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Effects of Exposure to Copper and Malathion on Metallothionein Levels and Acetylcholinesterase Activity of the Mussel *Mytilus edulis* and the Clam *Macoma balthica* from the Northern Baltic Sea

K. K. Lehtonen, S. Leiniö

Finnish Instiute of Marine Research, Post Office Box 33, FIN-00931, Helsinki, Finland

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In the Baltic Sea, biomarkers have been applied to the monitoring of biological effects of pollution to a very limited extent. Prior to their effective use, baseline information on the levels and ranges of biomarker responses in species suitable for monitoring is required. The constant brackish-water environment ranging from less than 2 PSU in the north to ca. 15 PSU in the south creates physiological stress to organisms of both marine and limnic origin. The ensuing adaptive physiological adjustments within the organisms may reflect in biomarker responses. Other abiotic and biotic features typical for the Baltic Sea, including those related to pollution load, call for basic research on biomarkers in this special environment.

Sessile benthic organisms are universally accepted as optimal bioindicators of environmental contamination. In the poor-diversity northern Baltic Sea, the hard-bottom filter-feeding mussel *Mytilus edulis* and the infaunal deposit-feeding clam *Macoma balthica* are among the very few benthic species feasible for environmental monitoring. In this area, the mussel lives at its low-salinity tolerance limits (ca. 5.5 PSU). The individuals grow slowly with a maximum length of only ca. 40 mm, and remain even smaller more close to their very limits of geographical distribution (e.g. central Gulf of Finland, northern Gulf of Bothnia).

In the present study the effects of copper (Cu) and the pesticide malathion (Mal) and their combinations on two common biomarkers in these bivalve species were studied using a short-term laboratory exposure. Acetylcholinesterase (AChE) inhibition is a well-established biomarker of neurotoxic effects and commonly used in marine biomonitoring, especially as the biomarker of pesticide pollution (e.g. Bocquené and Galgani 1998). AChE inhibition in organisms collected from areas with no pesticide contamination has also been observed, indicating that AChE has a wider spectrum of responsive potential than recognized previously, with other contaminant groups including metals, hydrocarbons and detergents possessing anti-ChE properties (Zinkl et al. 1991; Payne et al. 1996; Guilhermino et al. 1998). Neosynthesis of metallothioneins (MT) or metallothionein-like proteins (MTLP) represents a specific response of organisms to pollution by heavy metals such as Cu, Zn, Cd and Hg (e.g. George and Olsson 1994). No experimental studies on these biomarkers in benthic organisms of the northern Baltic Sea have been published.

Correspondence to: K. K. Lehtonen

MATERIALS AND METHODS

The bivalves were collected close to the Tvärminne Zoological Station (University of Helsinki) in the Gulf of Finland (northern Baltic Sea). *M. edulis* (shell length range 19-27 mm) were obtained by scuba diving from the depth of 4-7 m and *M. balthica* (15-19 mm) by bottom trawling from 35 m. In the laboratory the bivalves were acclimated at 10°C in local brackish water (ca. 5.5 PSU) for 7 d prior to the start of the experiments.

Semi-static aquarium exposures (without sediment) at 10°C, with 24-h periodical renewals of filtered (0.2 µm) brackish water and re-dosings of toxicants were used. Concentrations of 0.04 and 0.2 mg l^{-1} of Cu (in $CuCl_2$), 0.02 and 0.1 mg l^{-1} of Mal and their combinations (Cu+Mal) at the lower and higher concentrations were applied. Ethanol (EtOH) was used as vehicle in the Mal and Cu+Mal treatments and its concentration in the respective control group (CT-EtOH) was 0.02 vol-%, corresponding to the high-dose toxicant treatments. Tissue samples for the measurement of AChE activity and MT were taken at d 0, 3 and 7. To assess the general activity of *M. balthica* during the experiment the number of individuals with siphons extended in each treatment was recorded at d 3 and d 7.

