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BIOACCUMULATION
METAL
COPPER
METALLOTHIONEIN
STRESS SENSITIVITY
BIVALVE
MACOMA BALTHICA
ARCTIC

ABSTRACT. – Differences in performance and bioaccumulation of copper, metallothionein-like protein (MTLP) levels and resistance in Baltic clams, *Macoma balthica*, from Arctic, subarctic and temperate areas were determined during a stress period caused by starvation and exposure to copper. Although the conditions at the start were different, the losses of weight and mortality rates were in general comparable in clams from all areas. In contrast to expectation, the accumulation of copper in (sub)Arctic clams was much faster than in temperate specimens, whereas the level of MTLP in all populations hardly increased. Copper was primarily accumulated in insoluble form: MTLP has in clams no major role in copper sequestration. It is suggested that the differences in copper accumulation rates between populations might be related to genetic (racial) differentiation.

BIOACCUMULATION
MÉTAL
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RÉSUMÉ. – La performance et la bioaccumulation du cuivre, les niveaux de protéines type métallothionéine et la résistance des Bivalves *Macoma balthica* ont été comparées dans des spécimens de zones Arctique, subarctique et tempérée durant une période de stress dûe au jeûne et à l'exposition au cuivre. Bien que leur condition soit initialement différente, les pertes de poids et les taux de mortalité sont en général comparables dans les Bivalves des trois zones. Contrairement à ce qui était attendu, l'accumulation du cuivre dans les Bivalves subarctiques est plus rapide que dans les spécimens de la zone tempérée tandis que le niveau de PTM dans toutes les populations augmente peu. Le cuivre est principalement accumulé sous forme insoluble : les PTM n'ont pas un rôle majeur dans la séquestration du cuivre chez ces Bivalves. Il est suggéré que les différences interpopulationnelles dans les taux d'accumulation du cuivre pourraient être liées à une différenciation génétique (raciale).

INTRODUCTION

In a series of field surveys and experiments with the Baltic clam *Macoma balthica*, covering its total European distribution range from south (SW France; Bachelet 1980) to north (N. Russia; Hummel *et al.* 1997a) (Fig. 1), the sensitivity to stressors was determined. It was found that different types of stress, as e.g. starvation, extreme temperatures or copper exposure, could have the same impact (Hummel *et al.* 1995, 1996). Due to a stressor the performance of the clams was hampered in a similar way when expressed in ecophysiological terms as growth or survival. Even changes in the genetic constitution, e.g. out-selection of some specific genotypes, were similar under different types of stress.

Strong differences in the reactions to stress were obtained when testing clams from different regions in Europe. These differences were caused by comparing different genetic ecotypes (Fig. 1) or comparing specimens from a place central in the species distribution with those living at the limit of their distribution. Animals at the limits of their distribution are often thought to be more sensitive to disturbances than their conspecifics from areas more central in their distribution (Conover 1978, Hoffmann & Parsons 1991). Such is the consequence of living at the limits of a species' adaptation capacity, whereby the performance, such as growth or fitness, becomes poor. It was found that this hypothesis was valid too for the Baltic clam, at least at its southern distribution limit (Hummel *et al.* 1995, 1996, 1997a, 1998). However, in the northern (subarctic and Arctic) area the animals were equally or less sensitive to disturbance by heavy metals (copper) or by starvation than those from central places (Hummel *et al.* 1997a, 1998). Differences in metabolic rate, genetic constitution, or sequestration of metals in the animals were suggested as an explanation of the low sensitivity to stress for (sub-)Arctic animals.

Support for a low metabolism in Arctic specimens was found in their very low annual growth, but long lifespan (Hummel *et al.* 1998). Could it be that such a low metabolism is also reflected in slow accumulation kinetics of metals in (sub-)Arctic clams and thereby in a low sensitivity to copper exposure?

Additionally, Arctic specimens were shown to have different genetic traits from clams of temperate areas, being a different subspecies, whereas those of subarctic areas could be called a different race (Hummel *et al.* 1997a). Could it be that the different genetic constitutions coincide with different biochemical defence strategies as with regard to e.g. metallothioneins (MTs)? For the sequestration of metals, clams from temperate areas probably lack a well-developed physiological regulatory mechanism to control copper concentrations over the short term (weeks), although with regard to the presence of metallothioneins (MT) in clams conflicting results have been obtained (Langston & Zhou 1987, Bordin *et al.* 1994, 1997, Mouneyrac *et al.* 2000).

Yet, it might be that latitudinal gradients in the presence/absence of MT in clams could be the reason for the observed differences in sensitivity to contaminants.

