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Manuscript submission to: Chemosphere

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

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INHIBITION OF RAINBOW TROUT ACETYLCHOLINESTERASE BY AQUEOUS AND SUSPENDED PARTICLE-ASSOCIATED ORGANOPHOSPHOROUS INSECTICIDES

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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 μg 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 μg l^{-1}) was without significant effects. Previously reported peak levels of aqueous CPF in the LR (~0.2 μg 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 μg l^{-1}; AZP 0.14 μg 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.,

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

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

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

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

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

95 The aim of this study was to compare the effects of aqueous and particle-
96 associated OPs on rainbow trout, using inhibition of AChE activity as the endpoint.
97 AChE inhibition represents a well-accepted biomarker of exposure to anti-AChE
98 insecticides and is mechanistically related to their toxicity. The two OPs selected for
99 this study, azinphosmethyl (AZP) and chlorpyrifos (CPF), are among the most
100 commonly used insecticides in agricultural sites within the LR catchment area, and
101 the only anti-AChE insecticides used. The per annum use of AZP in orchards in the
102 Western Cape, South Africa, has been estimated at 52,000 kg active ingredient
103 (Schulz et al., 2002). Quantitative data on CPF use in the Western Cape are not
104 available; however, CPF has been estimated to be among the most intensively used
105 OPs in agriculture (Larson et al., 1997). Results obtained in indoor microcosms were
106 compared to in situ exposures of rainbow trout at three sites in the LR showing
107 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 km^2 and receives a mean annual rainfall of 915 mm. About 87% of its mean annual discharge of 35 x 10^6 m^3 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

133 located upstream of agriculturally used areas and previously shown to lack pesticide
134 contamination (Schulz, 2001a). Sites LR2 and LR3 were located within areas of
135 intense agriculture and have been found to show elevated levels of sediments and
136 nutrients, as well as contamination by pesticides of agricultural origin (Thiere and
137 Schulz, 2004).
138
139 *Microcosm studies*
140 Indoor microcosm experiments were carried out to assess the effects of aqueous-
141 and particle-associated OPs, simulating the conditions of spraydrift and runoff events,
142 respectively. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local
143 hatchery and allowed to acclimate to laboratory conditions for one week. In
144 experiments, trout (body length 10.9 ± 1.6 cm, body weight 14.6 ± 6.1 g) were
145 randomly assigned to treatments (10-13 individuals). Fish were exposed for one hour
146 to azinphos-methyl (AZP) or chlorpyrifos (CPF) in stream microcosms (length = 1.5
147 m, width = 0.21 m, height = 0.15 m). After exposure, fish were transferred into
148 microcosms with clean water and allowed to recover for 24 h before sampling. The
149 microcosms contained 30 l river water from LR1. In the recirculating systems, a
150 paddle wheel generated a water current (~0.11 m s^{-1}). pH, temperature, and oxygen
151 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
152 ammonium levels never exceeded 0.05 mg l^{-1}. Levels of toxic metals (aluminium,
153 copper, zinc, mercury and lead) and pesticides (CPF, AZP, fenvalerate, deltamethrin,
154 prothiofos and endosulfan) in the testing water were below detection limits (metals,
155 0.005-0.25 mg l^{-1}; pesticides, 0.01 µg l^{-1}).
156 For exposures to aqueous OPs, stock solutions were prepared in distilled water
157 and appropriate amounts added to microcosms. For exposures to suspended particle

158 (SP)-associated OPs, stock solutions of SP-OP were prepared that were 500-fold
159 concentrated with respect to both OP and SP. To 20 g oven-dried silt-loam sediment
160 (grain size < 20 µm, total organic carbon = 15.4 %) from a pristine tributary of the
161 LR, the appropriate amount of pesticide was added in 10 ml of acetone. After
162 evaporation of the solvent, 80 ml of dilution water were added and serial dilutions
163 using 250 g l^{-1} of dry sediment as the dilution medium. The obtained SP-OP stock
164 solutions were aerated for 36 h at 17°C in the dark, and then added to microcosms, to
165 give the desired nominal OP concentration and a final SP concentration of 0.5 g l^{-1}
166 which is characteristic for conditions during runoff-borne contamination events in the
167 LR (Schulz, 2001a). Control treatments consisted of untreated trout (exposure water
168 only) and uncontaminated SP (treated with acetone only).
169
170 *In situ exposures*
171 Groups of ten trout were exposed in 20 l plastic buckets with large openings
172 covered with 5-mm stainless steel mesh to allow water flow (0.15 m s^{-1}) through the
173 bucket. Exposure took place for 7 days (7/2/2002 to 14/2/2002).
174
175 *Sampling and water parameters for in situ experiments*
176 Selected physical and chemical parameters were recorded using electronic meters
177 from WTW (Weilheim, Germany) and Lange (Düsseldorf, Germany) between
178 4.12.2001 and 6.3.2002, either weekly (discharge, total suspended solids (TSS),
179 conductivity, oxygen, temperature and pH) or at 2-week intervals (concentrations of
180 orthophosphate, nitrate, nitrite and ammonium). Suspended sediment samplers used to
181 obtain SP samples (Liess et al., 1996) have been described elsewhere. Previous field
182 observations have shown that during the application season spraying of orchards in

