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RESEARCH REPORT

# An investigation on the disruptive effect of pollution in cold- and warm- adapted clam populations

**F Gagné[1], C André[1], C Blaise[1], J Pellerin[2], J Sherry[3], A Talbot[1,3]**

*[1]Fluvial Ecosystem Research Section, Environment Canada, 105 McGill, Montréal, Québec, Canada, H2Y 2E7*
*[2]ISMER-Université du Québec à Rimouski, Rimouski, 310 Grande Allée, Rimouski, Québec, Canada, G5L 3A1*
*[3]Environment Canada, National Water Research Institute, 867 Lakeshore Rd., PO Box 5050 Burlington, Ontario, Canada, L7R 4A6*

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## Abstract

The purpose of this study was to examine the relationship between mitochondrial activity and gonad lipid stores in clams exposed to anthropogenic pollution at cold- and warm-water sites. The balance between energy expenses and energy reserves was measured by mitochondrial electron transport (MET) activity and lipid content in the gonad. The activity of malate dehydrogenase (MDH) was measured as an intermediary between energy production and the production of lipids in gonadal tissues. The results revealed that intertidal clam populations at warm-water sites under no source of pollution had less heavy metal content (Ag, As, Cr, Hg and Ni), lower MDH activity and temperature-dependent MET than clams from cold-water sites. However, MDH activity measured at 6 $^{o}$C was higher at the warm-water sites. Lipid peroxidation in the gonad was higher in clams from the cold-water sites. The impacts of pollution differed among the study sites, clams from cold-water sites having increased MDH activity, temperature-dependent MET activity, higher lipid content and DNA strand breaks; clams from the warm-water sites had increased temperature-dependent MDH activity and lower gonadal lipid reserves. A multiple regression analysis revealed that gonad lipid reserves were positively correlated with MDH activity and negatively correlated with its temperature-dependent activity, suggesting that increased temperature sensitivity was negatively related to gonad energy reserves. The data show that pollution increases temperature sensitivity at the MET level in clams in cold water, while temperature sensitivity in MDH activity was observed in clams from warm-water sites. Discriminate function analysis revealed that pollution stress shows a tendency to be closer to clams adapted to warmer temperatures. In conclusion, pollution could increase MDH activity in cold-adapted clams which can lead to increased lipid stores in the gonad, oxidative stress and genotoxicity while pollution seems to increase the temperature dependence in MET. In warm-adapted clams, temperature dependent MDH activity was higher by pollution with decreased lipid content in the gonad tissues which was independent of gonad maturation and size.

**Key Words**: mitochondrial electron transport; malate dehydrogenase; gonad lipid; clam health

## Introduction

The St. Lawrence River is subjected to various sources of chemical pollution that are historically linked to a variety of industrial (aluminum plants and pulp and paper mills), municipal effluents and agricultural runoffs from adjacent basins and harbors. Clams are particularly at risk to these types of pollution since they are sessile, relatively long-lived (12-15 years for *Mya arenaria*), and in close contact with sediments, which are well-known sinks for substances such as tributyltin (TBT) and industrial contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals (Gagnon *et al.*, 2004; Yang *et al.*, 2006). The ecotoxicological impacts of pollution in this intertidal bivalve were extensively examined in the St. Lawrence and one of its largest tributaries, the Saguenay Fjord (Blaise *et al.*, 2003; Gagné *et al.*, 2008a, b; Pellerin *et al.*, 2009). In these studies, it was revealed that the density of intertidal clam beds was closely related to immunocompetence (hemocyte concentration and phagocytic activity) and mitochondrial electron

*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

transport activity (MET), a measure of respiration and energy expenditure. Moreover, pollution increased clam temperature dependence or sensitivity to electron transport activity in mitochondria (Gagné *et al.*, 2008b). Increased energy expenditure could occur at the cost of energy reserves in the form of lipids, sugars and proteins to a lesser extent (Smolders *et al.*, 2004). The balance between energy expenditure and reserves was introduced as the concept of cellular energy allocation, which proved a very predictive and sensitive endpoint of impacts at higher levels of biological effects such as survival, growth and reproduction in zebra mussels and daphnids (De Coen and Janssen, 2003). In a context of global warming and given the cumulative effects of environmental stressors, a recent study revealed that MET activity was sensitive to temperature changes and pollution readily increased this temperature sensitivity (Gagné *et al.*, 2007). Indeed, this study revealed that clams under pollution stress were more sensitive (i.e., spend more energy) to temperature increments than clams from less-contaminated sites. To further investigate the interaction of pollution with thermal adaptation and stress, we undertook a spatial survey of intertidal clams at cold-water sites (St. Lawrence Estuary) and from areas of warmer water in the Saguenay Fjord. The balance between energy expenditure (MET) and reserves in the gonad (gonad size and lipid content) was closely examined by tracking the transfer of precursors from the mitochondria to the cytoplasm to assist lipid synthesis. In cells, oxaloacetate is transferred in the cytosol to support lipid and amino acid synthesis through the malate shunt pathway, which involves NADH-dependent malate dehydrogenase (MDH).