On basis of the above suppositions it was hypothesized that when stressing Baltic clams by starvation and/or copper exposure, the specimens from temperate areas would show a better performance, e.g. lower mortality and a lower decrease of the weight, than Arctic specimens, whereas during a period with copper exposure Arctic clams would show lower copper accumulation (rates) and/or a more enhanced increase of MT-like proteins than temperate specimens. For subarctic specimens performance and intermediate copper accumulation (rates) are expected, since they belong to the same race as the temperate specimens (Hummel *et al.* 1997a), yet, live under almost similar extreme climatic conditions as the Arctic specimens.

Therefore, in this comparative study we tried to assess simultaneously for clams from Arctic, subarctic and temperate areas the differences in performance under stress (starvation and/or copper exposure) and differences in bioaccumulation strategies and metallothionein levels at copper exposure.

MATERIAL AND METHODS

Sampling and experimental exposure: In the framework of an INTAS mission (International Association for the Promotion of Cooperation with Scientists from the Independent States of the former Soviet Union; project 94-391), Baltic clams, *Macoma balthica*, were taken alive from the Arctic (29-7-1997; Khaypudyr, Pechora Sea; 68° 39.8' N, 59° 50.7' E; temperature 9 °C, salinity 32; Cu 1.6 ppm in sediment (Amiard *et al.* 1998)), the subarctic (11-8-1997; Kartesh, Chupa Bay, White Sea; 66° 0.2' N, 33° 39.0' E; temperature 12 °C, salinity 25; Cu 10.6 ppm in sediment (Amiard *et al.* 1998)) and a station in temperate areas (13-8-1997; Paulina, Westerschelde, the Netherlands; 51° 21.6' N, 3° 42.7' E; temperature 19 °C, salinity 15; Cu 7.9 ppm in sediment) (Fig. 1). During transport to the Netherlands Institute of Ecology at Yerseke, the animals were kept in wet tissue-paper at a temperature of 0 to 5 °C in a transportable cooling unit till the start of the experiment. On 21-8-1997 the bivalves were introduced into aquaria of 1 l with sea-

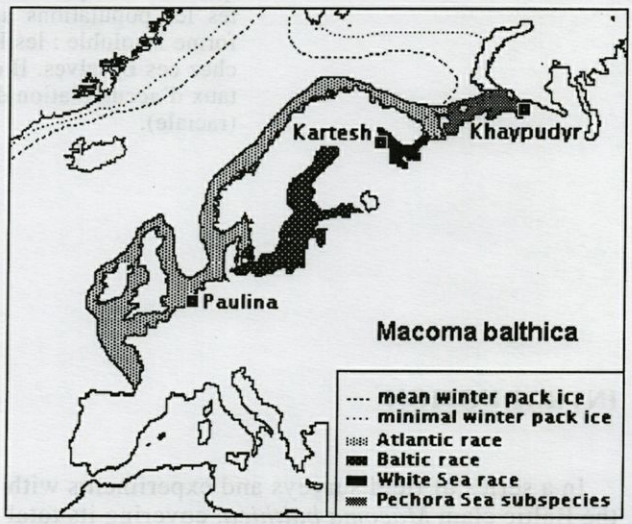

Fig. 1. – Distribution of the different ecotypes of the Baltic clam *Macoma balthica* in Europe and the location of sampling stations for animals used in the experiment on Cu accumulation and metallothionein.

Table I. – Sampling and experimental exposure to copper (Sm: clams sampled when reaching 50% mortality, S: clams sampled before 50% mortality was reached; in the controls no mortality occurred; 50, 100: exposure to 50 and 100 ppb Cu).

Exposure period (days)	Date	Paulina (temperate)			Kartesh (subarctic)			Khaypudyr (Arctic)	
	29/07/97							sampling	
	11/08/97				sampling				
	13/08/97	sampling							
	Starvation period	8 days			10 days			23 days	
0	21/08/97	control	50	100	control	50	100	control	100
		↓	↓	↓	↓	↓	↓	↓	↓
13	2-4/09/97	S	S	S	S	S	Sm	S	Sm
		↓	↓	↓	↓	↓			
18	8-9/09/97	↓	↓	Sm	S	Sm			
		↓	↓		↓				
28	18/09/97	S	Sm		S				

water of 30 ppt salinity at a constant temperature of 12 °C, and 6 hours later copper was added at nominal concentrations of 50 and 100 ppb to test the animals for their resistance to copper (Table I). The water in the aquaria was changed twice a week. No food was administered to prevent too strong complexation of copper. Due to complexation, e.g. adsorption to the walls, the average copper concentration in our aquaria is 50 to 75% of the nominal concentration (Hummel *et al.* 1995, 1997a).

The rather high Cu exposure concentrations were chosen since in preliminary experiments the sub-Arctic specimens were shown to survive more than 3 to 4 weeks at concentrations ranging from 25 to 75 ppb Cu.

Fifty specimens originating from the subarctic Kartesh and temperate region (Paulina) were exposed to each treatment in 2 duplicate aquaria. Due to the restricted number of specimens, twenty clams from the Arctic Khaypudyr were exposed to the highest dose only and the treatment was not duplicated. Identical groups of clams were kept under the same laboratory conditions as controls. Moreover, ten specimens from each site were taken at the start of the exposure period (Table I).