183 the LR catchment occurs almost every workday of the week (Schulz, 2001b). To
184 estimate potential water pollution by spraydrift, pesticide concentrations in water were
185 measured from composite samples taken at each site over a period of 5 h on a
186 workday, as described earlier (Schulz et al., 2001a).
187
188 *Pesticide analysis*
189 Sample extraction and GC analysis was carried out as described in detail
190 elsewhere (Schulz et al., 2001a). Detection limits were 0.01 µg l^{-1} and 0.1 µg kg^{-1} dry
191 mass for water and suspended sediments, respectively, and spiked recovery
192 efficiencies were between 79 and 106%.
193
194 *Biochemical analyses*
195 Prior to tissue sampling, fishes were stunned by a blow to the head and killed by
196 cervical dislocation. The whole brain and approximately 150 mg of skeletal white
197 muscle tissue were sampled immediately and stored at -20 °C in 1 ml buffer
198 (potassium phosphate, 0.1 mol l^{-1}, pH 7.4). For analyses, samples were thawed and
199 homogenized on ice using a Polytron homogeniser. The supernatant obtained after
200 centrifugation (10,000 g , 4 °C, 15 minutes), containing the low-salt soluble fraction
201 of cholinesterases (Massoulié and Bon, 1982), was diluted 1:20 in buffer and used in
202 enzymatic and protein measurements. AChE activity was determined at a temperature
203 of 25 °C by the method of Ellman et al. (1961) as adapted for 96-well microplates
204 (Sturm et al., 1999a). Cholinesterase activity in brain and white muscle of rainbow
205 trout consisted exclusively of AChE (EC 3.1.1.7, nomenclature of the International
206 Union of Biochemistry and Molecular Biology), as apparent from its lack of activity
207 on the selective butyrylcholinesterase (EC 3.1.1.8) substrate, butyrylthiocholine

208 iodide (data not shown). Protein concentrations were determined by the Coomassie
209 blue method using a commercial kit (Carl Roth, Karlsruhe, Germany) with bovine
210 serum albumin as the standard.
211
212 *Data analysis*
213 Bartlett's test indicated that the variances in some subsets of the data were non-
214 homogenous. Therefore, the non-parametric Kruskall-Wallis test was used to compare
215 AChE activities among treatments or sites. To obtain lowest observed effect
216 concentrations (LOECs) and no observed effect concentrations (NOECs), Dunn's
217 multiple comparison test was used (Zar, 1996). Differences were considered
218 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 $\mu g\ l^{-1}$ and 3.3 $\mu g\ l^{-1}$, while no significant effects were observed with the highest aqueous azinphos-methyl (AZP) concentration tested, 3.3 $\mu g\ l^{-1}$ (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 $\mu g\ l^{-1}$ / 1 $\mu g\ l^{-1}$ for aqueous CPF, 200 mg kg^{-1} / 660 mg kg^{-1} for SP-associated CPF and 20 mg kg^{-1} / 66 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,

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

262 During the study, we observed a noteworthy variability of muscle, but not brain,
263 AChE activities between trout kept in laboratory aquaria and in situ exposed fish (Fig.
264 4). Muscle AChE activities of trout exposed at reference site LR1 were higher than
265 those of most control groups of laboratory experiments (Fig. 4).
266

DISCUSSION

In this study, exposure of trout to 1 μg 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 μg l^{-1} AZP. The inhibition of brain AChE following acute exposures to low concentrations of OPs in the μg l^{-1} range has been reported for various fish species including salmonids. For instance, 96 h of exposure to 2.5 μg 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 μg l^{-1} (Jarvinen et al., 1983). After AZP exposure, Van Dolah et al. (1997) reported 24 h EC50s of 1.1 μg l^{-1} in mummichog and 5.3 μg l^{-1} in red drum, and Ferrari et al. (2004) found a 96 h EC50 of 0.4 μg 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 CFP concentration causing a similar degree of AChE inhibition, 3.3 μg 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-

292 associated AZP (66 mg kg^{-1}) to the highest tested aqueous concentration that was
293 without effect (3.3 µg l^{-1}). A more pronounced effect of SP on the toxicity of CPF
294 compared to AZP was expected, as CPF is more hydrophobic than AZP (CPF K_{oc} =
295 7000 - 25000 l kg^{-1}; AZP K_{oc} 1000 l kg^{-1};
296 http://pmep.cce.cornell.edu/profiles/extoxnet/) and thus would be more strongly
297 adsorbed to SP.