MDH catalyzes the reversible reduction of oxaloacetate to malate in the presence of the co-factor NADH in the mitochondria (Stryer, 1995): oxaloacetate + NADH → malate + $NAD^+$. Malate diffuses out the mitochondria and is re-oxidized into oxaloacetate in the cytosol by a cytosolic MDH. During this process, NADH is formed which, in turn, can be used to support lipid synthesis. Adaptation to cold or warm temperatures brings about changes in allelic MDH expression, selecting towards isoforms with various temperature dependence (Fields *et al.*, 2006). Indeed, adaptation to colder temperatures was associated with lower enzyme affinity for NADH, but the activity was more sensitive to temperature. Lower enzyme affinity for NADH will displace the reaction in favor of the oxidation of malate into oxaloacetate and NADH, hence is consistent with lipid production in organisms acclimated to colder temperatures. The production of oxaloacetate and malate will also depend on mitochondria respiration rate i.e. aerobic metabolism and the cytosolic activity of MDH. In another study, the affinity constant $K_M$ for NADH was less sensitive to high temperatures in bivalve species adapted to warmer temperatures (*L. scabra* and *L. gigantea*) than in those adapted to colder temperatures such as *L. scutum* or *L. pelta* (Dong and Somero, 2009). This change in enzyme affinity was associated with a single amino acid substitution of glycine to serine at position 291 in cytosolic MDH, a change that favors hydrogen bonding and reduced conformational entropy, hence less affected to temperature variations. These studies suggest that warm/cold adaptation calls for changes in protein/enzyme kinetic activity and sensitivity to temperature changes. Intertidal clams have to cope with air-temperature fluctuations during low tide which bring about shifts between anaerobic and aerobic metabolism and how clams handle these stresses in either cold- or warm-water habitats is not fully understood. Moreover, the interaction of pollution in these adaptation processes complicates the outcome of clam survival. There is a need to further study into how pollution stress and temperature interacts in feral clam populations.

The purpose of this study was therefore to examine the impacts of pollution on the relationship between MET, cytosolic MDH activity, lipid reserves in the gonad and the gonado-somatic index (GSI) of intertidal clam populations adapted to cold and warm water considering their relative distance from the shore (i.e., emersion time to air). Moreover, toxic tissue damage was concurrently investigated by tracking changes in lipid peroxidation (LPO) and the formation of DNA strand breaks. An attempt was made to relate the impacts of pollution on MET and MDH activity, including their temperature-sensitive properties and gonad lipid reserves in clams obtained from cold- and warm-water.

## Materials and Methods

*Spatial survey and clam collection*

*Mya arenaria* soft-shell clams were collected by hand on mud flats at morning low tide from two sites in the St. Lawrence Estuary and two sites in the Saguenay Fjord (Fig. 1). Sampling was made in later-half June of 2006 which corresponds to the pre-spawning period with gravid gametes and high GSI. Clams from the former area thrive in the colder water of the St. Lawrence (typically 3-6 $^oC$ compared to water temperatures of 5-12 $^oC$ at the fjord site) owing to the inputs of warmer river water draining nearby basins and adjacent tributaries (small streams and rivers, drainage of rainfall). Both areas are influenced by tidal rhythms and they are interconnected and relatively close to each other; no significant pH and conductivity differences were observed for the surface waters at the site of collection. Temperature measurements of the surface waters at the shore line were also determined during clam collection. Among the St. Lawrence Estuary sites, Baie Sainte-Catherine (BSC) is influenced by heavy traffic from sightseeing and whale-watching boats which is considered the cold temperature and polluted sites (CT+P); Baie-du-Moulin à Baude (BAU) was considered the cold-temperature reference site (CT) because of the absence of any direct source of pollution. As for the Saguenay Fjord sites, Anse Étienne site (ASE), located on the south shore 20 km upstream of the estuary, is under no direct source of anthropogenic activity and was thus considered as the reference site for the fjord for the warm water temperature site (WT); the Anse Saint-Jean (ASJ) site is exposed to the minimally treated (screening) urban wastewater of a population of about 2000 residents, located 40

