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Joint Effects of Copper Sulphate and Methidathion on Rainbow Trout (*Oncorhynchus mykiss*) EROD and AChE Activities

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Effects on aquatic organisms of simultaneous exposure to various contaminants, both organic compounds and heavy metals, have been rarely studied (Lemaire-Gony and Lemaire 1992). The measurement of the induction of fish ethoxy-resorufin-O-deethylase (EROD) activity, a cytochrome P450 monooxygenase, has been proposed for the biological monitoring of environmental contamination (Payne et al. 1987) and is frequently used to identify exposure to organic xenobiotics such as polycyclic aromatic hydrocarbons, dioxins, polychlorobyphenyls. Moreover, the inhibition of acetylcholinesterase (AChE) activity is used to detect exposure to organophosphates or carbamates (Bocquene et al. 1990). To improve the significance of such a biological monitoring we need to assess the effects of a complex mixture of both organic xenobiotics and metals on those biomarkers (Gagné and Blaise, 1993).

Trace metals and pesticides are common pollutants in the aquatic environment. For instance, high levels of copper sulphate (metal) and methidathion (organophosphate insecticide) can be measured in a french watershed covered by vineyards (Ardieres, South-East of france) (Table 1). Copper is a very toxic heavy metal which is involved in many metabolic pathways leading to a very complex toxicology (Sorensen 1991). Abiotic parameters (pH, conductivity) can modify the speciation and toxicity of copper (Brown et al. 1974). Methidathion is one of the more toxic organophosphates with a high 50 % lethal toxicity on rainbow trout: 96-hr LC50 = 10 µg/L (Montgomery 1993).

In some field conditions where EROD induction and AChE inhibition could be used for the monitoring of water pollution, the joint presence of metals and insecticides may lead to an inhibition of such a monitoring system. This work was carried out to increase the knowledge of those inhibition effects using copper and methidathion in laboratory conditions.

MATERIALS AND METHODS

Methidathion (99.7%) was obtained from Dr Ehrenstorfer BmbH; copper sulphate was purchased from E. Merck, Darmstadt, Germany; acetylthiocholine iodide (ATCI), dithiobisnitrobenzoic acid (DTNB), 7-ethoxyresorufin, S-naphthoflavone (5,6 benzoflavone), β-nicotinamide adenosine-diphosphate reduced form

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(NADPH) were purchased from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were of the highest available commercial grade.

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 6-11 g obtained from a local fish farm were used for all experiments. Immediately before the exposure, fish were intraperitoneally injected with 0.5 mg/kg of β-naphthoflavone in corn oil in order to induce EROD activity to a level comparable to that of fish exposed to environmental inducers. AChE activity was not modified by this pretreatement (data not shown). Then, groups of ten trout were exposed to continuous flow of copper (Cu) and methidathion (MD) in combination for 96 hr. Experimental concentrations were chosen considering field concentrations (Ardières) and acute toxicities on rainbow trout (Table 1):

Table 1. Choice of experimental concentrations of xenobiotics.

Pesticides	Field average concentration (µg/L)	Experimental range (µg/L)	Acute toxicity 96-h LC50 (µg/L)
Copper	10	0; 8; 18	105 (unpublished data)
Methidathion	7.8	0; 1; 5	10 (Montgomery 1993)

Cu and MD were added from stock solutions to the continuously flowing dilution water to give the required concentrations. The stock solutions were prepared daily. Cu and MD water concentrations were only controlled once the assay. The nominal and observed Cu concentrations were equal in value. The observed concentrations of MD were 40% lower than the nominal ones: this is maybe due to the low stability of the MD to light. All fish were tested at 17.0 ± 0.1 °C (conductivity 510 ± 10 µS/cm; pH 7.3 ± 0.1; dissolved oxygen 8.7 ± 0.4 mg/L). Temperature, dissolved oxygen, conductivity and pH were measured twice a day. Fish were fed twice daily during experiments.

Four days after injection, fish were sacrified, weighed, measured, and immediately dissected. Liver and muscle were removed. Each liver was rinsed in 150 mM KCI, homogenized in 100 mM phosphate buffer, pH 7.8 with 20% glycerol, poured into cryotubes. Liver homogenates and muscles were frozen in liquid nitrogen and kept for several days at -80 °C before enzymatic assays (Figure 1).