Mortalities were registered daily. No mortality occurred in controls. For each treatment and each geographical site, the experiment was stopped at the moment when 50% mortality was reached (Table I). Additionally some specimens were taken in the different experimental groups far before some mortality was found in order to unravel the copper accumulation kinetics (Table I). The samples were lyophilized till further determinations.

Compartmentation: The length of the shell, down to the nearest 0.1 mm by means of a caliper, and the dry weight of soft tissues, after 3 days of lyophilization, was determined in all the individuals. Soft tissues of bivalves were individually homogenized in 50 mM Tris-NaCl (pH = 8.6) buffer (25:1, v:w) in an ice bath. The soluble (S1) and insoluble fractions (P1) were separated by centrifugation at 25 000 g for 1 hr at 4 °C. Tris contained 10^{-5} mM ß-mercaptoethanol to avoid the formation of disulfide bridges between MT molecules. An aliquot of the supernatant was submitted to heat denaturation (75 °C for 30 min) and then centrifuged (15 000 g for 10 min at 4 °C). The supernatants S2 recovered after heat denaturation were stored (at least 1 week) at –20 °C until MTLP quantification.

Metallothionein-like protein (MTLP) analysis: The amount of MTLP has been determined by differential pulse polarographic analysis (DPP). This technique is based on –SH compound determination according to the Brdicka reaction (Brdicka 1933) as described by Thompson & Cosson (1984). The PAR Model 174 analyser, the PAR/EG&G Model 303 static mercury drop electrode (SMDE) and an X-Y recorder (RE 0089) were used. Metallothionein (MT) of rabbit liver (Sigma Chemical Co., St. Louis, MO) was used to carry out the calibration according to the method of standard additions. The system consisted of a bevelled capillary mercury working electrode, a platinum counter electrode and an Ag/AgCl reference electrode. The polarographic determination in heat-denaturated cytosol is an analytical procedure based on several characteristics of MTs but does not allow the assertion that the studied molecule is a true MT since purification and sequencing have not been carried out. Thus later on, the terminology of metallothionein-like protein (MTLP) will be preferred and results will be expressed as concentrations: mg of MTLP per gram dry weight of homogenized tissue.

Metal analysis: Nalgene bottles were used to store all reagents. All labware was soaked in 10% hydrochloric acid, rinsed 3 times with deionized water and dried in a desiccator sheltered from atmospheric dust. Pellets (P1) were heated (65 °C, 24 h) with nitric acid 65% (RPE Carlo Erba). After digestion, the volume was adjusted to 5 mL with deionized water. The supernatant was digested with nitric acid (1 mL per 1 mL S1) at 100 °C for 1h then at 120 °C till drying was completed. The residue was solubilized in 0.1 mL nitric acid 65% + 0.4 mL deionized water. Metal levels in these acid solutions were determined by flame (clams experimentally exposed to Cu) or flameless (controls) atomic absorption spectrophotometry using Zeeman effect (HITACHI Z 8 200). The laboratory is involved in procedures of internal quality controls based on standard reference materials (CRM 278 Mussel tissue, SRM 1566 Oyster tissue) and in external intercalibrations under the I.A.E.A.

Statistical treatment: Differences between stations or treatments were evaluated by analysis of variance (ANOVA) and post hoc comparisons assessed by the Multiple Ranges Test of Scheffé. These tests as well as paired t-tests and linear regressions were carried out using standard statistical packages (Systat (Wilkinson 1988) and StatView SE + Graphics^{tm}).

RESULTS

Mortality

Clams experienced different starvation periods, depending on the period of transport (Table I): Arctic (Khaypudyr) 23 days, subarctic (Kartesh) 10 days and temperate region (Paulina) 8 days. As it became clear from previous experiments (Hummel *et al.* 1995, 1996) different stressors may yield the same impact on the performance of Baltic clams, and therefore it might be that the starvation period during transport and the period of copper exposure should be added.

At 100 ppb copper the mean lethal time (MLT) of the animals from the Arctic Khaypudyr was 13 days (35 days including the starvation period), for those from the subarctic Kartesh 12 days (22 days including the starvation period), and from the temperate region (Paulina) 17 days (25 days including the starvation period). The survival period thus does not differ strongly between locations, being slightly higher at Paulina when excluding the starvation period, and higher at Khaypudyr when including the starvation period.

At 50 ppb the MLT of the subarctic Kartesh specimens was 18 days (28 days including the starvation period), and of those from the temperate region (Paulina) 28 days (36 days including the starvation period) (no animals from the Arctic Khaypudyr were available). At the lower copper concentration, the temperate specimens thus showed a slightly better performance.