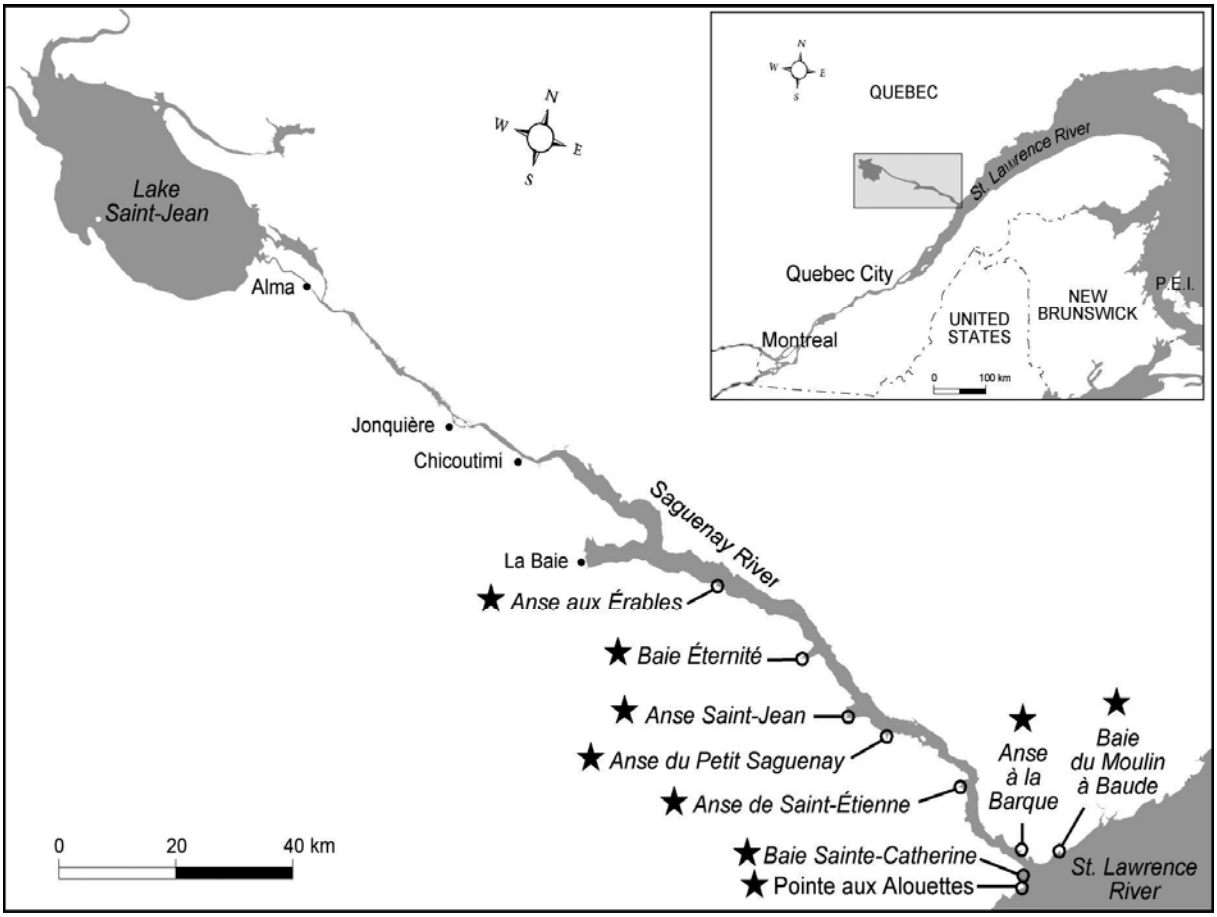


**Fig. 1** Clams were collected at four sites (identified by stars) in June 2006. The sites Anse de Saint-Étienne (ASE) and Baie du Moulin à Baude (BAU) were considered as the reference CT and WT sites respectively, while Anse Saint-Jean (ASJ) and Baie Sainte-Catherine (BSC) were the WT+P and CT+P sites respectively. Clams from ASE and ASJ sites were located in the warmer water temperatures of the Saguenay River; clams from BAU and BSC sites were located in the colder waters of the St. Lawrence Estuary.

km upstream of the estuary and was considered the warm-temperature polluted site (WT-P).