For the determination of AChE activity in *M. edulis*, 3-5 gill pairs were pooled in one sample, with 5 replicate samples per treatment group. For *M. balthica*, foot tissue samples from 7-10 individuals were pooled from each experimental group, with 4 replicate samples. In addition, 10 smaller (*M. edulis* 17-21 mm, *M. balthica* 11-14 mm) individuals from each treatment group were analyzed for whole soft tissue AChE activity. The analyses were performed essentially as described in Bocquené and Galgani (1998). A 96-well microplate reader was used for the spectrophotometric determination of the Ellman et al. (1961) reaction. Each sample was measured in quadruplicate. Tissue protein concentration was determined using the Bradford (1976) method with bovine gamma globulin as the standard. AChE activity values are expressed as equivalents of acetylthiocholine (ACTC) hydrolyzed (nmoles ACTC min^{-1} mg $protein^{-1}$), with 1 ΔO.D. corresponding the hydrolysis of 75 nmoles of ACTC.

For MT, the digestive gland (DG) of the bivalves was dissected. In *M. balthica*, the DGs from 6-8 individuals were pooled to obtain sufficient amounts for a single analysis, with 4 replicate measurements per treatment. In *M. edulis*, DGs from 7-10 individuals were pooled for 3 replicates. The concentration of MT was measured by the spectrophotometric determination of -SH groups (Viarengo et al. 1997). MT was not measured from individuals exposed to Mal only. The data was analyzed with 2-factor and single ANOVAs with Tukey's *post hoc* pairwise comparisons using SYSTAT® 9.0 statistical software package. The AChE activity data were square-root transformed prior to statistical analyses.

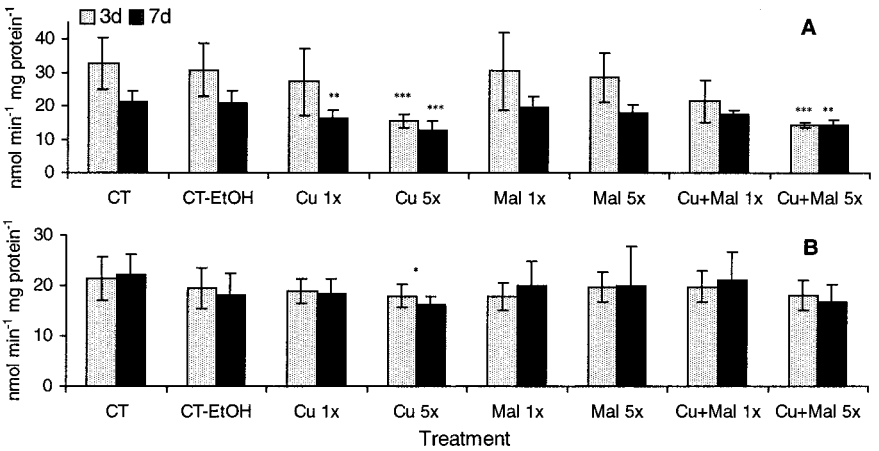

Figure 1. *M. edulis*. AChE activity (mean ± SD) during the experiment at d 3 and d 7. (A) gill tissue, (B) whole animals. Key: CT=control, CT-EtOH=ethanol-added control, Cu (copper) 1x=0.04 mg l^{-1}, Cu 5x=0.2 mg l^{-1}, Mal (malathion) 1x=0.02 mg l^{-1}, Mal 5x=0.1 mg l^{-1}. Statistically significant differences compared to appropriate control treatments: *p<0.05, **p<0.01, ***p<0.001. In (B), both time periods (3 and 7 d) were combined for statistical analysis.

RESULTS AND DISCUSSION

Both time and treatment affected the AChE activity levels in the gills of *M. edulis* (Table 1, Fig. 1). No significant relationship in the time vs. treatment interaction existed. The control treatments (CT and CT-EtOH) showed similar activity levels, both decreasing between d 3 and d 7 ($p<0.001$). Compared to the control group, a decrease in the AChE level was observed in mussels exposed to the high-dose of Cu (0.2 mg l^{-1}), both at d 3 ($p=0.001$) and d 7 ($p<0.001$). Exposure to the low-dose of Cu (0.04 mg l^{-1}) did not affect AChE at d 3 ($p=0.825$) but at d 7 a reduction was observed ($p<0.05$). Exposure to the high-dose combination of Cu+Mal (0.1+0.2 mg l^{-1}, respectively) led to a reduction in AChE in comparison with the appropriate control group (CT-EtOH) both at d 3 ($p<0.001$) and at d 7 ($p<0.01$). Treatment with Mal alone did not cause any significant effects. At d 3 the individuals exposed to the high-dose of Cu+Mal had a lower AChE level than those exposed to the high-dose Mal (0.1 mg l^{-1})($p<0.01$) but at d 7 no difference could be observed ($p=0.335$).