Homogenates or muscles were thawed at 4°C. Prior to AChE assay the muscle was homogenized in Tris HCI 0.1 M pH 7.8. Each liver or muscle homogenate was centrifuged at 9,000 g and the supernatant (S9) was used for enzymatic assays.

EROD activity of the liver S9 was determined at 20°C by a modified Pohl and Fouts (1980) procedure: EROD activity was assayed in a final volume of 1 mL containing 100 mM phosphate buffer pH 7.8, 5 µM NADPH, 0.2 µM ethoxyresorufin and S9 protein. The reaction was stopped by addition of 2 mL acetone.

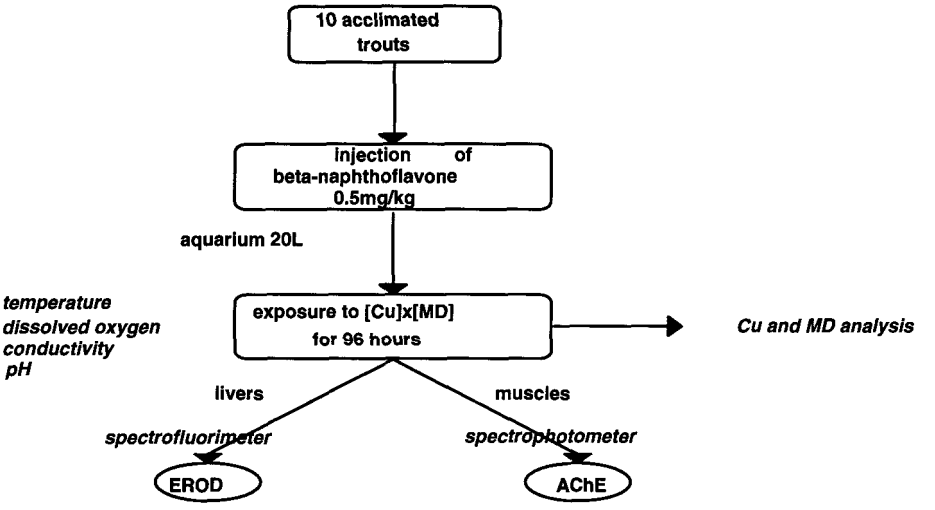

Figure 1. Experimental scheme.

EROD activities were measured using a spectrofluorimeter (Jobin Yvon JY3D) at 547 nm excitation wavelength and 585 nm emission wavelength.

AChE activity of the muscle S9 was measured according to Ellman et al. (1961): 1.75 mL tris 0.1 M pH 7.8, 50 µL DTNB 0.01 M, 50 µL S9 and 50 µL ATCI 0.1 M were successively added. AChE activities were determined spectrophotometrically (Uvikon 860 Kontron) at 405 nm.

Both enzymatic activities were reported on S9 protein concentrations which were measured by the method of Lowry et al. (1951) using a Sigma kit (procedure N° P5656).

Statistical procedures were performed using NCSS software (Number Cruncher Statistical System, Dr Jerry Hintze, Kaysville, Utah, USA) and Toxstat 3.0. (Gulley DD, Boelter AM, Bergman HL). Experimental data for EROD and AChE activities were log transformed to conform to the normality test (χ^2 test) and to the homogeneity of variance (Bartlett's test). All activities were compared by Student-t tests ($p<0.05$) and the general linear model ANOVA of the NCSS software was used to determine the influence of copper, methidathion and their interaction on the enzymatic activities.

RESULTS AND DISCUSSION

Copper or methidathion had no significant effect on basal EROD activity (3.5 ± 2.8). Pretreatement with β-naphthoflavone resulted in 23-fold induction of EROD activity (Table 2).

Preliminary studies (unpublished data) had showed that the exposure of rainbow trout to 18 µg/L and more of copper led to a 55% decrease of EROD induction but did not affect AChE activity. After exposure to methidathion both a significant decrease (-39% at 1 µg/L) of EROD induction and a significant inhibition of AChE

activity (-70% at 5 µg/L) occured. Those results were confirmed by the present study (Tables 2 and 3).

Table 2. Joint effect of copper and methidathion on rainbow trout induced EROD activity (pmol of resorufin/min/mg of protein) (± standard deviation). Concentrations are nominal ones.

