ISJ 7: 22-31, 2010 ISSN 1824-307X

RESEARCH REPORT

Change in metallothionein phosphorylation state in *Mya arenaria* clams: implication in metal metabolism and oxidative stress

F Gagné, M Gélinas, C Gagnon, C André, C Blaise

Fluvial Ecosystem Research, Aquatic Ecosystem Protection Research Division, Water Science and Technology, Environment Canada, 105 McGill Street, Montréal, Quebec, Canada H2Y 2E7

Accepted January 4, 2010

Abstract

The contamination of the benthic environment poses a threat to long-lived sessile organisms such as clams. The purpose of this study was to investigate metal contamination in tissues and changes in metallothioneins (MT) in respect to its redox status in *Mya arenaria* clams collected at three polluted sites. The phosphorylation state of MT was also investigated to determine whether this state is changed in clams collected at heavy-metal contaminated site and its involvement in cytoprotective signaling during stress contamination. The results show that clams collected at least one of the three polluted sites presented significantly higher concentrations of silver (Ag), arsenic (As), cobalt (Co), copper (Cu), mercury (Hg), nickel (Ni), tin (Sn) and lead (Pb) in tissues. In the visceral tissue, total MT levels and the reduced, metal-binding form of the protein were significantly induced at the sites. The phosphorylation of MT and mitochondrial activity, as determined by electron transport and cytochrome c oxidase activities, were also significantly reduced at the contaminated sites. Reduced phosphate levels in MT were negatively correlated with total MT levels, suggesting that decreased phosphorylation was involved in kinase-mediated signaling during cellular stress and could possibly alter the protein's affinity to confer cytoprotection against heavy metal contamination. These preliminary investigations revealed that the phosphorylation state could change in polluted environment and provide some clues on the modulation of binding affinities during heavy-metal and oxidative stress in clams.

Key Words: Phosphorylated metallothioneins; mitochondrial electron transport activity; cytochrome c oxidase; heavy metals; *Mya arenaria*

Introduction

Metallothioneins (MT) are low-molecular-weight (6–7 kDa) metal-binding proteins. They are ubiquitous in life forms, from bacteria to mammals, as well as being rich in cysteine (25–30%) and heat-stable owing to a lack of aromatic amino acids and hydrophobic regions (Klaassen *et al*., 1999). These characteristics make them efficient transitional metal-binding proteins that are capable of binding Ag(I), Au(I), Cd (II), Co(II), Cu(II), Hg(II), Pb(II) and Zn(II). Moreover, it was demonstrated that MT not only binds metals but also has the capacity to sequester reactive oxygen in the oyster (Andersen et al., 1999) and nitrogen species such as superoxide anion and nitric oxide (Atif *et al.*, 2006). Upon reacting with these radicals, the metal thiolate clusters of MT oxidize, liberating metals from the protein (Kang, 2006; Gagné *et al*., 2008). Hence, MT exists in both a reduced metal-binding form and in an oxidized metal-releasing form in the cytoplasm of cells. In contaminated *Mya arenaria* populations exhibiting lower clam-bed density, growth and increased mean age values, marked responses in MT, oxidative stress and gonad size were observed (Blaise *et al*., 2003). The same was also found in freshwater mussels, where Cd concentrations in the high-molecular-weight protein fraction, where MT intervenes to reduce the metal content of these fractions, was the biomarker response that was most frequently and strongly correlated with the population variables (Perceval *et al*., 2004). The MT biomarker is therefore considered a relevant biomarker of stress and has predictive value at higher levels of biological organization.

Recent studies suggest that ser/thr protein kinases (protein kinase C, or PKC) can phosphorylate

Corresponding author:
François Gagné
Fluvial Ecosystem Research
Section Environment Canada
105 McGill St., Montreal, Quebec, Canada H2Y 2E7
E-mail: Francois.Gagne@ec.gc.ca

