Bull. Environ. Contam. Toxicol. (1998) 61:397-404

Bulletin of Environmental Contamination and Toxicology

Effect of Copper and Lindane on Some Biomarkers Measured in the Clam *Ruditapes decussatus*

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Received: 9 April 1998/Accepted: 6 July 1998

Studies based on exposure biomarkers allow the evaluation of biochemical and physiological effects of pollutants and are of great interest because they permit the detection of pollutant effects before deleterious effects occur. The specificity of a biomarker to a certain class of pollutant is an advantage in monitoring but in most of the cases, pollutants are present together and could therefore interact between themselves leading to synergetic or antagonist effects. For this reason *in vivo* contamination experiments are needed to understand the joint action of various toxicants on marine organisms.

In this paper, the effects of two pollutants (copper and lindane) alone or in combination are reported for the Mediterranean clam *Ruditapes decussatus*. Copper uptake, metallothionein (MT) concentrations and acetylcholinesterase activity were determined in treated animals.

Among the major contaminants reaching the marine environment, copper is a very toxic metal. Although essential at low concentrations in the medium, effects of elevated concentrations are still important because it is used as algicid or in agriculture (viticulture). This trace metal is usually present in tissues of organisms as divalent cations, which are free or complexed to different classes of biological ligands. In addition, Cu may shift from a valence of 2 to 1, thus showing unique characteristics in speciation within the biological matrix (Viarengo 1989). Metallothionein induction may be considered as a biomarker of exposure to metals although this statement is still controversial (Amiard-Triquet and Hamza-Chaffai 1996; Cosson and Amiard 1997).

Insecticides cause serious ecotoxicological problems mainly due to their persistence and high toxicity. The use of lindane (isomer gamma hexachlorocyclohexane) is nowadays prohibited in most countries but this organochlorine persists in soils and may reach the marine environment through erosion processes. Acetylcholinesterase (AChE) is an enzyme essential to the

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correct transmission of nerve impulses. A reduction or inhibition of this enzymatic activity has been used to detect and measure the biological effects of organophosphorus and carbamates in the marine environment (Galgani and Bocquené 1988). Lindane is not an organophosphorus nor a carbamate compound. Nevertheless, Huve (1980) demonstrated that lindane, as well as ethyl-parathion and atrazine at low levels (2.10^{-8} 2.10^{-10} M), stimulated olfactory cells of fish. The site of action of lindane and other neurotoxins (cyclodienes, toxaphene, picrotoxinin) is the GABA receptor, which functions as a Cl^- channel through the nerve membrane (Walker *et al.* 1996).

MATERIALS AND METHODS

Samples of *Ruditapes decussatus* of standardized shell size (45-50 mm) were obtained from an aquaculture farm from Sète (France). Before the beginning of the experiments, they were kept for 3 days in an aerated polyethylene tank filled with natural sea water (S=38 P.S.U., 15 ± 1°C). Animals were exposed to contaminants for 5 days, a first set of animals was treated with 75 µg/L of copper, a second with 34.5 µg/L of lindane and finally the third group was submitted to copper/lindane mixture (75 µg/L and 34.5 µg/L respectively). For each condition, at least five animals were taken into consideration. During the experiments, 1 L of sea water was used per animal, sea water was changed and contaminants were added every 48 hours and the bivalves were not fed. Control animals were held in the same conditions. After 5 days, animals were killed. Then the digestive gland, gills and the remainder (comprising mantle, muscles and gonads) were dissected out and weighed.

As a reduction of survival time in air for molluscs may be considered as an index of a general stress syndrome due to marine pollution (Viarengo *et al.* 1995), a stress on stress experiment was performed. After contaminant exposure, 4x10 animals from each group (control and exposed clams = 160 animals) were subjected to anoxia by air exposure at 15 ± 1° C, in humidified chambers. Survival was assessed daily. Death symptoms were considered to be open valves and absence of muscular activity. Mean lethal time (± standard deviation) corresponding to 50% of dead animals in each group was calculated from the four groups of animals.

For acetylcholinesterase (AChE) activity measurements, fresh tissues (gills and remainder) were suspended in Tris buffer (0.1 M, pH=6.7) and homogenized with an ultra turrax. Extracts were then centrifuged at 9000 g for 30 min. AChE was determined spectrophotometrically in the supernatant S9, using acetylthiocholine as the substrate. The method of Ellman et al. (1961) as modified by Galgani and Bocquené (1991) was used. Results are expressed in arbitrary units (U) as defined by Bocquené *et al.* (1990) : variation of 1 optical

density (ΔO.D.) per minute and per mg protein = 1000 U mg protein^{-1}. Protein content was determined in S9 by using the method of Bradford (1976).

For metallothionein-like protein (MTLP) analysis, the digestive glands were homogenized in ice-cold 50 mM Tris solution and β-mercaptoethanol buffer pH 8.6, with an ultra-turrax. The homogenates were centrifuged (40000 g, 90 min, 4°C). The supernatants were separated from the pellet, heat denatured to precipitate the heat sensitive compounds. MTLPs were analyzed in the heat stable fraction according to Hamza-Chaffai *et al.* (1995) using differential pulse polarography. Copper analysis was performed in the homogenates before centrifugation at 40000 g and in the soluble and insoluble fractions resulting from the centrifugation. Determinations were carried out by atomic absorption spectrophotometry (flame).