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

307 One hour average aqueous concentrations of CPF of 0.19 to 0.2 µg l^{-1} have been
308 reported in two earlier studies describing runoff events in the LR (Schulz et al.,
309 2001a; Dabrowski et al. 2002). These levels are very close to the NOEC of 0.33 µg l^{-1}
310 (this study), suggesting that CPF can exert a significant risk on fish in the LR through
311 a water-borne route. Concerning AZP, previously determined maximal aqueous
312 concentrations in the LR are 1.5 µg l^{-1} during a runoff event (Schulz et al., 2001a) and
313 1.7 µg l^{-1} AZP (in a tributary to the LR) following contamination by spraydrift
314 (Schulz et al. 2001a). Thus, the highest aqueous AZP concentrations reported in
315 samples from the LR system are only about 2-fold lower than 3.3 µg l^{-1}, a
316 concentration at which there were at least some indications that decreased AChE

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

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

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

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

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

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

383

384 ## Conclusions

385

386 ● Comparatively high levels of suspended particulate-associated OPs (~1 mg l^{-1}),
387 having been previously reported in the Lourens River (LR), were without effect on
388 trout AChE activities in 1 h-exposures and probably pose no acute risk for fish.
389 ● The measurement of brain AChE inhibition after the short-term (1 h) exposure of
390 trout to different concentrations of water-borne CPF yielded a NOEC of CPF
391 (0.33 µg l^{-1}) that was close to previously reported CPF levels during

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

395 ● One-week in situ exposures of trout in the LR during the OP application period
396 provoked AChE inhibition at one of two agricultural sites when compared to an
397 upstream reference site. However, aqueous OP levels at the agricultural sites were
398 too low to explain AChE inhibition. This discrepancy seems to reflect the
399 difficulty in appropriately modelling the highly dynamic exposure conditions in
400 streams. Because of their integrative nature, biomarkers such as AChE may be
401 useful in scenarios where environmental concentrations are highly variable.

402 ● Compared to AChE activities of trout maintained in the laboratory, muscle, but
403 not brain, AChE activities in cage-exposed trout at the reference site were
404 significantly increased. Caution should be applied in using muscle AChE in future
405 studies with trout, and where possible, AChE activities should be measured in
406 brain.

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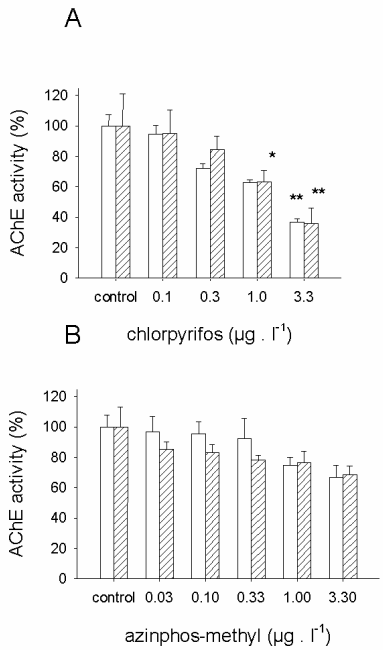
Fig. 1. Percentage of AChE inhibition in white muscle (open bars) and brain hatched bars) of rainbow trout after exposure (1 h) to aqueous-phase chlorpyrifos (A) and azinphos-methyl (B). Values are the mean and SEM of enzyme activities from 8 to 13 fish. Significantly different from control group: *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

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), $P < 0.01$.

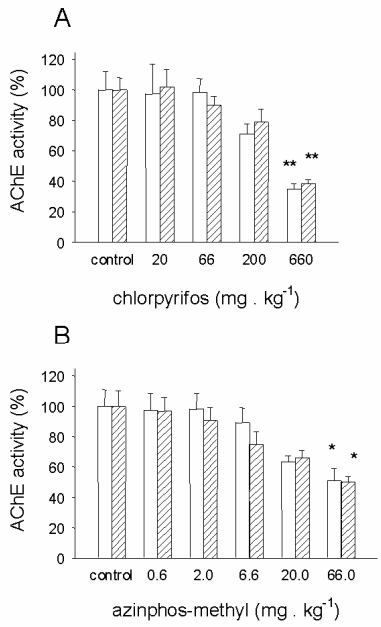
Fig. 4. AChE activities in rainbow trout from laboratory control groups (E1 to E4) and trout exposed in situ (reference site LR1). Panel A, brain. No significant differences among groups were observed. Panel B, white muscle. Columns having no common letter differ significantly ($P < 0.05$). Values are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