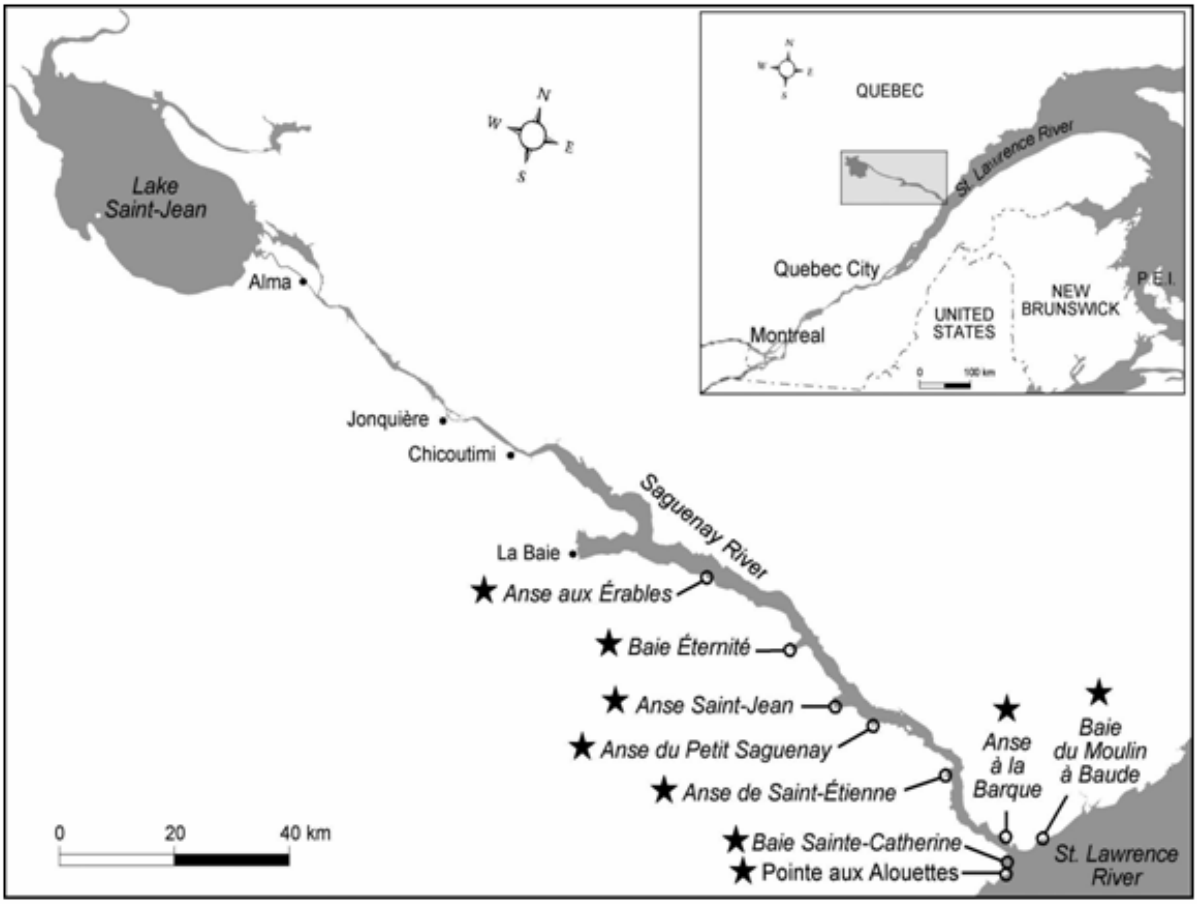

Fig. 1 Clams were collected at four sites (identified by stars) in June 2007. The site Baie du Moulin à Baude (BAU-R) was considered as the reference site; Anse Saint-Jean (ASJ-E), Baie Sainte-Catherine (BSC-T) and Baie Éternité (BE-M) were the pollution-impacted sites.

MT in neuronal cells (Aras *et al*., 2009). PKC is a family of enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of aliphatic ser and thr amino acids. Indeed, Zn-induced metal-responsive element activation was significantly decreased in neuronal cells expressing a recombinant MT-1 devoid of its phosphorylation site, while cells expressing normal MT-1 had enhanced expression (Aras *et al*., 2009). This suggests that PKC signaling could influence the phosphorylation of MT, which could, in turn, change its affinity for Zn. However, it was recognized that the phosphorylation of proteins causes them to be degraded by the ATP-dependent ubiquitin/proteasome pathway. These target proteins become substrates for particular E3 ubiquitin ligases only when they are phosphorylated. It is interesting to observe that ubiquitin is activated by the formation of a thioester bond between the C-terminal carboxyl group of ubiquitin and a (metal sensitive?) cys residue of the E1 enzyme, a process requiring ATP as an energy source. At present, the function of MT phosphorylation is unclear despite its apparent involvement in PKC signaling and possible ubiquitinylation proteasome tagging. In another study, MT was seemingly involved in protein phosphorylation signaling: the re-activation of cysteine proteinase like caspases or other phosphatases. To show this, MT-III prevented the activation of caspase-3 and -9 and the release of mitochondrial cytochrome c to the cytoplasm in neuronal cells (Kim *et al*., 2009). MT-III also increased the activation of Akt, a ser/thr protein kinase, the phosphorylation (and degradation) of Ikappa B, which can activate either an inflammatory or immune response, a cell survival response or cellular proliferation. These studies reveal that the phosphorylation state of MT might provide some clues about its involvement in cell signaling during cellular stress.

This study represents the first attempt to track changes in MT-phosphate levels in *M. arenaria* clam populations obtained at polluted sites. The levels of total phosphate bound to MT and the proportion of oxidized/reduced MT collected at three sites contaminated by heavy metals were determined. The implication of mitochrondrial respiration (metabolic activity) was also examined by tracking mitochondrial electron transport and cytochrome c oxidase activities, the terminal enzyme

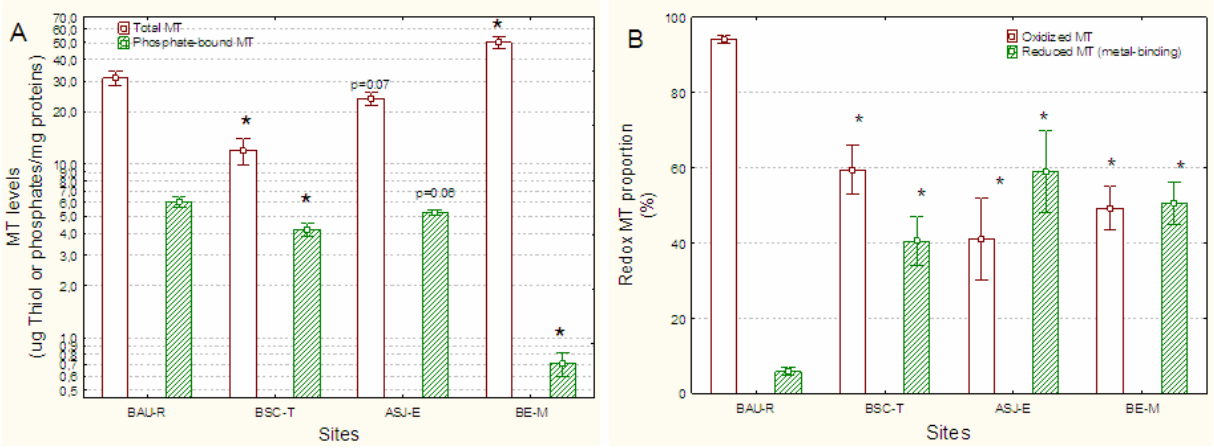

Fig. 2 Change in MT characteristics in clams from the St. Lawrence Estuary and Saguenay Fjord. Clams were analyzed for MT in the visceral mass tissues. The total and phosphate-bound MT (A) and the redox status of MT (B) were determined.

before the formation of ATP, which is the main source of reactive-oxygen species formation in cells. An attempt was made to examine the influence of MT phosphate level, total MT and redox status on clam's condition status.

