pesticides. In: Hoffman, D.J.,
 442 Rattner, B.A., Burton, G.A., Cairns, J. (Eds.). Handbook of ecotoxicology.
 443 Lewis, Boca Raton, Fl., USA, pp. 243-273.
- 444 Jarvinen, A.W., Nordling, B.R., Henry, M.E., 1983. Chronic toxicity of Dursban
 445 (chlorpyrifos) to the fathead minnow (*Pimephales promelas*) and the resultant
 446 acetylcholinesterase inhibition. Ecotox. Environ. Safe. 7, 423-434.
- 447 Kreuger, J., 1998. Pesticides in stream water within an agricultural catchment in
 448 southern Sweden, 1990-1996. Sci. Total Environ. 216, 227-251.
- 449 Larson, S.J., Capel, P.D., Majewski, M.S., 1997. Pesticides in Surface Waters:
 450 Distribution, Trends, and Governing Factors. Ann Arbor, Chelsea, MI.
- 451 Liess, M., Schulz, R., Neumann, M., 1996. A method for monitoring pesticides bound
 452 to suspended particles in small streams. Chemosphere 32, 1963-1969.
- 453 Liess, M., Schulz, R., Liess, M.H.D., Rother, B., Kreuzig, R., 1999. Determination of
 454 insecticide contamination in agricultural headwater streams. Water Res. 33,
 455 239-247.
- 456 Ludke, J.L., Hill, E.F., Dieter, M.P., 1975. Cholinesterase (ChE) response and related
 457 mortality among birds fed ChE inhibitors. Arch. Environ. Con. Tox. 3, 1-21.
- 458 Main, A.R., 1964. Affinity and phosphorylation constants for the inhibition of
 459 esterases by organophosphates. Science 144, 992-993.

- 460 Massoulié, J., Bon, S., 1982. The molecular forms of cholinesterase and
461 acetylcholinesterase in vertebrates. *Ann. Rev. Neurosci.* 5, 57-106.
- 462 Pope, C.N., 1999. Organophosphorus insecticides: Do they all have the same
463 mechanism of action? *J. Toxicol. Env. Heal. B* 2, 161-181.
- 464 Ryhanen, R., Kajovaara, M., Harri, M., Kaliste-Korhonen, E., Hanninen, O., 1988.
465 Physical exercise affects cholinesterases and organophosphate response. *Gen.*
466 *Pharmacol.* 19, 815-818.
- 467 Sandahl, J.F., Jenkins, J.J., 2002. Pacific steelhead (*Oncorhynchus mykiss*) exposed to
468 chlorpyrifos: Benchmark concentration estimates for acetylcholinesterase
469 inhibition. *Environ. Toxicol. Chem.* 21, 2452-2458.
- 470 Schulz, R., 2001a. Rainfall-induced sediment and pesticide input from orchards into
471 the Lourens River, Western Cape, South Africa: Importance of a single event.
472 *Water Res.* 35, 1869-1876.
- 473 Schulz, R. 2001b. Comparison of spraydrift- and runoff-related input of
474 azinphosmethyl and endosulfan from fruit orchards into the Lourens River,
475 South Africa. *Chemosphere* 45, 429-437.
- 476 Schulz, R., 2003. Using a freshwater amphipod in situ bioassay as a sensitive tool to
477 detect pesticide effects in the field. *Environ. Toxicol. Chem.* 22, 1172-1176.
- 478 Schulz, R., Peall, S.K.C., Dabrowski, J.M., Reinecke, A.J., 2001a. Spray deposition of
479 two insecticides into surface waters in a South African orchard area, J.
480 *Environ. Qual.* 30, 814-822.
- 481 Schulz, R., Peall, S.K.C., Dabrowski, J.M., Reinecke, A.J., 2001b. Current-use
482 insecticides, phosphates and suspended solids in the Lourens River, Western
483 Cape, during the first rainfall event of the wet season. *Water SA* 27, 65-70.