Biometric data

Clams originating from the temperate region had a higher shell length than specimens taken from Arctic (Khaypudyr) and subarctic (Kartesh) sites (Fig. 2). The intersite differences were enhanced when the weight of soft tissues was considered (Fig. 2). Yet, a proper comparison should be made on basis of the weight-index, i.e. the weight per volume ratio (estimated by DW / (length)^3; Hummel *et al.* 1996, 1997b), which is a better indicator of the (differences in) condition of an animal. The weight-indices of the clams differed significantly between stations (Table II; $p < 0.001$). Clams from the temperate region showed the highest weight index, whereas subarctic and Arctic specimens had a 40% lower weight index (Fig 2).

With an increase of the experimental period the weight-index significantly decreased (Fig. 2; Table II). With exposure to copper, the weight-index showed a significant stronger trend to decrease (Fig. 2, Table II). However, the degree of change in the weight-index is not different between clams from different locations.

Table II. Significance of the effects of origin (=station), exposure to copper (=copper) and exposure periods (=period) on the length, weight-index, soluble and insoluble copper and metallothionein concentrations in clams (ANalysis Of VAriance, General Linear Model (Wilkinson 1988); –: non significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

	Stations	Copper	Period
Length	***	–	–
Weight-index	***	**	***
Soluble copper	***	***	***
Insoluble copper	***	***	***

Bioaccumulation of copper

Whatever the geographic origin, exposure to copper induced a bioaccumulation of this element in soft tissues. Copper concentrations reached in Arctic and subarctic samples were approximately three times higher than in temperate clams (Fig. 3). Such can not have been caused by the difference in weight index, which was in (sub-)Arctic specimens 40% lower; thus would have accounted for only 1.7 times higher copper concentrations (and not 3 times higher).

When exposed to a twofold higher copper concentration in the external medium (100 vs 50 ppb Cu), the subarctic clams showed after 13 days the same ratio in their soft tissues (167 vs 86 ngCu.mg^{-1}), whereas in specimens originating from the temperate region, copper accumulation under both regimes remained limited (45 vs 31 ngCu.mg^{-1})(Fig. 3).

A preferential storage of copper in insoluble form was observed in clams originating from all three sites (Fig. 4; at start as well as in controls and in specimens exposed to copper; paired t-tests, $p < 0.001$, in controls $p = 0.01$).

Both soluble and insoluble copper concentrations in clams differed significantly between stations (Table II). Concentrations were significantly higher in subarctic (Kartesh) clams compared to specimens from the temperate site (Paulina) or the Arctic site Khaypudyr (Fig. 4; as well as during the start as in controls and in copper exposed animals; $p < 0.001$ for both Cu doses and both physico-chemical forms of storage). Only when exposed to 100 ppb copper, the Arctic clams even reached the same insoluble copper concentrations as subarctic clams (for both being significantly higher ($p < 0.001$) than temperate specimens) (Fig. 4).