AChE measured from the whole-animal soft tissues of *M. edulis* showed differences between the treatments but no effects of time (Table 1). Combined data from d 3 and d 7 showed variation between the treatments ($F_{7,143}=2.823$, $p<0.01$) but the only significant difference ($p=0.010$) was found between the control and the high-dose Cu exposure (0.2 mg l^{-1}).

AChE measured from the foot of *M. balthica* showed no differences between the treatments or time (Table 1). The groups exposed to the higher Cu and Mal con-

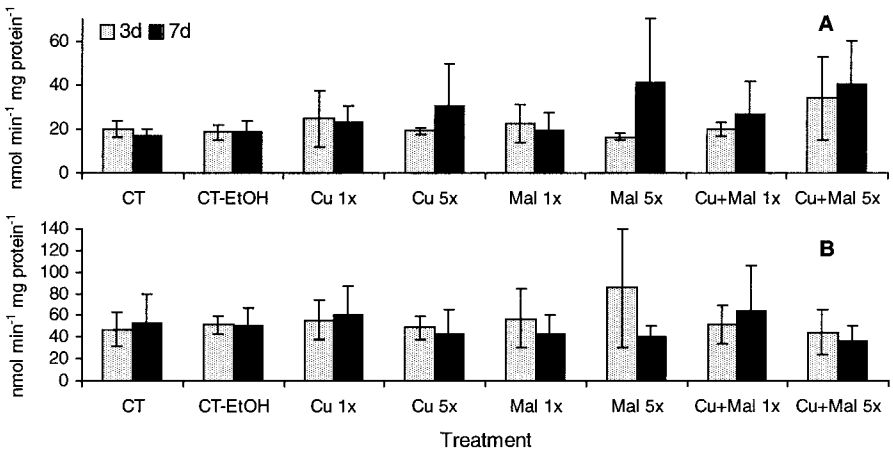

Figure 2. *M. balthica.* AChE activity (mean ± SD) during the experiment at d 3 and d 7. (A) foot tissue, (B) whole animals. Key: see figure 1.

centrations and to the Cu+Mal combinations showed marked variability in activity compared to the respective control treatments (Fig. 2). AChE measured from the whole soft tissues showed no effect of treatment but the individuals measured at d 7 had generally a lower activity level than those measured at d 3. However, the control individuals measured at d 3 and d 7 showed equal levels.

Exposure to Cu reduced considerably the siphon activity of *M. balthica*, while Mal alone had a stimulating effect at d 7 compared to the control treatment (Fig. 3). That the clams had possibly remained closed for extended periods during the Cu and Cu+Mal treatments would mean that they were not effectively exposed to the toxicants, which may have affected the biomarker responses measured.

Table 1. ANOVA table. Effect of time and treatment on AChE activity of *M. edulis* and *M. balthica.*

Mytilus edulis		Gill tissue			Whole animal	
	df	F ratio	P	df	F ratio	P
Treatment	7	10.93	0.000***	7	2.69	0.012*
Time	1	46.76	0.000***	1	0.14	0.710
Treatment x Time	7	1.81	0.094	7	0.58	0.774
Error	96			135		
Macoma balthica		Foot tissue			Whole animal	
	df	F ratio	P	df	F ratio	P
Treatment	7	1.79	0.106	7	1.58	0.148
Time	1	3.59	0.063	1	3.93	0.049*
Treatment x Time	7	0.98	0.452	7	1.31	0.251
Error	61			128		