Materials and Methods

Spatial survey and clam collection

Soft-shell clams (*Mya arenaria*) were hand-collected at low tide in the morning from three pollution-impacted sites and one reference (i.e., under no direct source of pollution) site located in the Saguenay Fjord and the St. Lawrence Estuary (Figure 1). In the Saguenay Fjord, the Anse-Saint-Jean site is located 40 km upstream from the estuary and receives the primary-treated (screened) effluent of about 2,000 residents (ASJ-E). The Baie Éternité site, which is located 15 km farther upstream, is historically recognized to be contaminated by heavy metals (BE-M) (Blaise *et al*., 2002). In the St. Lawrence Estuary, the Baie Sainte-Catherine site was chosen because it is heavily impacted by local commercial and pleasure-boat traffic from sightseeing and whale-watching operations, and has displayed a history of contamination by organotin (BSC-T) compounds and heavy metals (Gagné *et al*., 2005). The Baie du Moulin à Baude site is located 3 km downstream along the north shore of the St. Lawrence Estuary; it was selected as the reference site because of the absence of any direct source of pollution (BAU-R).

A total number of 30 clams were collected from clam beds at each site during early morning low tide. The clams were processed for biomarker analysis. Clam age was estimated by the number of major grooves on the shell. Clam weights and maximum longitudinal shell length were determined. The soft and gonad tissues were dissected out at 4 ^{o}C to determine the gonadosomatic index (GSI: wet weight of gonad/wet weight of soft tissues), condition factor (CF: clam weight/shell length) and growth index (GI: shell length/age). Gender was determined by microscopic examination of gonad smears at 400 times magnification. Parasitism in gonad tissues was seldom observed; any infected clams were discarded when present. The clam tissue samples were then frozen using dry ice for transportation to the laboratory and stored at -85 ^{o}C until analysis. After thawing on ice, the visceral tissues containing the gonad were crushed with a Teflon pestle tissue grinder and homogenized in a 50 mM Hepes-NaOH buffer, pH 7.4, containing 150 mM NaCl, 10 µg/mL apoprotinin and 0.5 mM dithiothreitol at a 1:5 volume/volume ratio (five passes). The dithiothreitol concentration was selected to stabilize the homogenates against MT oxidation during tissue processing (Minkel *et al*., 1980). Samples (100 µl) of each homogenate were collected for total protein determinations (Bradford, 1976). The remaining homogenates were centrifuged at 1,500 x *g* (20 min at 2 ^{o}C) and the supernatant was centrifuged at 10,000 x *g* for 20 min at 2 ^{o}C to isolate the mitochondria in pellet. The mitochondria were resuspended in two volumes of the homogenization buffer. The remaining supernatant was centrifuged at 15,000 x *g* (20 min at 2 ^{o}C) for MT characterization, as described below. All biomarkers were normalized with total protein levels in the corresponding fractions. Heavy metals and metalloids (i.e., Ag, Al, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Sn, and Zn) were analyzed in whole soft tissues (three pools of ten individuals per site) according to standard methods of the National Laboratory for Environmental Testing (NLET, 1994). The data were expressed as ng of metals or metalloids/g of dry weight.

Metallothionein characterization

The levels of total, oxidized and reduced MT were determined using a modified version of the spectrophotometric method of Viarengo *et al*. (1997), as described elsewhere (Gagné *et al*., 2008). The original methodology uses a series of

solvent fractionation steps to isolate the MT fraction from high-molecular-weight proteins and peptides such as glutathione. The modification includes a step for the complete reduction of MT (with the highly potent reducer Tris[2-carboxyethyl]phosphine) in the S_{15} fraction, which was stabilized at the homogenization step using a small amount of dithiothreitol, which was not sufficient to further reduce MT in the homogenates (Minkel *et al*., 1980). The S_{15} samples were divided between two tubes for total MT and metallic (reduced) MT evaluations. For the former, 200 µL of the S15 fraction was pre-treated with 50 µL of 50 mM Tris(2-carboxyethyl)phosphine (Sigma Chemical Company, MO, USA) for 30 min before the addition of an acidic ethanol/chloroform solution and subsequent ethanol fractionation steps to obtain the total level (reduced and oxidized) of MT. For the metal-binding (reduced) MT form, 200 µL of the S15 fraction was mixed with 50 µl of water alone with no reduction step. A sample of the MT pellet was set aside for total phosphate determinations according to the phosphomolybdate methodology of Stanton (1968) after treating the MT pellet in 1 M NaOH for 30 min to liberate inorganic phosphate. The data were expressed as µmol of thiol (GSH) equivalents/mg total protein for total and reduced MT or as µg phosphates/mg total proteins in the S_{15} fraction for phosphate MT. The oxidized fraction of MT was calculated as follows: MToxidized = MTtotal (phosphine treated) - MTmetallic. Analysis of the fractionated MT samples by high-resolution polyacrylamide gel electrophoresis with Coomassie blue staining revealed a major band around 10 kDa that is characteristic of MT, with no contaminating high-molecular-weight protein bands apparent.

Mitochondrial activity

Energy expenditures were determined by measuring mitochondrial electron transport (MET) activity (Smolders *et al*., 2004). MET activity was determined in the mitochondrial fraction (resuspended 10,000 x *g* pellet) using the *p*-iodonitrotetrazolium dye method (King and Packard, 1975; Smolders *et al*., 2004). Briefly, 100 µL of the resuspended mitochondrial pellet was mixed with 100 mM Tris-HCl, pH 8.5, containing 100 µM $MgSO_4$, 0.1% Triton X-100 and 5% polyvinylpyrrolidone for 1 min on ice. The reaction mixture was mixed with 1 mM and 0.2 mM NADH and NADPH, respectively, on ice. The reaction was initiated with the addition of 50 µl of 5 mM p-iodonitrotetrazolium for 30 min at 20 ^{o}C. Absorbance readings were measured at 15-min intervals at 520 nm. The data were expressed as the loss of absorbance at 20 ^{o}C/30 min/mg total proteins in the mitochondria. Cytochrome c oxidase (CCOx) was determined by a spectrophotometric cytochrome c oxidation assay (Sakai *et al*., 1988).

Data analysis

A total of 12 clams were analyzed for each site. The homogeneity of variances was determined using Bartlett's test. Where the data proved to be heterogeneous they were log-transformed. The data were subjected to an analysis of variance using the Benferroni *t* test for comparison of the biomarker data against the reference site (i.e., BAU-R). A Pearson product-moment analysis was performed to determine if there were any correlations within the metal and biomarker of effects data. Factorial and discriminant function analyses were also performed to examine the interrelatedness among the data, the biomarkers and the metal loads in tissues in order to discriminate among and to examine the sites. Significance was set at $p<0.05$. All statistical tests were performed using Statistica software (version 8).