A total of 20 clams were collected at each site for the biomarker analyses. Clam weights, soft tissue mass and shell length were determined on site and in the laboratory for GSI (gonad wet weight /soft tissues wet weight), condition factor (clam weight/shell length), and growth index (shell length/age ratio) determinations. Age was determined by counting the number of major grooves on the shell. Sex and gonad maturity were determined by microscopic analysis of gonad smears at 400X. Parasitism was rarely observed but there were some occurrences of trematode infection; clams so infected were discarded. The clams retained for analysis were frozen under dry ice for transport to the laboratory and stored at -85 $^{o}$C until the analyses. After thawing on ice, the visceral mass containing the gonad tissues were removed and homogenized with a Teflon pestle tissue grinder apparatus in 25 mM Hepes-NaOH buffer, pH 7.4, containing 145 mM NaCl, 10 µg/ml apoprotinin and 0.1 mM dithiothreitol at a 1:5 volume/volume ratio (five passes). Samples (100 µl) of each homogenate were collected for total protein determinations (Bradford, 1976), lipid peroxidation (LPO), total lipid content and DNA damage. The remaining homogenate was centrifuged at 1,500x*g* (20 min at 2 $^{o}$C) to remove cell debris and nuclei (pellet). The supernatant was centrifuged at 10,000x*g* (20 min at 2 $^{o}$C) to isolate the mitochondria (pellet) for mitochondrial electron transport (MET) activity, and the remaining supernatant was kept for the post-mitochrondrial malate deshydrogenase (MDH) activity evaluations. The mitochondria pellet was resuspended in 2 volumes of ice-cold homogenization buffer before measuring MET activity during the same day.

*Energy status*

Cellular energy allocation was determined by measuring mitochondrial electron transport (MET) activity and lipid content in gonad. MET activity was determined in the mitochondrial fraction (10,000x*g* pellet) using the p-iodonitrotetrazolium dye method (King and Packard, 1975; Smolders *et al.,* 2004). Briefly, 100 µl of the supernatant was mixed with 100 mM Tris-HCl, pH 8.5, containing 100 µM $MgSO_4$,

**Table 1** Heavy metal content in *M. arenaria* clams and concordance analysis with the measured biomarkers

| Metals (µg/g) | BSC | BAU | ASJ | ASE |
|---|---|---|---|---|
| Ag | **0.14±0.02*** | 0.03±0.05 | **0.58±0.25** | 0.09±0.02 |
| As | **0.91±0.05*** | 0.30±0.01 | **0.84±0.04** | 0.48±0.03 |
| Cd | 0.06±0.01 | 0.04±0.02 | 0.11±0.01 | 0.09±0.01 |
| Cr | **7±1.4*** | 4 ±0.9 | **4±0.8** | 1.3 ±2* |
| Cu | 1.35±0.3 | 1.3±0.26 | 1.5±0.03 | 1.28±0.26 |
| Hg | **0.03±0.005*** | 0.008±0.002 | **0.03±0.003*** | nd |
| Ni | **4±0.8*** | 0.8±0.2 | 1.7±0.3 | 1.6±0.3 |
| Pb | 0.04±0.003 | 0.06±0.1 | 0.09±0.02 | 0.065±0.01 |
| Sn | **0.12±0.05*** | 0.03±0.01 | -- | -- |
| Zn (x10) | 1.1±0.2 | 1±0.2 | 1.7±0.1 | 1.3±0.3 |
| Sum | 24.65 | 16.6 | 27.6 | 17.9 |

* indicates significance at $p<0.05$

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 and then divided into two portions, one being equilibrated at 6 $^{o}$C and the other at 25 $^{o}$C. The reaction was initiated with the addition of 50 µl of 5 mM p-iodonitrotetrazolium for 30 min. Absorbance readings were measured at 15-min intervals at 520 nm. The data were expressed as the loss of absorbance at 6 $^{o}$C and 25 $^{o}$C/30 min/mg total proteins in the corresponding supernatant. Temperature-dependent MET ($MET_T$) was determined and the rate difference in MET at 25 $^{o}$C and 4 $^{o}$C was divided by the temperature change (25-6 $^{o}$C = 19 $^{o}$C) as determined previously (Gagné *et al.*, 2007). The data were expressed as the rate change (normalized against total proteins)/unit temperature in $^{o}$C. Gonad lipid levels were determined in gonad homogenates following the phosphovanillin method (Frings *et al.*, 1972). The detergent Triton X-100 was used for calibration. The data were expressed as µg lipid equivalents/mg protein. Malate dehydrogenase (MDH) activity in the mitochondrial fraction (S3) was determined by tracking the formation of NADH in the presence of 0.2 mM malate and $NAD^+$ for 30 min at 20 $^{o}$C. The reaction buffer was 50 mM Hepes-NaOH, pH 7.4, containing 100 mM NaCl and 0.1 mM EDTA. The appearance of NADH was measured by fluorescence (excitation 360 nm; emission 450 nm). Temperature-dependent MDH was also determined as described with $MET_T$. The kinetic constants for MDH ($K_M$ and $V_{max}$) were also determined at both temperatures using the classic Lineweaver_Burk plot method (Stryer, 1995).