- 484 Schulz, R., Thiere, G., Dabrowski, J.M., 2002. A combined microcosm and field
485 approach to evaluate the aquatic toxicology of azinphosmethyl to stream
486 communities. *Environ. Toxicol. Chem.* 21, 2172-2178.
- 487 Sturm, A., da Silva de Assis, H.C., Hansen, P.-D., 1999a. Cholinesterases of marine
488 teleost fish: Enzymological characterization and potential use in the
489 monitoring of neurotoxic contamination. *Mar. Environ. Res.* 47, 389-398.
- 490 Sturm, A., Wogram, J., Hansen, P.-D., Liess, M., 1999b. Potential use of
491 cholinesterase in monitoring low levels of organophosphates in small streams:
492 Natural variability in three-spined stickleback (*Gasterosteus aculeatus*) and
493 relation to pollution. *Environ. Toxicol. Chem.* 18, 194-200.
- 494 Sturm, A., Wogram, J., Segner, H., Liess, M., 2000. Different sensitivity to
495 organophosphates of acetylcholinesterase and butyrylcholinesterase from
496 three-spined stickleback (*Gasterosteus aculeatus*): Application in
497 biomonitoring. *Environ. Toxicol. Chem.* 19, 1607-1615.
- 498 Sveistrup, H., Chan, R.Y.Y., Jasmin, B.J., 1995. Chronic enhancement of
499 neuromuscular activity increases acetylcholinesterase gene expression in
500 skeletal muscle. *Am. J. Physiol. Cell* 269, C856-C862.
- 501 Thiere, G., Schulz, R., 2004. Runoff simulation with particle-associated
502 azinphosmethyl in multispecies stream microcosms: Implications for the field.
503 *Environ. Toxicol. Chem.* 23, 1984-1990.
- 504 Van Dolah, R.F., Maier, P.P., Fulton, M.H., Scott, G.I., 1997. Comparison of
505 azinphosmethyl toxicity to juvenile red drum (*Sciaenops ocellatus*) and the
506 mummichog (*Fundulus heteroclitus*). *Environ. Toxicol. Chem.* 16, 1488-1493.
- 507 Weiss, C.M., Gakstatter, J.H., 1964. Detection of pesticides in water by biochemical
508 assay. *Journal of the Water Pollution Control Federation* 36, 240-253.

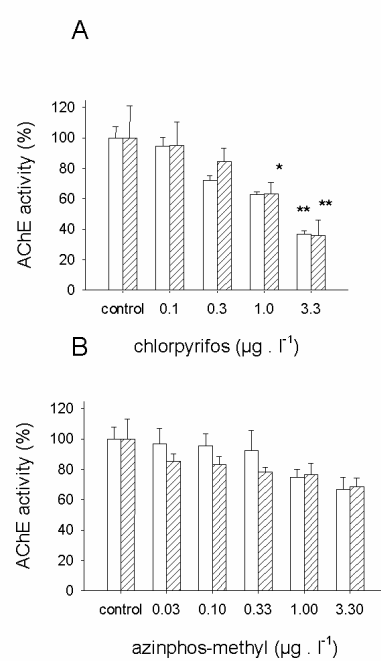
- 509 Williams, R.J., Brooke, D., Matthiesen, P., Mills, M., Turnbull, A., Harrison, R.M.,
510 1995. Pesticide transport to surface waters within an agricultural catchment. J.
511 Inst. Water Env. Man. 9, 72-81.
- 512 Wilson, I.B., Hatch, M.A., Ginsburg, S., 1960. Carbamylation of acetylcholinesterase.
513 J. Biol. Chem. 235, 2312-2315.
- 514 Zar, J.H., 1996. Biostatistical Analysis. Prentice Hall, New Jersey, USA.
- 515 Zinkl, J.G., Shea, P.J., Nakamoto, R.J., Callman, J., 1987. Technical and biological
516 considerations for the analysis of brain acetylcholinesterase of rainbow trout.
517 T. Am. Fish. Soc. 116, 570-573.
- 518
- 519
- 520

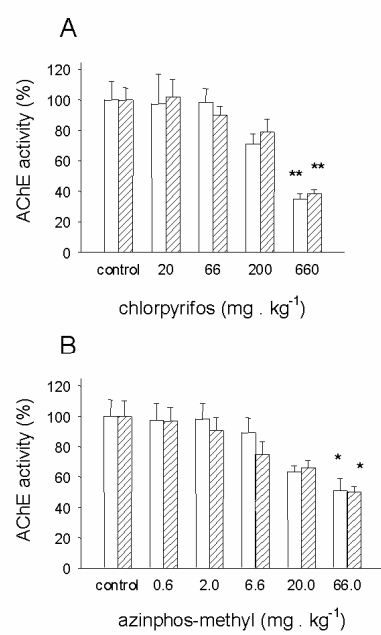
Fig. 1. Percentage of AChE inhibition in white muscle (open bars) and brain hatched bars) of rainbow trout after exposure (1 h) to aqueous-phase chlorpyrifos (A) and azinphos-methyl (B). Values are the mean and SEM of enzyme activities from 8 to 13 fish. Significantly different from control group: * $P < 0.05$; ** $P < 0.001$.