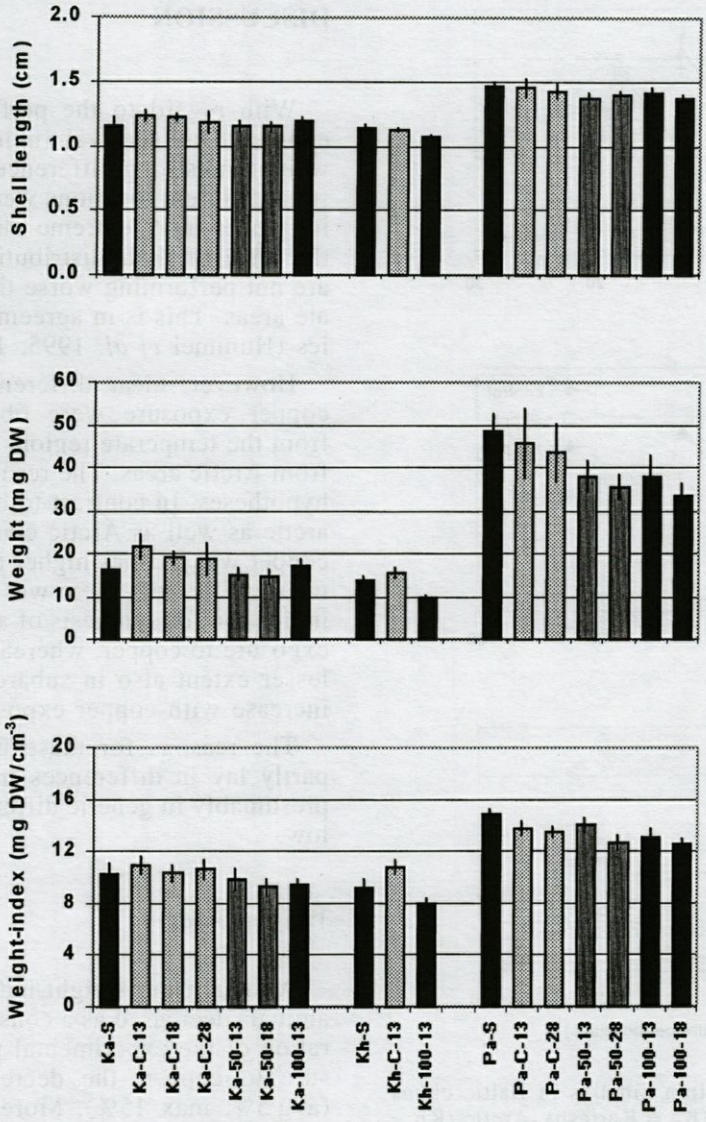

Fig. 2. – Mean shell length, lyophilized soft tissue weight and weight-index of Baltic clams (standard error) originating from subarctic (Ka = Kartesh), Arctic (Kh = Khaypudyr) and temperate areas (Pa = Paulina), and exposed or not to copper (C = control, 50 = exposed to 50 ppb Cu, 100 = exposed to 100 ppb Cu) for different periods (S = start = specimens lyophilized at the beginning of the experiment, 13,18,28=days of experimental exposure).

MTLP levels

As for copper concentrations, MTLP levels differed significantly between stations (Table II), and the subarctic clams (Kartesh) were characterized by the highest levels of MTLP (Fig. 5).

For all the three studied sites, MTLP levels at start were significantly higher than the level determined in controls maintained under experimental conditions over the different durations (Fig. 5; $p < 0.001$).

Significant higher MTLP levels were found in specimens exposed to either of the copper doses than in the controls (Fig. 5; Table II). These results were partly corroborated by the relationships between copper and MTLP concentrations in the animals (Fig. 6). In the specimens from the temperate region (Paulina) (apart from the specimens measured at the start) a strong positive correlation ($n = 72$, $r = 0.41$, $p < 0.001$) was shown. Higher MTLP levels coincided with higher internal copper concentrations. Specimens from the subarctic Kartesh showed only a weakly significant relation between copper and MTLP tissue levels ($n = 62$, $r = 0.26$, $p = 0.05$) (Fig. 6). Yet, the slightly higher MTLP level for specimens from the subarctic Kartesh at Start, in comparison to the level of

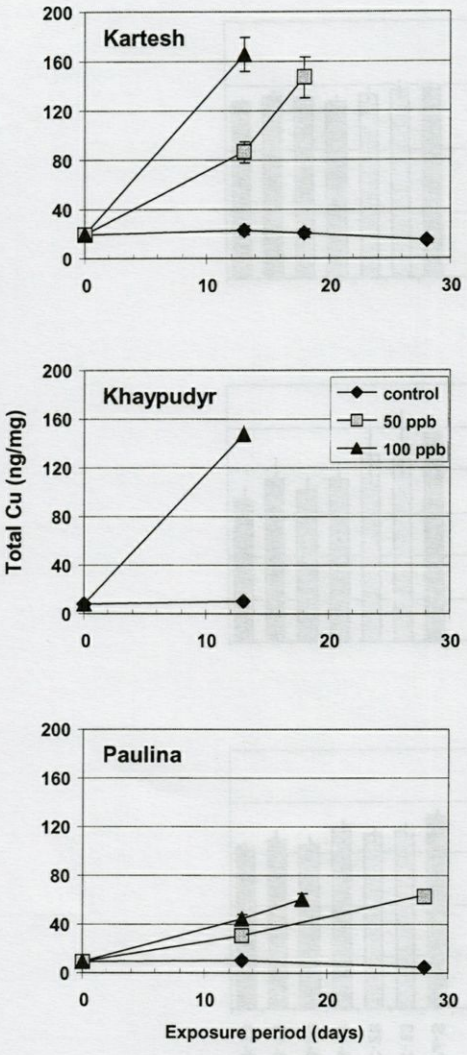

Fig. 3. – Copper accumulation kinetics in Baltic clams originating from subarctic (Ka = Kartesh), Arctic (Kh = Khaypudyr) and temperate areas (Pa = Paulina), and exposed or not to copper (means and standard errors).

specimens from the other locations (Fig. 5), may thus be related to the higher copper concentration in those clams (Fig. 3). Such a slightly higher copper concentration for animals from the area of Kartesh was already observed by Amiard-Triquet *et al.* (1998) and Regoli *et al.* (1998), and could be contributed to a higher copper concentration in the sediment (see also M&M) as a consequence of moderate industrial wastewater discharges in the near neighbourhood.

Specimens from the Arctic Khaypudyr showed a non-significant trend-line between copper and MTLP tissue levels ($n = 16$, $r = 0.32$, $p > 0.10$).

Irrespective the increase due to copper exposure, in all cases the increased MTLP levels due to copper exposure remained far below the MTLP level in the specimens directly at Start.