	EROD activity (pmol/min/mg protein)		
Concentrations	**0 µg Cu/L**	**8 µg Cu/L**	**18 µg Cu/L**
0 µg MD/L	82.2 ± 54.8	68.6 ± 40.0	51.1 ± 15.7
1 µg MD/L	75.9 ± 33.6	65.4 ± 18.2	52.6 ± 18.5
5 µg MD/L	31.9 ± 19.1*	21.6 ± 14.3*	25,1 ± 11.1*

* significantly different ($p<0.05$) from control.

Rainbow trout injected with β-naphthoflavone and exposed to 18 µg/L of copper showed a 38% decrease of EROD activity but this was not significant ($p=0.19$). A decrease has already been observed with copper on the rat monooxygenase activities (Asokan et al. 1985). With cadmium, Fair (1986) reported a reduction of black sea bass benzo(a)pyrene (BaP) hydroxylase activity which had been previously induced by BaP.

EROD induction was significantly reduced (-61%) after exposure to 5 µg/L of methidathion ($p<0.05$). This could be due to a covalent binding of the oxon form (P=O) of the organophosphate (P=S) on the P450 protein leading to a decrease of the monooxygenase activities (Levi et al. 1988). Simon et al. (1984) found a reduction of the cytochrome P450 levels of carp exposed for 2 d to methidathion. To assess a possible effect of methidathion on the P450 synthesis, it would have been useful to measure the level of specific cytochrome P450 1A1 in our experiment.

In our study, only methidathion caused a significant ($p<0.05$) inhibition (-90% at 5 µg/L) of the AChE activity (Table 3): AChE activities of trout exposed to copper did not significantly differ from control group.

Table 3. Joint effect of copper and methidathion on rainbow trout AChE activity (nmol of ATCI hydrolyzed/min/mg of protein) (± standard deviation). Concentrations are nominal ones.

	AChE activity (nmol/min/mg protein)		
Concentrations	**0 µg Cu/L**	**8 µg Cu/L**	**18 µg Cu/L**
0 µg MD/L	261.2 ± 41.3	212.8 ± 72.4	252.3 ± 50.6
1 µg MD/L	203.1 ± 46.5*	208.5 ± 70.4	201.7 ± 81.2
5 µg MD/L	24.69 ± 16.74*	14.33 ± 10.35*	23.94 ± 14.44*

* significantly different ($p<0.05$) from control.

It is often considered that heavy metals inhibit AChE activity (Olson and Christensen 1980) and many surveys on several species in various experimental conditions show inhibition by copper (Nemcsok and Hugues 1988; Szabo et al. 1992). Inhibition was already observed in the serum of carp exposed for 96 hr to methidathion at 35% of the 96-hr LC50 (Szabo et al. 1992).

In our experimental conditions of joint exposure, an ANOVA analysis showed that copper ($p=0.04$) and methidathion ($p<0.01$) had an additive effect on the reduction of EROD induction: no interaction term could be identified as being a significant factor ($p=0.49$). Moreover, only methidathion ($p<0.01$) inhibited AChE activity. Copper ($p=0.18$) did not affect this inhibition and no interaction term was found ($p=0.14$).

Organophosphates need to be oxidized by monooxygenases to further inhibit AChE activity in vivo by phosphorylation (De Bruijn et al. 1993) and we artificially increased the natural metabolization capacity by injecting β-naphthoflavone before exposing fish to methidathion. Thus the toxicity of methidathion could have been accentuated. In fact we found no difference between AChE activities of trout, injected or not with β-naphthoflavone, and exposed to 5 µg/L of methidathion ($p=0.8$).

The present study shows that a joint exposure to copper and methidathion decreased the induced EROD activity. Exposure to copper leads to a decrease of EROD induction but methidathion has a more severe effect. Thus, in field conditions, detection of specific inducers of EROD activity might be hidden by simultaneous presence of such xenobiotics. Copper, at environmental concentrations, does not modify AChE inhibition by methidathion. As a consequence, methidathion can be detected even if copper is present. Freshwater fish EROD and AChE activities may be very useful to detect sublethal toxic pollution in the field but our study illustrates the need to clearly establish the limits of field use of those biomarkers. According to Goksoyr et al. (1991), it is of interest to measure the P450 levels by immunological techniques with a view to complementing the measurement of EROD activities in an environmental situation.

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