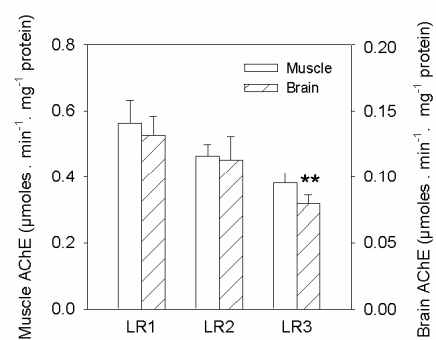
Fig. 2. Percentage of AChE inhibition in white muscle (open bars) and brain (hatched bars) of rainbow trout after exposure (1 h) to suspended particle-associated chlorpyrifos (A) and azinphos-methyl (B). Particles of the indicated contamination level were added to exposure water at 0.5 g l^{-1} . Values are the mean and SEM of enzyme activities from 4 to 13 fish. Significantly different from control group: * $P < 0.05$; ** $P < 0.001$.

Fig. 3. AChE activities in white muscle and brain of rainbow trout after seven days of in situ exposure at different sites in the Lourens River, South Africa. Values are the mean and SEM of enzyme activities from 7 to 10 fish. ** Significantly different from site LR1 (reference), $P < 0.01$.

Fig. 4. AChE activities in rainbow trout from laboratory control groups (E1 to E4) and trout exposed in situ (reference site LR1). Panel A, brain. No significant differences among groups were observed. Panel B, white muscle. Columns having no common letter differ significantly ($P < 0.05$). Values are the mean and SEM of enzyme activities from 8 to 16 fish.

544 **Fig. 1**

546 **Fig. 2**

548 **Fig. 3**

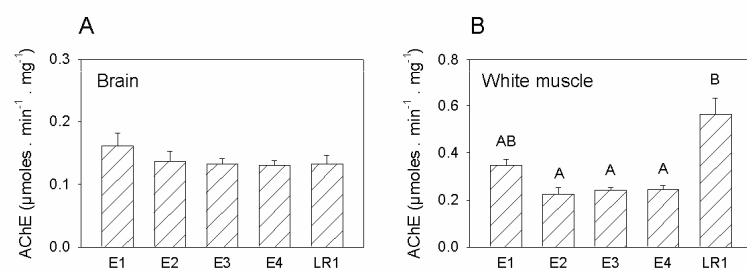
550 **Fig. 4**

Table 1. Water-quality parameters (average \pm SEM) at field sites in the Lourens River, South Africa, based on measurements from 12/2001 to 03/2002.

	site		
	LR1	LR2	LR3
Width (m)	7.1 \pm 0.0	7.9 \pm 0.1	11.8 \pm 0.2
Discharge (m ³ . s ⁻¹)	0.25 \pm 0.08	0.65 \pm 0.17	0.71 \pm 0.20
Total suspended solids (mg . l ⁻¹)	3.0 \pm 2.0	76.8 \pm 23.3	63.8 \pm 23.0
Conductivity (μ S)	513 \pm 37	1,090 \pm 53	1,141 \pm 62
Oxygen (mg . l ⁻¹)	7.81 \pm 0.27	7.84 \pm 0.34	7.60 \pm 0.28
Temperature (°C)	18.3 \pm 0.5	20.3 \pm 0.5	21.1 \pm 0.5
PH	6.0 \pm 0.10	6.4 \pm 0.05	6.6 \pm 0.04
PO ₄ ³⁻ (mg . l ⁻¹)	0.14 \pm 0.03	0.22 \pm 0.04	0.23 \pm 0.03
NO ₃ ⁻ (mg . l ⁻¹)	0.29 \pm 0.18	1.86 \pm 0.55	2.29 \pm 0.71
NO ₂ ⁻ (mg . l ⁻¹)	< 0.005	0.13 \pm 0.06	0.17 \pm 0.07
NH ₄ ⁺ (mg . l ⁻¹)	< 0.02	< 0.02	< 0.02
Total organic carbon (%)	1.46	1.96	2.90

Table 2. Concentrations of chlorpyrifos (CPF) and azinphos-methyl (AZP) in water and suspended particle samples collected from sites in the Lourens River, South Africa, during an in situ exposure of rainbow trout. Malathion was not detected in any of the samples.

Site	Water ($\mu\text{g l}^{-1}$) ¹		Suspended particle ($\mu\text{g kg}^{-1}$) ²	
	CPF	AZP	CPF	AZP
LR1	ND	ND	ND	ND
LR2	ND	0.04	59.2	34.6
LR3	0.01	0.14	74.4	57.9

¹ Integrated 5 h pesticide concentrations determined on one workday within the 7 day in situ exposure (07-13/02/2002).

² Sampling interval 01-15/02/2002.