DISCUSSION

With regard to the performance during stress, expressed as survival (mortality) or (changes in) weight, no strong differences between Baltic clams from different locations were found. Thus even being under more extreme climatic conditions, or at the edge of their distribution, (sub-)Arctic clams are not performing worse than clams from temperate areas. This is in agreement with previous studies (Hummel *et al.* 1995, 1996, 1997a, 1998).

However, clear differences in the reaction to copper exposure were observed between clams from the temperate region, those from subarctic or from Arctic areas. The results were opposite to our hypotheses. In contrast to our expectations in subarctic as well as Arctic clams the accumulation of copper was 3 times higher than in temperate specimens. Moreover, there was in Arctic specimens no indication for synthesis of additional MTLP during exposure to copper, whereas in temperate, and to a lesser extent also in subarctic, specimens a slight increase with copper exposure was observed.

The reasons for these unexpected results may partly lay in differences in the weight index and presumably in genetic differences, as explained below.

Weight-index

Although the weight-index of the experimental animals decreased as a consequence of both the duration of the experimental period as well as exposure to copper, the decrease remained minimal (avg 5%, max 15%). Moreover, the weight index did not reach the level observed in clams living at the limits of their adaptation capabilities (Hummel *et al.* 1995, 2000). A weight index of 5 mg DW/cm^3 was suggested as the minimum value below which the metabolic energy balance of Baltic clams becomes negative, and clams die. In all cases the weight-index remained 8 mg DW/cm^3 or higher. The observed subtle changes in the weight index during the experiment therefore do not help to explain differences in Cu accumulation.

Copper accumulation and MTLP

The lowest bioaccumulation rates of both cytosolic and insoluble copper were determined in clams originating from the temperate site. Similar low copper accumulation (and elimination) rates in Dutch (= temperate) Baltic clams, lasting for several weeks before reaching equilibrium, were observed by Absil *et al.* (1996). At the other hand, the (sub-)Arctic clams showed higher accumulation rates.

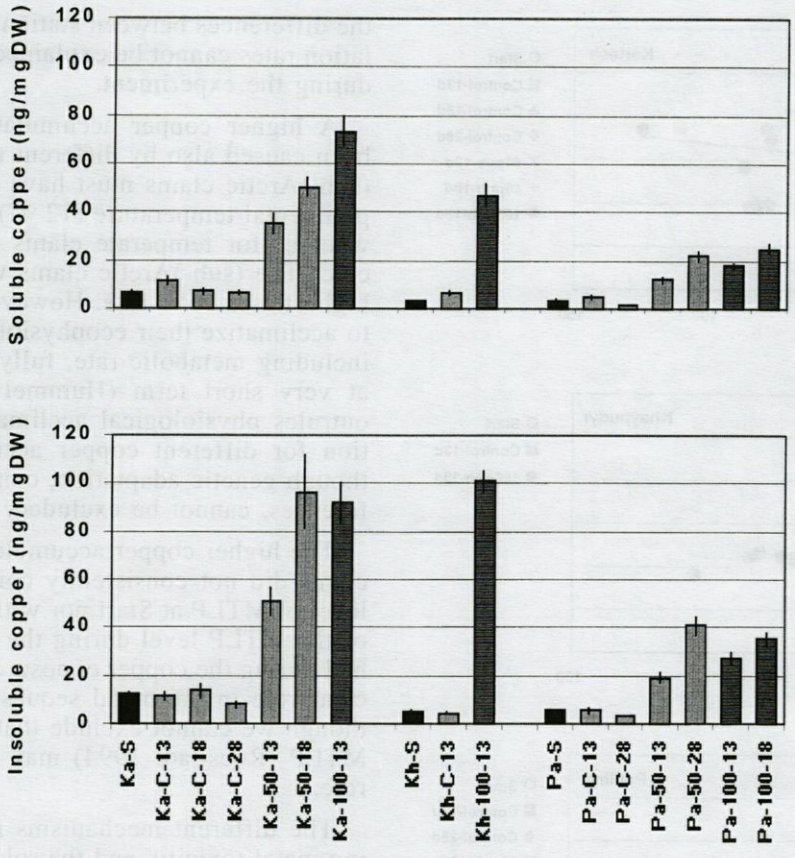

Fig. 4. – Concentrations of soluble and insoluble copper ($ng.mg^{-1}$) in soft tissues of Baltic clams originating from subarctic (Ka = Kartesh), Arctic (Kh = Khaypudyr) and temperate areas (Pa = Paulina), and exposed or not to this metal (abbreviations as in Fig. 2).