Statistical significance: *p<0.05, ***p<0.001

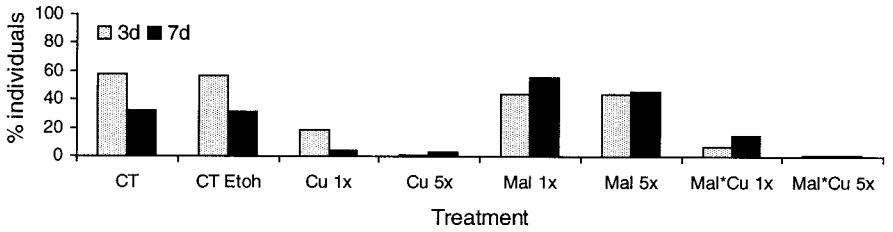

Figure 3. *M. balthica.* Siphon activity (% of individuals) during the experiment at d 3 (n=103-111 in each treatment) and d 7 (n=63-71).

The results show that the effects of toxicant exposure on the AChE activity differ between the two species. In Cu-exposed *M. edulis*, a dose-dependent inhibition was observed. Cu has been shown to inhibit the AChE activity of mussels *in vitro* in whole tissue homogenates (Najimi et al. 1997). In the clam *Ruditapes decussatus* a ca. 60% inhibition of AChE was observed during a 5-d exposure to 75 ug l^{-1} of Cu (between the concentrations used here)(Hamza-Chaffai et al. 1998). The Mal treatment alone had no effect on AChE but the response to combined Cu+Mal exposure was similar to that observed in exposure to Cu. In copepods (*Tigriopus brevicornis*) exposed to 0.02 mg l^{-1} of Mal, Forget et al. (1999) recorded a >50% inhibition, while exposure to Cu+Mal showed a strong synergistic effect. *In vitro* studies by Perret et al. (1996) on the zebra mussel (*Dreissena polymorpha*) showed a slight (25%) inhibition of carboxyl esterases in considerably higher Mal concentrations. Galgani and Bocquené (1990) showed that AChE in mussels is less sensitive to organophosphates compared to fish and crustaceans.

The effects of laboratory keeping or starvation might have caused the marked reduction in AChE activity of gill tissue observed at d 7. Due to technical problems, the 7 d gill samples were analyzed 6 mo later than the 3 d samples, which may have led to a partial loss of enzyme activity. However, the pattern of the AChE response observed at d 3 was the same in the d 7 samples, although the differences between the treatments are smaller. The marked inhibition observed in the gill tissue of the mussels exposed to the high doses of Cu and Cu+Mal was not found in the whole tissue homogenates although a similar trend was clear. Thus, the use of whole soft-tissue homogenates for measuring AChE in *M. edulis* cannot be recommended here. In *M. balthica* the whole-tissue preparations showed a considerably higher AChE activity compared to the foot tissue (52 and 20 nmoles ACTC min^{-1} mg $protein^{-1}$, respectively; all control treatments pooled). The whole-tissue levels of AChE of *M. balthica* were over two-fold compared to *M. edulis* (50 and 22 ACTC min^{-1} mg $protein^{-1}$, respectively; all control treatments pooled).

At d 0, the background levels of MT were two-fold higher in *M. balthica* compared to *M. edulis* (288 ± 65 and 148 ± 12 μg g wet wt^{-1}, respectively)(Fig. 4). In the non-exposed *M. balthica*, the level of MT elevated markedly during the experiment, reaching 462 ± 94 μg g wet wt^{-1} at d 7. In *M. edulis* no significant elevation could be observed in the control group.

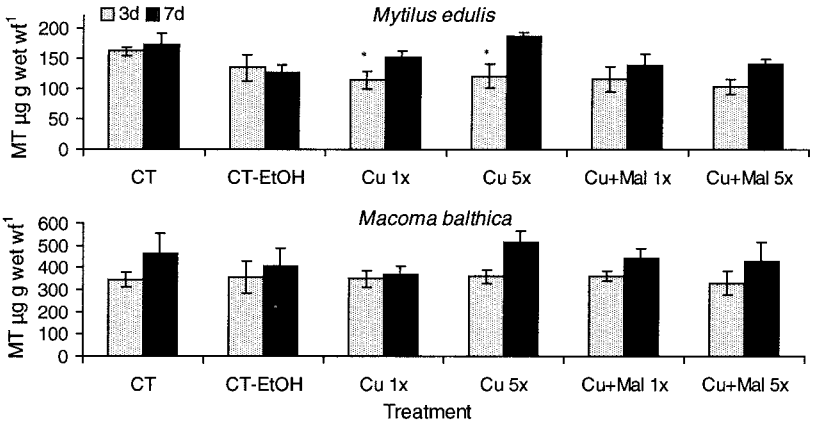

Figure 4. MT levels (mean ± SD) in digestive glands of *M. edulis* and *M. balthica* during the experiment at d 3 and 7. Key: see figure 1.