544 **Fig. 1**


A
AChE activity (%)
120
100
80
60
40
20
0
control
0.1
0.3
1.0
3.3
*
**
**
chlorpyrifos (µg . l-1)
B
AChE activity (%)
120
100
80
60
40
20
0
control
0.03
0.10
0.33
1.00
3.30
azinphos-methyl (µg . l-1)

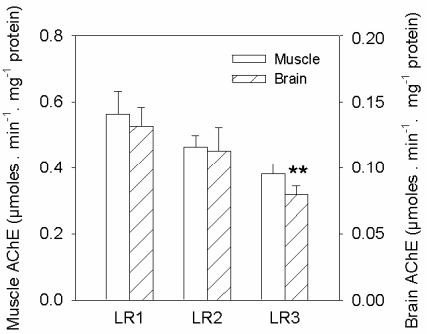
545

546 **Fig. 2**


A
AChE activity (%)
120
100
80
60
40
20
0
control
20
66
200
660
**
**
chlorpyrifos (mg . kg-1)
B
AChE activity (%)
120
100
80
60
40
20
0
control
0.6
2.0
6.6
20.0
66.0
*
*
azinphos-methyl (mg . kg-1)

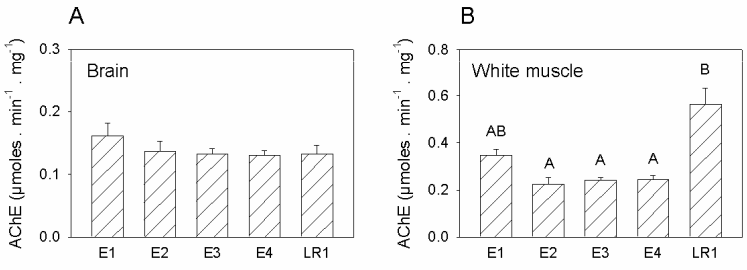
547

548 **Fig. 3**


Muscle AChE (µmoles . min-1 . mg-1 protein)
Brain AChE (µmoles . min-1 . mg-1 protein)
Muscle
Brain
0.8
0.6
0.4
0.2
0.0
0.20
0.15
0.10
0.05
0.00
**
LR1
LR2
LR3

549

550 **Fig. 4**


A
Brain
AChE (µmoles. min-1 . mg-1)
0.3
0.2
0.1
0.0
E1
E2
E3
E4
LR1
B
White muscle
AChE (µmoles. min-1 . mg-1)
0.8
0.6
0.4
0.2
0.0
AB
A
A
A
B
E1
E2
E3
E4
LR1

551

552 **Table 1.** Water-quality parameters (average ± SEM) at field sites in the Lourens
553 River, South Africa, based on measurements from 12/2001 to 03/2002.
554

	site		
	LR1	LR2	LR3
Width (m)	7.1 ± 0.0	7.9 ± 0.1	11.8 ± 0.2
Discharge ($m^3 . s^{-1}$)	0.25 ± 0.08	0.65 ± 0.17	0.71 ± 0.20
Total suspended solids ($mg . l^{-1}$)	3.0 ± 2.0	76.8 ± 23.3	63.8 ± 23.0
Conductivity (µS)	513 ± 37	1,090 ± 53	1,141 ± 62
Oxygen ($mg . l^{-1}$)	7.81 ± 0.27	7.84 ± 0.34	7.60 ± 0.28
Temperature (°C)	18.3 ± 0.5	20.3 ± 0.5	21.1 ± 0.5
PH	6.0 ± 0.10	6.4 ± 0.05	6.6 ± 0.04
PO_4^{3-} ($mg . l^{-1}$)	0.14 ± 0.03	0.22 ± 0.04	0.23 ± 0.03
NO_3^- ($mg . l^{-1}$)	0.29 ± 0.18	1.86 ± 0.55	2.29 ± 0.71
NO_2^- ($mg . l^{-1}$)	< 0.005	0.13 ± 0.06	0.17 ± 0.07
NH_4^+ ($mg . l^{-1}$)	< 0.02	< 0.02	< 0.02
Total organic carbon (%)	1.46	1.96	2.90

555
556
557

558 **Table 2.** Concentrations of chlorpyrifos (CPF) and azinphos-methyl (AZP) in water
559 and suspended particle samples collected from sites in the Lourens River, South
560 Africa, during an in situ exposure of rainbow trout. Malathion was not detected in any
561 of the samples.
562

	Water ($\mu g\ l^{-1}$)[1]		Suspended particle ($\mu g\ kg^{-1}$)[2]	
Site	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

563 [1] Integrated 5 h pesticide concentrations determined on one workday within the 7 day
564 in situ exposure (07-13/02/2002).
565 [2] Sampling interval 01-15/02/2002.
566
567
568