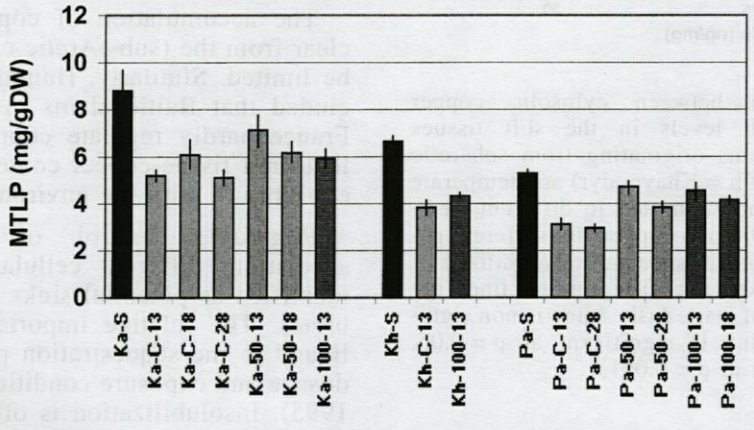

Fig.5. – Metallothionein-like-protein concentrations (MTLP; $mg.g^{-1}$) in soft tissues of Baltic clams originating from subarctic (Ka = Kartesh), Arctic (Kh = Khaypudyr) and temperate areas (Pa = Paulina), and exposed or not to copper (abbreviations as in Fig. 2).

It might be thought that the higher accumulation rates of (sub-)Arctic clams could be related to a higher mortality rate. However, for such we thus have no indication. Similarly, in Baltic clams originating from the Bay of Somme and the Loire estuary (France), specimens that survived Ag or Hg exposure at LT50 did not protect themselves against metal toxicity by accumulating a significantly lesser amount of these metals than clams that did not survive metal stress (Boisson *et al.* 1998).

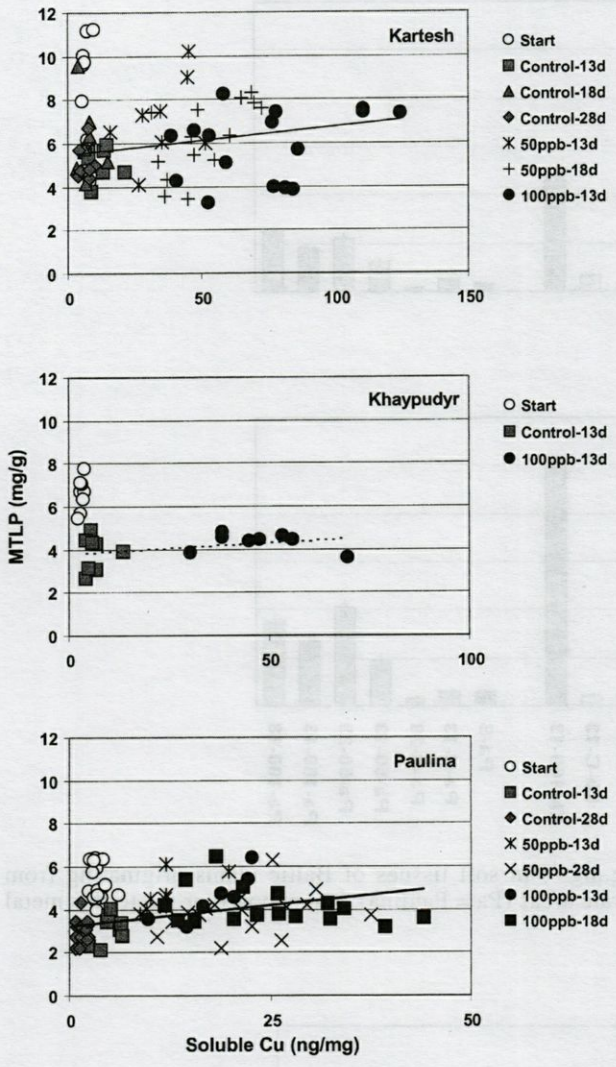

Fig. 6. – Relationship between cytosolic copper (ng/mgDW) and MTLP levels in the soft tissues (mg/gDW) of Baltic clams originating from subarctic (Ka = Kartesh), Arctic (Kh = Khaypudyr) and temperate areas (Pa = Paulina), and submitted to different treatments (control, 50 and 100 ppb copper) for different periods (13,18,28 days; start = specimens lyophilized at the beginning of the experiment)(regression lines are calculated without data of start, dashed line is non significant trendline, thin line is significant at $p = 0.05$, strong line is significant at $p < 0.001$).

In the short-term (within days or some weeks), changes in tissue copper concentrations may also be caused by changes in the weight-index. Meagre specimens were shown to have a higher Cu concentration, and heavier specimens lower Cu concentrations, in such a way that the Cu body burden in clams from a certain population was similar when viewed within a short period (Hummel *et al.* 1997b). However, because in this study changes in the weight-index remained minimal for all stations, the differences between stations in copper accumulation rates cannot be explained by weight changes during the experiment.