Results

Based on the investigation on the primary sequences of MT from various species in public medline database (PubMed), the sequences reveal the substantial presence of serine (ser) and threonine (thr), which can be potential aliphatic phosphorylation sites (Table 1). The proportion of {ser+thr} amino acids represents about 17% of the amino acids in various species, ranging from protozoans to mammals. In bivalves, the proportion of {ser+thr} represents between 14% (*M. edulis*) to 18% (*Dreissena polymorpha*) of the amino acids of the MT protein. The highest proportion was found in rainbow trout (*Oncorhynchus mykiss*) MT-1 with 23% of the amino acids as either ser or thr residues. The proportion of {ser+thr} residues was significantly correlated with the proportion of cys ($r=0.78$; $p<0.01$) and independent of the total number of amino acids of MT. Moreover, ser and thr residues were often located in the vicinity of cys residues, frequently forming -cys-ser-cys- or –cys-thr-cys- sequences. This suggests that MT could undergo phosphorylation and perhaps influence the protein's capacity to bind heavy metals and/or reactive oxygen species, which is well-established in MTs. The investigation of MT sequences also revealed that these proteins contains lysine residues which are possible ubiquitinylation sites. Indeed, MT typically has 10% lysine residues, which suggests possible ubiquitinylation sites (Table 1). The proportion of lysine appears to be independent (i.e., not correlated) of the proportion of ser+thr, of cys and of the total number of amino acids in the various species of MT.

Clams were harvested at one reference and one polluted site in the St. Lawrence Estuary (BAU-R and BSC-T, respectively) and two sites located upstream in the Saguenay Fjord (ASJ-E and BE-M, respectively). In an attempt to characterize the quality of each site, total heavy-metal contents were determined in *M. arenaria* clams (Table 2). The data revealed that As and Co were significantly increased at the BSC-T sites while Cu was elevated at BE-M sites ($p<0.05$ level). Cr was significantly higher at the ASJ-E and BE-M sites. Ni loads in the clam tissues were significantly higher at site ASJ-E. Pb was significantly higher at all sites relative to the reference site BAU-R. Mercury, Pb and Sn were significantly higher at the BSC-T site, already known to be contaminated by organotin compounds (Viglino *et al*., 2006). Zn was significantly reduced at this site. A correlation analysis revealed that Ag was significantly correlated with Cd ($r=0.67$; $p<0.001$), Cu ($r=0.68$; $p<0.001$) and Hg ($r=0.89$; $p<0.001$). Tissue

Table 1. Characteristics of MT in different species.

Organism	Species	Proportion of ser and thr amino acids[1]	Proportion of lysine residues[2]	Proportion of cysteine (%)	Total amino acids
Protist	*Tetrahymena pyriformis*	16	14	29	107
Mussels	*Mytilus galloprovincialis*	15	7	26	72
	Psychotria viridis	16	5	28	75
	Mytilus edulis	14	10	25	73
Clams	*Dreissena polymorpha*	18	8	28	73
	Musca lusoria	14	12	28	76
Gastropods	*Helix pomatia*	16	12	24	67
Nematodes	*Caenorhabditis elegans*	11	13	25	63
Fish	*Oncorhynchus mykiss*	23	12	31	61
	Perca fluviatilis	20	10	33	60
Human	*Homo sapiens*	20	11	31	61
	Mean±SD	*16.6±3.4*	*10.4±2.7*	*28±3*	*71±13*

[1] Potential phosphorylation sites as estimated by the percentage of number (ser+thr)*100/total number of amino acids of MT.
[2] Potential ubiquitinylation sites as calculated by the percentage of lysine residues: number lys (*100)/total number of amino acids of MT.

Al content was significantly correlated with As (r=0.44; p<0.05), Pb (r=0.4; p=0.05) and Sn (r=0.83; p<0.001). Tissue As levels were significantly correlated with Co (r=0.61; p=0.001), Cr (r=0.51; p=0.01), Cu (r=0.52; p<0.01) and Pb (r=0.66; p<0.001). Cd levels in tissues were significantly correlated with Cr (r=0.53; p<0.01), Cu (r=0.53; p<0.01), Zn (r=0.72; p<0.001) and Hg (r=0.66; p<0.001). Co tissue levels were significantly correlated with Pb (r=0.78; p<0.001) only. Cr levels were significantly correlated with Cu (r=0.47; p<0.05) and Zn (r=0.47; p<0.05). Tissue Cu levels were significantly correlated with Sn (r=-0.44; p<0.05), Zn (r=0.66; p<0.001) and Hg (r=0.63; p=0.001). Tissue Sn levels were significantly correlated with Hg (r=-0.50; p=0.01). Zn tissue levels were significantly correlated with Hg (r=0.44; p<0.05). It is noteworthy that most correlations were positive, with the exception of Sn levels in tissue, which were negatively correlated with metals such as Ag, Cu and Hg.

Clam condition factor (CF) varied significantly across all sites (ANOVA p<0.001). The CF was significantly reduced (1.3-fold) at the BSC-T and ASJ-E (1.5-fold) sites. No significant change was observed in CF at the BE-M site (Table 3). The GI was significantly reduced by 1.2 at only one site (BSC-T). GSI also significantly affected (ANOVA p<0.001) at one site and was significantly increased 1.4-fold at the BE-M site. A correlation analysis revealed that CF and GSI were significantly correlated (r=0.31; p<0.05). Correlations between biomarkers of effects were included in table 4 while correlations with the heavy metal tissue loadings were cited in the text. Clam CF was significantly correlated with tissue Ag (r=0.51; p=0.01), Cu (r=0.51; p=0.01) and Hg (r=0.57; p<0.05). The growth index was negatively correlated with tissue Co (r=-0.42; p<0.05) and Sn (r=-0.4; p=0.05) levels and positively correlated with tissue Cu (r=0.48; p<0.05) and Zn (r=0.50; p=0.01) levels. The GSI was not correlated with any of the metal burdens in tissue.