*Biomarkers of toxic effects*

Toxic effects were determined by tracking LPO and DNA strand breaks in gonad. LPO was determined in gonad homogenates using the thiobarbituric acid method (Wills, 1987). Standard solutions of tetramethoxypropane were used for calibration and fluorescence was measured at 520 nm excitation and 600 nm emission (Chameleon-II, Multireader, Bioscan, USA). Given that the reagent could react with other aldehydes, results were expressed as µg of thiobarbituric acid reactants (TBARS)/mg of homogenate protein. DNA damage was determined by the alkaline precipitation assay developed by Olive (1988), with fluorescence quantitation of DNA strand breaks in the presence of detergents and an alkaline pH (Bester *et al.*, 1994). The assay principle is based on the potassium-detergent (SDS)-assisted precipitation of protein-linked genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. The number of DNA strand breaks results from the DNA repair of DNA adducts and alkali-labile sites. The results were expressed as µg of DNA/mg proteins. Calibration was achieved with salmon sperm DNA (Sigma Chemical Company, USA).

*Data analysis*

A total of 20 clams were analyzed for each study site. The homogeneity of variances was determined using Bartlett's test. Where the data proved heterogeneous, they were log-transformed. The data were subjected to a two-way ANOVA, with gender and site of collection as the main factors. A discriminant analysis was performed to identify if the sites could be correctly classified using the biomarker

**Table 2** Clam morphological characteristics

| Site | Clam weight/shell length | Growth index (shell length/age) | Age | Gonado-somatic index |
|---|---|---|---|---|
| ***Saguenay Fjord*** | | | | |
| ASE (reference) | 10±0.31 | 0.49±0.022 | 6.04±0.25 | 0.12±0.01 |
| ASJ | 11.5±0.33 | 0.48±0.01 | 5.6±0.17 | 0.12±0.005 |
| ***St. Lawrence Estuary*** | | | | |
| BAU (reference) | 12±0.28 [b] | 0.57±0.02 [b] | 5.62±0.12 | 0.06±0.005 [b] |
| BSC | 9.2±0.38 [a,c] | 0.47±0.02 [a] | 6.43±0.22 [a,c] | 0.04±0.003 [a,c] |

[a] Significance at p<0.05 between the polluted site relative to the corresponding reference site
[b] Significant difference between the reference sites in cold- and warm-water sectors
[c] Significant difference between polluted sites in cold- and warm-water sectors

test battery and which measurements contributed the most to site discrimination. Correlation (Pearson-moment) and multiple regression analyses were also performed. Significance was set at $p < 0.05$ but marginal effects ($0.1 < p < 0.05$) were also provided. All statistical tests were performed using the Statistica version 8 software package.

## Results

*Clams contamination and morphological characteristics*

The surface water pH, conductivity and temperature were measured in surface waters from the Saguenay fjord and the St-Lawrence Estuary (Fig. 1). Clams collected at the St-Lawrence area were exposed to colder surface water temperatures while those collected in the Saguenay fjord were exposed to warmer surface water temperatures. Indeed, the water temperature was significantly ($p = 0.02$) higher at the Saguenay fjord ($10 \pm 4$ $^{o}$C) than the Estuary ($5 \pm 1$ $^{o}$C) confirming the temperature differences between these two sectors. The pH and water conductivity did not significantly changed albeit a marginal ($p = 0.07$) increase in conductivity for the St-Lawrence Estuary sites. Moreover, clams were analyzed for heavy metal content to confirm that they were indeed exposed to pollution stress (Table 1). The data revealed that clams at the heavy-traffic harbor were more contaminated in arsenic (As), silver (Ag), chromium (Cr), mercury (Hg), nickel (Ni) and total tin (Sn). Clams exposed to urban wastewaters from a township of 2,000 inhabitants had the highest levels on Ag with higher amounts of As, Cr, and Hg. These data corroborates previous findings (Gagnon *et al.*, 2004; Yang *et al.*, 2006) and confirms that the organisms were significantly more contaminated with some metals at least.