The results of AChE activities and MTLP and Cu analyses in the four groups of animals (controls, Cu, lindane and Cu + lindane) were statistically tested for homogeneity of variance and for normal distribution. One-way analysis of variance (ANOVA) was performed. Pairwise comparisons were made (Scheffe F-test) to determine which values differed significantly when a significant overall ANOVA was found.

The analytical procedure was quality assured by using a standard reference material, the lobster hepatopancreas (Tort-1, provided by the National Research Council of Canada), chosen as a representative matrix for animal material. Our results gave a copper concentration of 420 ± 20 µg Cu/g (n = 5) in Tort-l comparable to the certified values of 439 ± 22 µg Cu/g.

RESULTS AND DISCUSSION

The stress on stress experiment was performed on animals previously exposed (Cu, lindane and Cu + lindane) for five days to assess globally the toxicity of copper, lindane and copper + lindane. Mean lethal time for 50% of individuals (LT50) was determined as explained in materials and methods. The effects of various contaminant exposures on anoxic survival time in clams are shown in Table 1.

Treatment	LT50 (days) for each group of 10 animals				Mean LT50 ± s.d. (days)
Control	9	10	10	9	9.75 ± 0.5
Cu	3	3	2	4	3.00 ± 0.8
Lindane	6	6	6	7	6.25 ± 0.5
Cu + Lindane	3	3	3	4	3.25 ± 0.5

The results demonstrate that copper (LT50 = 3.00 ± 0.8) and copper + lindane (LT50 = 3.25 ± 0.5) exposures caused a significant decrease in survival compared to the treatment with lindane alone (LT50 = 6.25 ± 0.50) or to controls (LT50 = 9.75 ± 0.50). At the tested concentrations, the toxicity of copper at 75 µg/L (1.15 µM) was clearly higher than that of lindane at 34.5 µg/L (0.12 µM) and there is no apparent synergistic effect of both contaminants added together to the medium.

Acetylcholinesterase (AChE) activity is shown in Table 2.

Table 2. Mean AChE activity (U/mg proteins) ± 1 standard deviation in the gills and remainder of the clam *Ruditapes decussatus* submitted to different treatments.

Treatment	Gills	Remainder
Control	78 ± 16 (n = 11)	86 ± 39 (n = 13)
Cu	32* ± 14 (n = 7)	31* ± 11 (n = 7)
Lindane	15* ± 6 (n = 9)	15* ± 9 (n = 5)
Cu + Lindane	16*+ 6 (n = 5)	21* + 3 (n = 5)

Values of AChE (Table 2) are of the same order in the gills and remainder of control and treated animals. A significant inhibition of AChE activity in both the gills and the remainder was observed in the three cases of contamination, compared to controls. However the inhibition was greater in the case of lindane and copper/lindane treatments (Scheffé F-test significant at $p<0.01$ for comparison of means of these treatments with those of copper). No synergistic effect was noted when copper and lindane were added jointly into the medium. Such an inhibition of AChE activity was noted previously for two species of mussels, *Mytilus galloprovincialis* and the African mussel *Perna perna,* treated *in vivo* with copper (Najimi *et al.* 1997). The authors reported that inhibition was higher in samples exposed to copper or iron compared to cadmium or zinc. A decrease in AChE activity was also observed (Najimi *et al.* 1997) in both species collected from the Atlantic coast and containing high metal levels in the whole soft body compared to animals with low levels. Bocquené *et al.* (1990) showed that AChE activity was strongly reduced with 10^{-3}M $CuSO_4$ and $5x10^{-5}$M $CuCl_2$ in four marine species including *Mytilus serratus.* Heavy metals, and particularly copper, have a pronounced preference for sulphur donor groups, and may therefore inhibit enzyme by binding to SH residues of proteins (Viarengo 1989). Inhibition of AChE by lindane was reported to be low in the muscle of the fathead minnow *Pimephales promelas* (Olson and Christensen 1980). The significant effect that *we* observed in the organs *of Ruditapes decussatus* indicates that lindane may act on neurotransmission in molluscs in a way involving GABA receptors (Walker *et al.* 1996) or other ionic channels.

Our control values were compared to data reported in the literature for other molluscs. Bocquené (1996) showed that AChE activity was low, less than 2000 U/mg protein in *Crassostrea gigas* and in *Mytilus edulis* compared to two fish species (plaice and mackerel : 13250 and 14230 U/mg protein, respectively). Le Bris *et al.* (1995) reported that total tissue AChE activity of the control clam *Ruditapes philippinarum* ranged from 78 to 107 U/mg protein. Our results are close to those of Bocquené (1996) and Le Bris *et al.* (1995).

The MTLP levels (Table 3) in digestive glands of control clams do not differ significantly from that in the treated animals.