The levels of MT in the clam gonad/visceral mass were determined (Fig. 2A and B). The results revealed that the total levels of MT were significantly affected at the polluted sites (Fig. 2A). Total MT levels decreased at BSC-T while they were significantly elevated at the BE-M site. At the ASJ-E site, only a marginal increase was observed (p=0.07). The phosphate levels of the MT fraction were significantly reduced at the BSC-T and BE-M sites, while a marginal decrease was found at site ASJ-E (p=0.06). A correlation analysis revealed that total MT was negatively correlated with the amount of phosphate bound to MT (r=-0.69; p<0.001) and positively so with As (r=0.50; p=0.05), Cu (r=0.83; p<0.001), Zn (r=0.79; p<0.001) and Hg (r=0.49; p=0.07). The phosphate associated with MT levels was negatively correlated with As (r=-0.65; p<0.01), Co (r=-0.61; p<0.01), Cu (r=-0.50; p<0.05) and Pb (r=-0.64; p<0.01). The redox status of MT was also appraised (Fig. 2B). The proportion of the reduced and metal-binding forms of MT rose significantly at all three polluted sites. The proportion of reduced metal-binding MT was significantly correlated with phosphorylated MT (r=-0.43; p=0.05) and Cr (r=0.47; p<0.05). This indicates that MT phosphorylation follows the formation of oxidized (metal-releasing) MT at the expense of the metal-binding (reduced) fraction of MT.

Table 2. Heavy-metal content of *M. arenaria* clams from the St. Lawrence Estuary and Saguenay Fjord.

Metals (µg/g)	BAU-R	BSC-T	ASJ-E	BE-M
Ag	0.212±0.02	0.044±0.006	0.122±0.022	0.36±0.14*
Al	91±13	122±24	96±6	109±5
As	0.63±0.04	0.83±0.02*	0.77±0.001	0.96±0.1*
Cd	0.08±0.008	0.06±0.003	0.09±0.002	0.094±0.009
Co	0.115±0.02	0.2±0.01*	0.12±0.009	0.18±0.006*
Cr	0.4±0.09	0.48±0.08	0.8±0.05*	0.74±0.05*
Cu	1.2±0.05	0.7±0.06*	1.16±0.07	1.65±0.2*
Ni	0.37±0.04	0.39±0.05	0.51±0.03*	0.46±0.04
Pb	0.03±0.001	0.06±0.005*	0.04±0.002	0.05±0.002*
Sn	0.02±0.02	0.117±0.02	0.043±0.02	0.03±0.02
Zn	14±1	10±1*	14±1	15±0.5
Hg	0.05±0.005	0.027±0.004*	0.03±0.001	0.064±0.01*

* Indicates statistical significance from the reference site at $p<0.05$ (ANOVA and least-square-difference tests).

Table 3. Values of morphometric parameters at four study sites.

Site	BAU-R	BSC-T	ASJ-E	BE-M
Parameter				
Condition Factor (CF)	0.67±0.02	0.54±0.01*	0.44±0.01*	0.70±0.02
Growth index	9.3±0.3	7.6±0.1*	9.2±0.3	8.8±0.3
GSI	0.08±0.01	0.09±0.01	0.080±0.003	0.110±0.004*
Sex ratio (1= all males; 2= all females)	1.5±0.1	1.67±0.14	1.75±0.10	1.6±0.2

* Indicates significance at $p<0.05$ level.

The influence of metabolic activity and oxidative stress on the expression of MT was determined by following mitochondrial electron transport activity (a measure related to oxygen production in mitochondria) and CCOx, the last enzyme complex involved in electron transport activity (Fig. 3A and 3B). MET activity was readily reduced at the ASJ-E (2.8-fold) and BE-M (2.3-fold) sites. MET activity was significantly correlated with phosphates bound to MT ($r=0.58$; $p<0.01$), Co ($r=0.51$; $p<0.05$), Cr ($r=-0.64$; $p<0.01$), Cu ($r=-0.54$, $p<0.05$) and marginally correlated with Ni ($r=-0,43$; $p=0.09$). The activity of CCOx was also affected in mitochondria (Fig. 3B). Its activity was significantly decreased at the BSC-T (1.6-fold) and BE-M (2.2-fold) sites, significantly correlated with phosphorylated MT ($r=0.50$; $p=0.05$) and marginally so with redox MT ($r=0.41$; $p=0.07$ for oxidized MT). CCOx was not significantly related to any of the measured metals in tissues.

The various tissue biomarker and metal loads were analyzed using factorial and discriminant function analyses to identify the major biomarkers that could explain most of the data responses and identify site-specific characteristics (Fig. 4A and 4B). A factorial analysis revealed that most of the variance (60%) was explained by three factors. The biomarkers with the highest factorial weights were Ag, Cu, Hg, Pb, Sn, Zn, CF and total MT. These metals formed a cluster close to total MT, CF, growth and GSI (Fig. 4A), indicating that these metals were statistically related to diminished clam condition and altered gonad development. Phosphorylated MT was located at the opposite side of this cluster and was closely located with CCOx activity and MET endpoints. A discriminant function analysis revealed that all sites were well discriminated at 100% accuracy (Fig. 4B). The first root function was able to discriminate between sites BE-M and ASJ-E, with the following biomarkers having the highest factorial weights: CF, MT-P and total MT. The second root function well discriminated the reference site BAU-R from the other sites, with the following biomarkers having the highest factorial weights: CF, MT-P and tissue Pb levels. These analyses revealed that the phosphorylation state of MT is an important factor relating to clam morphological status and health condition.

Table 4 Correlation analyses of the measured endpoints.