A higher copper accumulation rate may have been caused also by different metabolic rates. The (sub-)Arctic clams must have experienced the experimental temperature (12 °C) as relatively warm, whereas for temperate clams it would have been cold. The (sub-)Arctic clams would have then the highest metabolic rate. However, clams are shown to acclimatize their ecophysiological performance, including metabolic rate, fully to a new condition at very short term (Hummel *et al.* 2000). This outrules physiological acclimation as an explanation for different copper accumulation rates, although genetic adaptation, connected to racial differences, cannot be excluded.

The higher copper accumulation in (sub-)Arctic clams did not consistently coincide with a higher level of MTLP at Start nor with a stronger increase of the MTLP level during the experiment. MTLPs had during the copper exposure experiment thus no clear role in the metal sequestration of clams, although we cannot exclude that a rapid turnover of MTLP (Roesijadi 1994) may have obscured their role.

The different mechanisms involved in preventing metal toxicity, and the role of metallothionein, have been reviewed before (Mason & Jenkins 1995, Amiard & Cosson 1997) and fall broadly into one of two basic strategies: limiting metal accumulation and controlling intracellular metal speciation.

The accumulation of copper, as was at least clear from the (sub-)Arctic clams, seems hardly to be limited. Similarly, Hummel *et al* (1997b) concluded that Baltic clams in the Netherlands and France hardly regulate copper accumulation and that their tissue copper content is in (partitioning) equilibrium with the environment.

Regarding control of intracellular metal speciation, different cellular ligands have been identified as potential sinks for metals in invertebrates. The relative importance of each type of ligand in the sequestration process can vary with dosage and exposure conditions (Mason & Jenkins 1995). Insolubilization is often the major way of detoxication. In this study the storage of copper in clams was also mainly in the insoluble fraction of the soft tissues. This coincides with the general observation that in European clams MTs seem not to play a substantial role in metal complexation (storage) in clams, whereas the major part of the incorporated metal becomes associated with high molecular weight proteins or mineral granules (Langston & Zhou 1987, Hummel *et al.* 1997b). Lately the presence of excess copper in lysosomes of clams from France exposed to copper showed that these organelles are able to play a role in detoxifying

ions in excess in the medium through precipitation (Jeantet, pers Comm). Yet, in a previous study on Arctic bivalves a relationship between MTLP and copper levels was shown, suggesting that a detoxication process was at work in the studied species, including *M. balthica* (Amiard-Triquet *et al.* 1998). Moreover, in Baltic clams originating from the same site in the Netherlands and exposed to a mixture of 100 ng $Cd.mL^{-1}$, 100 ng $Cu.mL^{-1}$ and 600 ng $Zn.mL^{-1}$, Bordin *et al.* (1994, 1997) showed increased concentrations of metal-binding proteins displaying several of the main characteristics of MTs. The conditions differed from those used in the present study by the presence of noticeable amounts of Cd and Zn besides Cu but the duration of exposure was considerably shorter (2 days). At longer exposure the threshold level of metals to induce effects generally decreases (Amiard *et al.* 1987). Yet, in our study with a much longer exposure period and still a similar amount of copper as used by Bordin *et al.* the increase of MTLP levels was still modest.

Thus, although MTLP production by clams has been shown also to occur in our study, as in some other studies, the minor role of MT(LP)s in the sequestration of copper remains most apparent. The fact that MTLP levels at Start were even higher than those exposed to copper for 4 weeks contributes to this thought.

The decrease of MTLP concentrations in the control animals, when compared to the concentrations at start, may have been caused by the long period of transport and the subsequent submersion in clean seawater. Indeed, MTLP is reported to be formed also in response to general stress factors including injuries, handling, starvation and laboratory manipulation (George 1990, Roesijadi 1992, Kägi 1993). During the transport, being out of the water, intracellular processes and the excretion of (MTLP) degradation products will have been hampered. Only during the course of the experiment, by submersion in water, the animals might have eliminated the stored MTLP again. Similarly, Wrench (1978) had shown that controls of the oyster *Ostrea edulis* submerged in water had significantly reduced soluble protein level of both gills and digestive gland. Therefore, the long transport of the clams used in our study by itself may have generated a higher MTLP level.

CONCLUSION

When taking the starvation (= transport) period into account, the results showed that the mortality and changes in weight-index during the copper exposure experiment were comparable for temperate as well as subarctic and Arctic clams. Such is in accordance with our hypothesis. However, in opposite to our hypotheses, copper is accumulated faster in (sub-)Arctic clams than in temperate clams and MTLPs have no major role in the sequestration of copper. At all stations, copper is accumulated primarily in insoluble form. Since we could not exclude genetic adaptation as a differentiating mechanism, we conclude that racial differences between the populations, as observed by Hummel *et al.* (1997a), may be causative for the comparable performance of (sub-)Arctic clams irrespective of a much higher copper accumulation rate. Further studies are needed to unravel the ecophysiological (detoxification and tolerance) mechanisms that differentiate the temperate and (sub-)Arctic clams.

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