In both species the MT levels exhibited significant temporal variability during the experiment (Table 2). However, only in *M. edulis* the effect of treatment could be recorded, with a reduction in the MT levels of the Cu-exposed groups at d 3 ($p<0.05$) and a subsequent elevation at d 7. In *M. balthica* both Cu-exposure concentrations at d 3 resulted in MT levels similar to the control group. The Cu exposures resulted in similar responses in both species at d 7 with the MT levels being higher in the high-dose treatment compared to the low-dose treatment but intermediate in the control individuals. In both species the Cu+Mal exposures resulted in reduced MT levels, similar to those observed in CT-EtOH group.

Table 2. ANOVA table. Effect of time and treatment on MT levels in *M. edulis* and *M. balthica*.

	Mytilus edulis			*Macoma balthica*		
	df	F ratio	P	df	F ratio	P
Treatment	2	4.32	0.022*	2	0.06	0.943
Time	1	19.06	0.000***	1	22.29	0.000***
Treatment x Time	2	4.89	0.014*	2	0.01	0.993
Error	32			42		

Statistical significance: *p<0.05, ***p<0.001.

The tissue level of MT varies depending on various natural abiotic and biotic factors (George and Olsson 1994), e.g. season and weight (*M. balthica*: Bordin et al. 1997; Mouynerac et al. 2000). Because of this the changes in MT levels during a short-term exposure to metals may be difficult to distinguish, especially in *M. balthica*. In *M. edulis* the background levels are lower, enabling a more sensitive detection of pollution-induced changes. The reason for the reduced MT levels in the exposed *M. edulis* observed at d 3 is unclear. At d 7, the level of MT is elevated at the higher Cu concentration. A decrease in the MT level at the early stage of exposure to Cu has been noted in *R. decussatus*, followed by a rapid elevation during a week's exposure and remaining constant for almost a month (Geret et al.

2002). In *M. edulis*, similar kinetics seems to be in operation although the present experiment lasted only for one week and therefore the plateau was probably not reached.

In *M. balthica*, no effects of treatments on MT levels were observed at d 3. In the control group the levels elevated with time, indicating acclimation to experimental conditions (no sediment). In the Cu-exposed group the effects of different concentrations began to manifest at d 7. Langston and Zhou (1987) recorded no induction of MT or MTLP in *M. balthica* exposed to Cd. Bordin et al. (1997) observed a marked rise in the levels of MTLP after a 3 d exposure to 0.1 mg l^{-1} of Cu in *M. balthica* from the North Sea. These results imply that genotypic characteristics of populations in different geographic areas, and possibly those modified by a long-term (>several decades) pollution history may direct the response of organisms in experiments. However, no differences in MTLPs of *M. balthica* from Loire estuary and a reference area were found by Mouneyrac et al. (2000).

Both the duration of exposure and the concentration of contaminants are important in determining the direction of the responses. The use of AChE as a biomarker of contaminant stress in *M. edulis* seems useful also in mussels from the northern Baltic Sea. In *M. balthica* the responses in AChE activity to short-term pollutant exposure are less sensitive and complex; the large variability observed in the AChE activity in exposed individuals indicates disturbances caused by exposure to toxicants. In both species a short-term exposure to the low Cu concentration (0.04 mg l^{-1}) produced a differential response in MT compared to the higher concentration (0.2 mg l^{-1}). The general levels of MT and the differential responses to a short-term exposure to Cu of the two bivalve species are probably caused by the different natural habitats of the two species, with *M. balthica* being naturally more exposed to heavy metals present in the sediment.

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