The morphological characteristics of clams collected at two impacted sites, and their corresponding reference sites, were examined (Table 2). Globally, no significant changes in the sex ratio were observed throughout the study sites (Kruskal-Wallis rank ANOVA, $p = 0.51$). The condition factor (clam weight/shell length) changed significantly according to site of collection. Condition factor was significantly reduced at CT+P site compared to its corresponding reference CT site. The condition factor was significantly lower at WT than at CT reference sites. The growth index (shell length/age) significantly changed across the sites but gender was not significant. The growth index was significantly reduced at the CT+P sites. Growth was significantly lower at WT than at CT sites. Correlation analysis revealed that CF was significantly correlated with growth index ($r = 0.26$; $p = 0.005$) and shell length ($r = 0.2$; $p = 0.03$). Gametogenic activity was determined by measuring the GSI. It was significantly affected by site location with no significant effects with clam's gender. Moreover, GSI increased significantly with clam age. The GSI was significantly reduced at site WT+P site and somewhat increased at site CT+P only when corrected for age differences. Clams from the WT reference site had higher GSIs than those from the CT reference site. A correlation analysis revealed that GSI was positively correlated with shell length ($r = 0.19$; $p = 0.04$), and age ($r = 0.21$; $p = 0.03$).

*Energy status*

Clam energy status in the gonad was determined by measuring temperature-dependent MET at 25 and 6 $^{o}$C, MDH at 25 and 6 $^{o}$C), and gonad lipid content (Figs 2A, B, C). MET at 25 $^{o}$C was significantly increased at CT+P site, with no change in MDH activity at the same temperature (Fig. 2A). However, MDH activity at 25 $^{o}$C was significantly higher at the WT reference site compared to the CT site. No significant differences in MET activity at 25 $^{o}$C were observed between the warm- and cold-water sites. At colder temperatures (6 $^{o}$C), there was no significant difference between the clean and polluted sites and both reference sites (Fig. 2B). The temperature sensitivity of MET activity ($MET_T$), but not MDH activity ($MDH_T$), was readily