	Total MT	Oxidized MT	Reduced MT	MT-P	MET	CCOx	GSI	Growth	CF
Total MT		0.25 p>0.1	-0.25 p>0.1	**-0.68** **p=0.001**	-0.17 p>0.1	-0.33 p=0.1	0.51 p<0.01	0.22 p>0.1	**0.67** **p<0.001**
Oxidized MT				**0.40** **p=0.05**	0.15 p>0.1	0.44 p<0.05	-0.14 p>0.1	0.04 p>0.1	**0.42** **p<0.05**
Reduced MT				**-0.40** **p=0.05**	-0.15 p>0.1	**-0.44** **p<0.05**	0.14 p>0.1	-0.05 p>0.1	**-0.42** **p<0.05**
MT-P					**0.58** **p<0.01**	**0.50** **p<0.05**	**-0.56** **p=0.001**	0.05 p>0.1	**-0.43** **p=0.01**
MET						-0.04 p>0.1	-0.1 p>0.1	-0.19 p>0.1	0.1 p>0.1
CCOx							**-0.41** **p<0.05**	0.08 p>0.1	*-0.32* *p=0.09*
GSI								0.09 p>0.1	**0.31** **p<0.05**
Growth									0.11 p>0.1

Discussion

The phosphorylation state of MT in clam tissues appears to be related to the catabolism of MT in cells. Our data support this hypothesis since MT-phosphate was negatively related with the total amount of MT in cells. The increase in MT-phosphate was also related to the proportion of oxidized MT in cells. Since mitochondria are the principal source of reactive oxygen species in cells (Abele *et al*., 2002), we examined their activity by tracking electron transport and CCOx activities. The data revealed that MT-phosphate was closely related to changes in both MET and CCOx activity in tissues, suggesting that 1) MT phosphorylation is coupled in some way with mitochondrial electron transport activity and/or 2) MT is oxidized by increased mitochondrial respiration rates and then phosphorylated for removal, perhaps through the ubiquitin/proteasome pathway. The latter hypothesis was consistent with the observation that MT-phosphate was negatively related to total MT levels and positively related to oxidized MT, MET and CCOx activities. Protein phosphorylation is a prerequisite for tagging proteasome degradation and ubiquitin requires lysine residues for binding to the phosphorylated proteins which are found in various MT sequences of different species (Table 1). However, this would have to be verified by more specific experiments (i.e., finding ubiquitylated MT in bivalve tissues by western blot analysis using anti-ubiquitin antibodies). It is noteworthy that the decrease in MT-phosphate was associated with clams with lower CFs and altered gonad weights. Clams contaminated by metals from polluted sites had decreased phosphate bound to MT with a concomitant elevation in the metal-binding form of MT and total MT levels. This indicates that clams from metal-contaminated sites increase the levels of MT for metal sequestration, perhaps at the expense of oxygen radical scavenging. This was corroborated by a previous study in which the metal-binding form of MT was more closely associated to oxidative stress and tissue damage than was the oxidized form of MT (Gagné *et al*., 2008). Perhaps this explains, at least in part, why MET and CCOx activity tended to be lower at the polluted sites. Moreover, it has long been established that phosphorylation plays an important role in controlling mitochondrial metabolism, where a delicate balance between electron transport for respiration and protection against the production of reactive oxygen species is required (Foster *et al*., 2009; Sokolova *et al*., 2005). MET and related enzyme complexes involved in respiration (CCOx) would release reactive oxygen species that are less sequestered by MT and thus likely to do damage. This was consistent with increased lipid peroxidation in the gonadal homogenates of clams at the polluted sites in this study (results not shown). It is noteworthy that Sn in tissues were negatively related to Ag, Cu and Hg in clams. According to the factorial analysis, Sn tissue loadings were relatively close to the MT-phosphate and CCOx cluster. A possible explanation for would be that Sn competes with Ag and Hg to Cu (And Zn) binding sites. Moreover, organotin compounds are well-known to have a high affinity for hemoproteins which could decouple electron flow in mitochondria (Simionatto et al., 1984).

To the best of our knowledge, this is the first report of the modulation of MT phosphorylation in clam populations from polluted sites as determined by increased heavy-metal tissue loadings. Ser and thr protein kinases are also ubiquitous in bivalves (Dailianis and Kaloyianni, 2004). The external face of

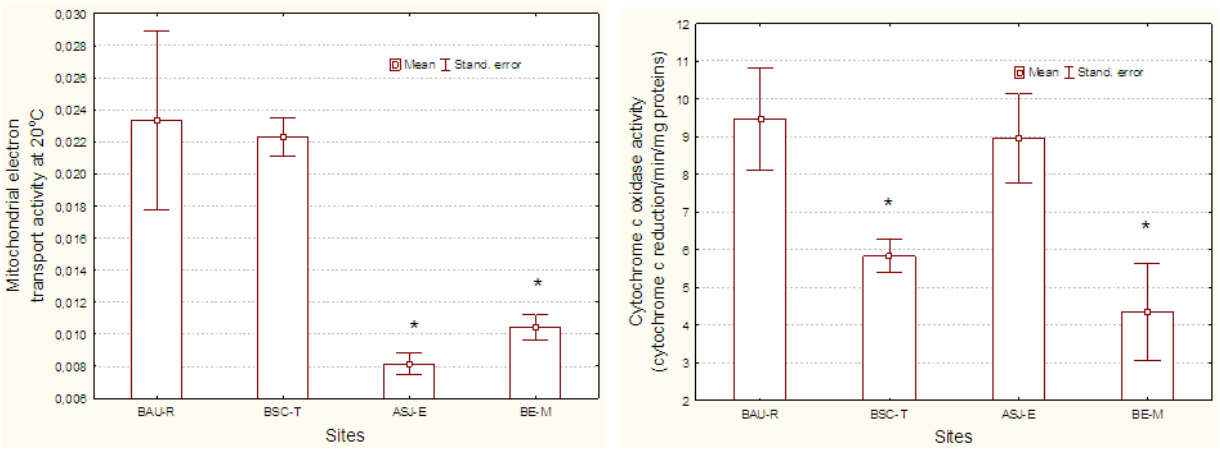

Fig. 3 Mitochondrial electron transport activity in selected clam populations. Mitochondrial electron transport (A) and cytochrome c oxidase (B) activities were measured in isolated mitochondria from feral clam populations. Asterisks (*) indicate significance at $p<0.05$