Table 3. MTLP concentrations (mg/g) in the digestive gland of *Ruditapes decussatus.*

MTLP	Control	Cu	Lindane	Cu + Lindane
Mean	0.52 ± 0.17 (n = 13)	0.62 ± 0.13 (n = 7)	0.53 ± 0.16 (n = 9)	0.42 ± 0.07 (n = 4)

Metallothioneins, induced by metals (Ag, Cd, Cu, Hg, Zn), affect homeostasis of essential metals. They constitute the main mechanisms for storage of metals in fish. In invertebrates, the structure and mechanisms of metal regulation are less understood than in fish and other systems may compete with MT for metal sequestration. Romeo and Gnassia-Barelli (1995) observed a weak MT induction in the gills of *Ruditapes decussatus* exposed to copper. Induction measured in the whole soft parts and in the digestive gland was higher for the same animals exposed to cadmium (Bebianno *et al.* 1993).

Copper concentrations in the homogenates i.e. in the digestive gland before subcellular fractionation are shown in Table 4. Copper concentrations differed according to the treatment. In the copper experiment, the Cu concentration was significantly higher than in controls whereas in the lindane treatment, copper concentration was significantly lower. There is no significant difference between copper concentrations in the digestive gland of controls and in that of copper + lindane treated organs. For the subcellular fraction, copper in control clams is mainly associated with the soluble heat stable fraction (HSF) containing MTLPs. In the copper treatment, although the copper amount is high (3.68 µg/g) in the MTLP fraction, MTLP levels did not change significantly (0.62 µg MT/g, Table 3) compared to controls. Thus the increase of copper in the soluble fraction (HSF) is not correlated with an induction of MTLPs. Copper may exert a toxic effect, the concentration in the medium being so high as to inhibit the animals ability to synthesize MTs. Such a phenomenon was reported by Harrison and Lam (1986) in the liver of the bluegill fish exposed to increased soluble copper under field and laboratory conditions. On another hand, copper has been shown to increase the lipid peroxidation of membranes in the digestive gland of *R. decussatus* (Romeo and Gnassia-Barelli 1997), this toxic phenomenon may

facilitate copper precipitation so that less copper is then available for MTLP induction. The stress on stress experiment revealed that animals treated with copper presented a much lower LT 50 than control animals (Table 1).

Table 4. Copper concentration (µg/g w.w.) in the homogenates and in the soluble (heat-stable: HSF and heat-denaturated: HDF) and insoluble (IF) fractions of the digestive gland of *R. decussatus* exposed to the three treatments. Percentages of copper found (calculated from the homogenates) in IF are also shown.

Fraction	Control	Cu	Lindane	Cu + Lindane
Homogenate	3.16 ± 0.88	6.51** ± 1.63	2.17* ± 0.67	3.07 ± 1.07
	n = 14	n = 7	n = 9	n = 5
HSF(MTLP)	2.06 ± 0.80	3.68** ± 0.94	1.48 ± 0.54	1.69 ± 0.44
HDF	0.27 ± 0.11	0.60** ± 0.28	0.16* ± 0.09	0.17 ± 0.10
IF	0.84 ± 0.22	2.22** ± 0.71	0.53* ± 0.18	1.21 ±0.66.
% of Cu	27%	34%	24%	40.6%

Scheffé F-test significant at *p< 0.05 and at **p<0.01 compared to the corresponding controls.

In the case of lindane treatment, animals lost a part of their copper content. This phenomenon may be related to ionic transport since lindane was shown to act upon Cl^- channel. The pattern of distribution is similar to that of controls, but with less copper in all fractions. When animals are exposed to the mixture copper + lindane, the amount of copper in the MTLP fraction was not significantly different from that of controls. In this case, the bioavailabiliy of copper may be changed, compared with the experiment with copper alone, by the presence of lindane in the medium. Unfortunately copper speciation in the medium could not be evaluated. The stress on stress experiment demonstrated that the mixture provoked a significant decrease in LT 50 comparable to the experiment with copper alone. In an experiment performed with sea bass, *Dicentrarchus labrax*, treated with copper and benzo(a)pyrene (BaP), Roméo *et al.* (1997) demonstrated that the effect of both contaminants provoked an increase in hepatic MTLP content. This phenomenon was attributed by the authors to the toxic action of BaP which disturbs the structure of hepatocytes by altering the plasma membrane. The liberated copper could then induce MTLPs. In the present work, lindane, in contrast to BaP, does not appear to have any effect upon MTLP induction. MTLP induction was not observed in any of the three conditions studied in this work.

In conclusion, the stress on stress experiments showed that copper alone or in combination with lindane provoked apparent toxic effects in the clam *Ruditapes decussatus*. Lindane but also copper and the mixture of both toxic compounds

significantly decreased AChE activity measured in the organs (gills and remainder) of *R. decussatus.* Copper accumulation in the digestive gland was observed only in the case of copper-treated animals. Lindane decreased copper accumulation to a lower level than that of controls, while the copper + lindane mixture resulted in an unchanged copper content compared to controls. No significant MTLP induction was observed compared to controls. In the environment, organic and metallic pollutants may interact, altering in one way or another the responses of biomarkers (AChE and MTLP) known to individually detect these two kinds of pollutants.

Acknowledgments. Work was supported by a French-Tunisian cooperation agreement.

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