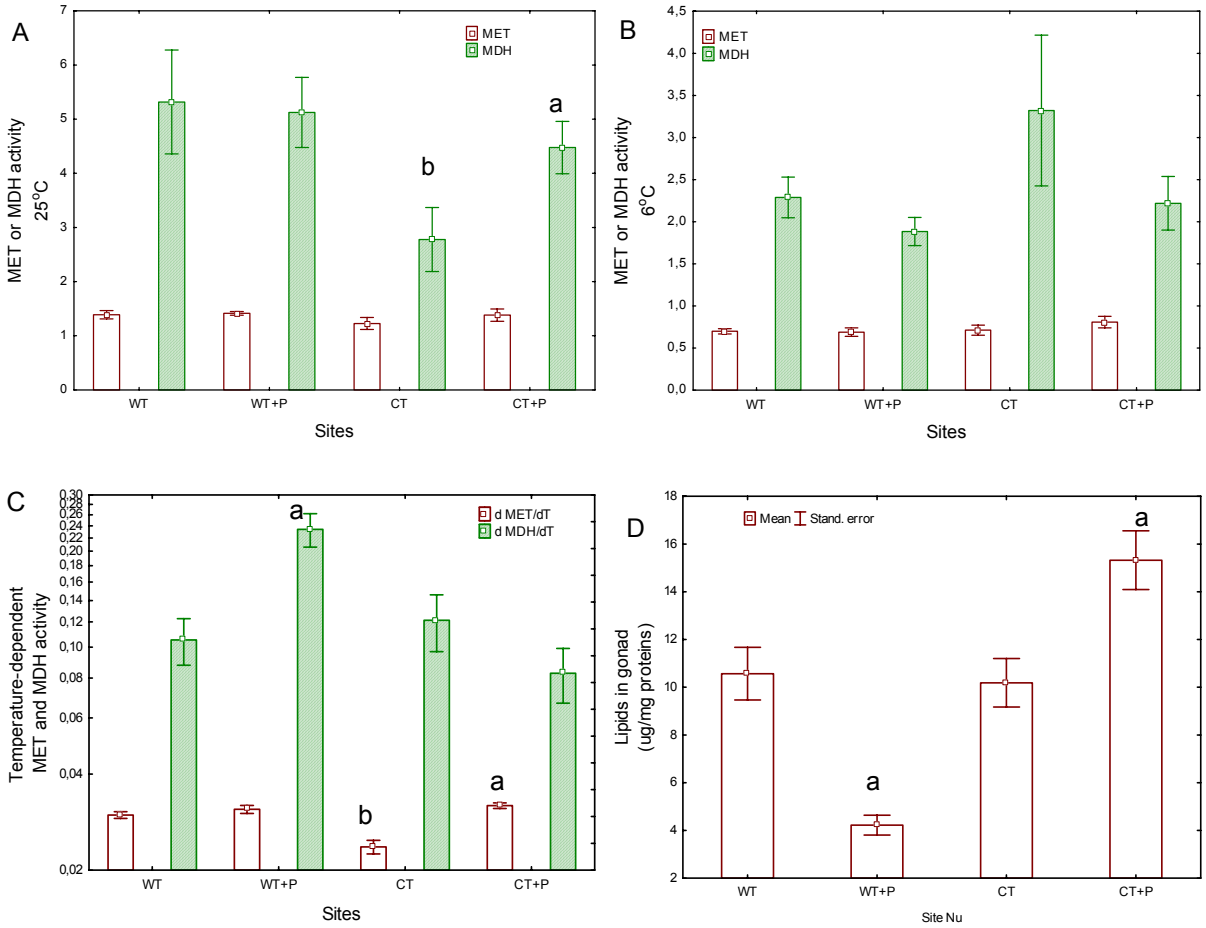


**Fig. 2** Temperature-dependent energy status in selected clam populations. The effects of clam length (size) and site location of *M. arenaria* clams are shown for MET and MDH at 25 °C (A), MET and MDH at 6 °C (B), temperature-dependent MET and MDH (C) and gonad lipid reserves (D). MET activity was expressed as the increase in dye reduction (520 nm)/min/mg proteins and MDH activity as the increase in NADH fluorescence/min/mg proteins. The letter 'a' indicates significance relative to each control sites (ASE or BAU) from the Saguenay Fjord and St. Lawrence Estuary. The letter 'b' denotes the significance between the cold-water and warm-water reference sites.

increased at the CT+P site relative to the corresponding CT reference site (Fig. 2C). $MET_T$ but not $MDH_T$ was significantly higher at the WT site compared to the CT site. Lipid levels in gonad were significantly reduced at the WT+P site while they were increased at the CT+P site (Fig. 2D). No significant change in lipid content was observed between the cold- and warm-water reference sites (i.e., CT and WT). Correlation analysis revealed that MET at 25 °C was correlated with growth index ($r = -0.3$; $p = 0.001$), MET at 6 °C ($r = 0.59$; $p < 0.001$), gonad lipids levels ($r = -0.24$; $p = 0.011$) and $MET_T$ ($r = 0.77$; $p < 0.001$). MET at 6 °C was significantly correlated with CF ($r = -0.21$; $p = 0.02$), growth index ($r = -0.34$; $p < 0.001$), MDH at 6 °C ($r = -0.25$; $p = 0.01$) and $MET_T$ ($r=0.53$; $p<0.001$). MDH at 25 °C was significantly correlated with GSI ($r=0.3$; $p=0.005$) and $MDH_T$ ($r = 0.84$; $p < 0.001$). MDH at 6 °C was significantly correlated with MET at 6 ° C ($r = -0.25$; $p = 0.01$), and $MET_T$ ($r = -0.40$; $p = 0.002$). $MDH_T$ was significantly correlated with GSI ($r = 0.38$; $p = 0.003$), CF (albeit marginally; $r = 0.23$; $p = 0.08$), gonad lipids ($r = -0.7$; $p < 0.001$) and MDH at 25 °C ($r = 0.84$; $p < 0.001$).

Because of the relationships between MDH activity and MET (energy expenditure) and gonadal lipids (energy reserves), the kinetic properties of this enzyme complex were characterized at cold and warm temperatures (Table 3). The enzyme has a higher affinity for $NAD^+$ than for malate, regardless of the water quality and thermal history of the site. The enzyme affinity at 25 °C for malate remains the same for both reference sites (WT and CT), but affinity increases in clams taken from the CT+P site. No changes were observed for $NAD^+$ when determined at 25 °C. However, at 6 °C, the enzyme affinity for both malate and $NAD^+$ increased at the CT site, whereas this was not found in clams at the CT+P site. Temperature had more influence on the maximum rate of reaction (Vmax) for malate and $NAD^+$. At cold temperatures (6 °C), Vmax was increased at the CT+P site but this was lost at