the outer mitochondrial membranes is filled with various intracellular receptors and serves as a site for signals implicated in steroidogenesis, cytoprotection and energy demands, where PKC-interacting proteins are found (Smith *et al*., 2006; Poole *et al*., 2004). This family of protein kinases is also involved in Zn signaling in neuronal cells, which confers tolerance towards Zn^{2+} and suggests that PKC acts directly on the intracellular source of Zn and the expression of Zn-regulated genes such as MT (Aras *et al*., 2009). In MT, a protein kinase C phosphorylation site was identified at serine 32 regardless of the species examined. Moreover, MT phosphorylation appears to modulate the Zn-binding efficiency of MT, as shown by the reduction in Zn^{2+}-induced metal-responsive element in cells expressing a mutated MT that is devoid of a phosphorylation site. This was explained by the phosphate-associated MT, which lowers the binding affinity for Zn and favoring the subsequent release of Zn^{2+} for the activation of metal-responsive elements and the production of *de novo* Zn-dependent genes such as MT. Our data revealed an inverse relationship between phosphate-bound MT and total MT/metal-binding MT, which suggests that decreased MT phosphorylation might have intervened in the MT forms in handling metal homeostasis in clams from metal-contaminated sites. In another study, copper-induced MT-1 transcription was regulated by PKC and a metal transcription factor (Mattie and Freedman, 2004). The role of MT in the maintenance of redox homeostatis appears to recall other protein phosphatases such as protein kinase B, leading to Ikappa B degradation which, in turn, activates the expression of NF-kappaB activity during the inflammatory response and oxidative stress (Kim *et al*., 2009). The inhibition of several intracellular protein kinases such as protein kinase A and C, MAPK and calmodulin kinase-II eliminated the neuroprotective effect of MT, highlighting the interplay between metal-binding capacity, oxidative stress and protein phosphorylation (Asmussen *et al*., 2009). In *Mytilus galloprovincialis*, Cd and Zn caused an increase in superoxide anion production, with Cd being more potent (2.2-fold) than Zn (1.5-fold) (Koutsogiannaki *et al*., 2006). In addition, the metal effect was reversed in the presence of calphostin, a PKC inhibitor, and an amiloride analogue that blocks Na/K-exchanges. Conversely, Cd induced pyruvate kinase activity and a rise in intracellular pH was augmented by phorbol esters, a potent activator of PKC (Dailianis and Kaloyianni, 2004). This is in agreement with the increase in metal affinity in less phosphorylated-MT hypothesis, which confers cytoprotection to Cd and Zn but perhaps at the expense of radical oxygen scavenging. Taken together, these studies reveal a hormone-like effect of divalent metals such as Cd and Zn at the β-adrenergic and PKC signal transduction pathways in *M. galloprovincialis*. However, GSI was not correlated with any of the biomarkers in this study suggesting perhaps that hormonal signaling involved in gametogenesis (i.e., steroids) had no important effects on the phosphorylation state of MT. We should bear in mind that these sites were not only contaminated by heavy metals but with organic contaminants such as polyaromatic hydrocarbons which could also produce oxidative stress and perhaps influence the phosphorylation state of MTs. More research is required to better understand the physiological role of MT phosphorylation in the handling of heavy metals and oxygen radicals in bivalves.

In conclusion, *M. arenaria* clams from heavy metal-contaminated sites show increased total and metal-binding MT with decreased MET and CCOx activities. Total MT and the metal-binding (reduced) form of MT were closely related with levels of Ag, Cu, Hg and Zn in tissue. Moreover, MT phosphorylation was also reduced at the contaminated sites (less-phosphorylated MT would have a stronger metal-binding capacity), suggesting

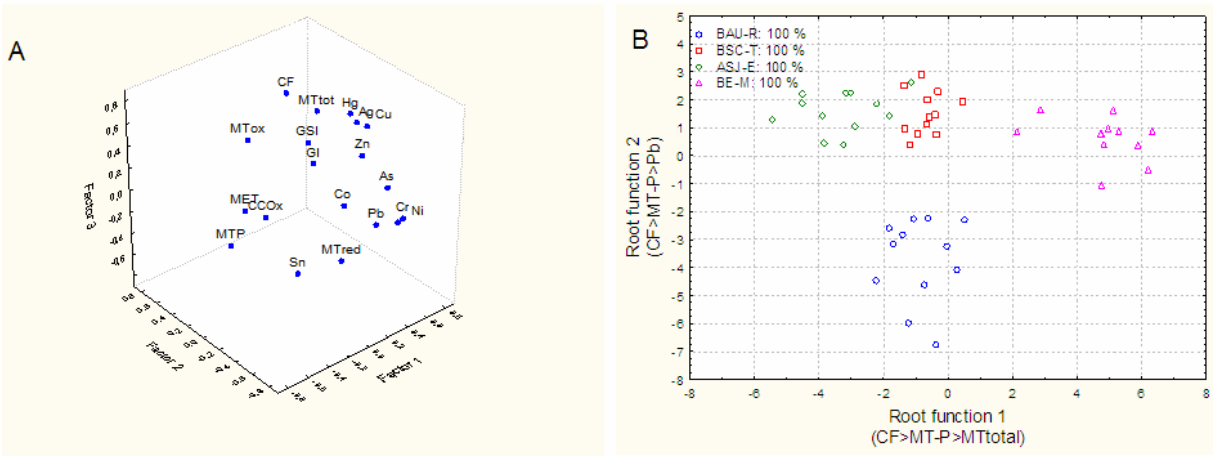

Fig. 4 Factorial and discriminant function analyses of the biomarker responses. The factorial analysis was performed with the principal component extraction procedure (A). The discriminant function analysis was also performed to examine the capacity of the biomarkers used in this study to identify the sites (B).

the involvement of PKC signaling pathways that can change the metal-binding affinity of MT towards exposure to various metals. The measurement of phosphates bound to the MT fraction might provide some clues on the hormone-like effects of metals and the modulation of binding affinities towards metal contamination of the environment.

Acknowledgements

The authors thank Sophie Trépanier for her technical support with the biomarker analyses. This work was funded by Environment Canada under the CEPA initiatives. The manuscript was edited by Patricia Potvin of Environment Canada.

References

Abele D, Heise K, Portner HO, Puntarulo S. Temperature dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205: 1831–1841, 2002.

Anderson SR, Patel KM, Röesijadi G. Oyster metallothionein as an oxyradicals scavenger: implication for hemocyte defense responses. *Dev. Comp. Immunol* 23:443–449, 1999.

Aras MA, Hara H, Hartnett K A, Kandler K, Aizenman E. Protein kinase C regulation of neuronal zinc signaling mediates survival during preconditioning J. Neurochem. 110:106-117, 2009.

Asmussen JW, Von Sperling ML, Penkowa M. Intraneuronal signaling pathways of metallothionein. J. Neurosci. Res. 87: 2926–2936, 2009.

Atif F, Kaur M, Yousuf S, Raisuddin S. *In vitro* free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, *Channa punctata* Bloch. Chem. Biol. Interact. 162: 172–180, 2006.

Blaise C, Gagné F, Pellerin J, Hansen PD, Trottier S. Molluscan shellfish biomarker study of the Quebec, Canada, Saguenay Fjord with the softshell clam *Mya arenaria*. Environ. Toxicol. 17: 170–186, 2002.

Blaise C, Gagné F, Pellerin J. Bivalve population status and biomarker responses in *Mya arenaria* clams (Saguenay Fjord, Québec,Canada). Fresenius Environ. Bull. 12: 956–960, 2003.

Bradford MM. A sensitive method for the total protein determination using the principle of protein-dye binding. Anal. Biochem. 72: 249–251, 1976.

Dailianis S, Kaloyianni M. 2004. Cadmium induces both pyruvate kinase and Na^+/H^+ exchanger activity through protein kinase C mediated signal transduction, in isolated digestive gland cells of *Mytilus galloprovincialis* (L.). J. Exp. Biol. 207: 1665–1674, 2004.

Foster DB, Van Eyk JE, Marban E, O'Rourke B. Redox signaling and protein phosphorylation in mitochondria: progress and prospects. J. Bioenerg. Biomembr. 41: 159-168, 2009.

Gagné F, Blaise C, Pellerin J, Pelletier E, Strand J. Health status of *Mya arenaria* bivalves collected from contaminated sites in Canada (Saguenay Fjord) and Denmark (Odense Fjord) during their reproductive period. Ecotoxicol. Environ. Saf. 64: 348–361, 2005.

Gagné F, André C, Blaise C. The dual nature of metallothioneins in the metabolism of heavy metals and reactive oxygen species in aquatic organisms: Implications of use as a biomarker of heavy-metal effects in field investigations. Biochem. Insights 1: 31–41, 2008.

Kang YJ. Metallothionein redox cycle and function. Exp. Biol. Med. 231: 1459–1467, 2006.

Kim HG, Hwang YP, Han EH, Choi CY, Yeo CY, Kim JY, *et al*. Metallothionein-III provides neuronal protection through activation of Nuclear Factor-κB via theTrkA/Phosphatidylinositol-3 kinase/Akt signaling pathway. Toxicol. Sci. 112: 435-449, 2009.

King F, Packard TT. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20: 849–854, 1975.

Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect against cadmium toxicity. Ann. Rev. Pharmacol. Toxicol. 39: 267–294, 1999.

Koutsogiannaki S, Evangelinos N, Koliakos G, Kaloyianni M. Cytotoxic mechanisms of Zn^{2+} and Cd^{2+} involve Na^{+}/H^{+} exchanger (NHE) activation by ROS. Aquat. Toxicol. 78: 315–324, 2006.

Mattie MD, Freedman JH. Copper-inducible transcription: regulation by metal- and oxidative stress-responsive pathways. Am. J. Physiol.-Cell Physiol. 286: 293–301, 2004.

Minkel DT, Poulsen K, Wielgus S, Shaw CF, Petering DH. On the sensitivity of metallothioneins to oxidation during isolation. Biochem J. 191: 475–485, 1980.

NLET – National Laboratory for Environmental Testing. 1994. Manual of Analytical Methods. National Laboratory for Environmental Testing, Environment Canada, Burlington, Ontario, Canada, 1994.

Perceval O, Couillard Y, Pinel-Alloul B, Giguère A, Campbell PGC. Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses. Aquat. Toxicol. 69: 327–345, 2004.

Poole AW, Pula G, Hers I, Crosby D, Jones ML. PKC-interacting proteins: from function to pharmacology. Trends Pharmacol. Sci. 25: 528–535, 2004.

Sakai Y, Tanaka A, Ikai I, Yamaoka Y, Ozawa K, Orii Y. Measurement of cytochrome c oxidase activity in human liver specimens obtained by needle biopsy. Clini. Chim. Acta 176: 343–346, 1988.

Simionatto CS, Anderson KE, Sassa S, Drummond GS, Kappas A.. Fluorometric measurement of tinprotoporphyrin in biological samples. Anal. Biochem. 141: 213–219, 1984.

Smith FD, Langeberg LK, Scott JD. The where's and when's of kinase anchoring. Trends Biochem. Sci. 31: 316–323, 2006.

Smolders R, Bervoet L, De Coen W, Blust R. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ. Pollut. 129: 99–112, 2004.

Sokolova IM, Sokolov EP, Ponnappa KM. 2005. Cadmium exposure affects mitochondrial bioenergetics and gene expression of key mitochondrial proteins in the eastern oyster *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). Aquat. Toxicol. 73: 242-255, 2005.

Stanton MG. 1968. Colorimetric determination of inorganic phosphate in the presence of biological material and adenosine triphosphate. Anal. Biochem. 22: 27-34, 1968.

Viarengo A, Ponzanon E, Dondero F, Fabbri R. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Mar. Environ. Res. 44: 69–84, 1997.

Viglino L, Pelletier E, Lee J. 2006. Butyltin species in benthic and pelagic organisms of the Saguenay Fjord (Canada) and imposex occurrence in common whelk (*Buccinum undatum*). Arc. Env. Cont. Toxicol. 49: 1–17, 2006.

Wills ED. Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell K; Mullock B (eds), Biochemical Toxicology: A Practical Approach,: IRL Press, Washington DC, USA, pp 127–150, 1987.