**Table 3** MDH* kinetic characteristics in *M. arenaria* clams

| | Substrate | Km 6°C | Vmax 6°C | Km 25°C | Vmax 25°C | Enzyme turnover 25°C (Vmax/Km) | Enzyme turnover 6°C (Vmax/Km) | Temperature sensitivity for enzyme affinity** | Temperature sensitivity for reaction rate** |
|---|---|---|---|---|---|---|---|---|---|
| **WT** | **Malate** | **9.7** | **0.2** | **28** | **0.6** | **0.02** | **0.02** | **18** | **0.4** |
| | *NAD* | *1.7* | *0.2* | *3* | *0.6* | 0.19 | 0.11 | 1.3 | 0.4 |
| **WT+P** | **Malate** | ***7.8*** | ***0.52*** | ***25*** | ***0.6*** | **0.02** | **0.07** | **17** | **0.06** |
| | *NAD* | *2.4* | *0.3* | *2.9* | *0.4* | 0.6 | 0.12 | 0.5 | 0.2 |
| **CT** | **Malate** | **5.4** | **0.2** | **27** | **0.5** | **0.02** | **0.03** | **22** | **0.3** |
| | *NAD* | *0.1* | *0.1* | *1.2* | *0.7* | 0.58 | 1 | 1.1 | 0.6 |
| **CT+P** | **Malate** | **8.2** | **0.5** | **12** | **0.4** | **0.03** | **0.06** | **4** | **-0.1** |
| | *NAD* | *2.6* | *0.3* | *1.5* | *0.5* | 0.34 | 0.11 | -1.1 | 0,2 |

* MDH activity: malate + $NAD^+$ → oxaloacetate + NADH
** Km or Vmax at 25 °C - Km or Vmax at 6 °C

higher temperatures (25 °C). The turnover number ($Vmax/K_M$) at 6 °C was readily increased in clams from the CT+P site. A concordance analysis revealed that lipid gonad levels were significantly correlated with $K_M$ and Vmax, turnover number for $NAD^+$ at 6 °C ($p < 0.001$), $K_M$ for $NAD^+$ at 25 °C ($p = 0.02$), $K_M$ and Vmax, turnover number for malate at 6 °C ($p < 0.001$) and the turnover number at 25 °C ($p < 0.001$). The GSI was significantly correlated with $K_M$ at 6 °C and 25 °C, Vmax at 25 °C and temperature-dependent $K_M$ and Vmax for malate. For $NAD^+$, the GSI was significantly correlated with $K_M$ and Vmax at 25 °C ($p = 0.02$), turnover number at 6 °C ($p = 0.02$), temperature-dependent $K_M$ and Vmax ($p = 0.02$). It appears therefore that gonad lipids levels are more dependent on the respective activities at 6 °C (which is a function of clam immersion in cold water), while GSI is related more to temperature dependence, which is favored at the warm-water sites (and exposed to surface air temperature). The temperature-dependent MDH activity and MDH activity were correlated (as expected) with temperature-dependent Vmax and Vmax at 25 °C, respectively.

Tissue damage was investigated by measuring the level of DNA strand breaks and LPO in gonad (Figs 3A, B). LPO tended to be higher at the polluted sites but the increase was only significant for WT+P site (ANOVA $p < 0.01$, least squares difference test). LPO at the CT site was significantly higher than the reference WT site in the Saguenay fjord. The levels of DNA strand breaks in the gonad were significantly higher at site WT+P site, reaching 1.9-fold the levels of the reference WT site.

In an attempt to determine the interaction between pollution and energy production and transfers to lipid reserves and gonadal mass in gonad tissues, a discriminate function analysis was performed (Fig. 4). The analysis revealed that sites were correctly classified in increasing order: CT+P site (68 %), WT site (68 %), WT+P site (70 %) and CT site (94 %). The following biomarkers were significantly correlated with the root 1 function of the X-axis, in descending order: GSI, gonad lipid, $MDH_T$ and LPO in gonad. For the second function of the Y-axis, the following biomarkers were significantly correlated, in descending order: $MET_T$, gonad lipid, condition factor and $MDH_T$. This indicates that temperature sensitivities of MDH and MET activity are key variables of site discrimination. At the colder sites (CT and CT+P), the effects of pollution displaced the data towards the warmer site (ASE). The effects of pollution at the warmer sites (ASE, ASJ) displaced the data away from the colder sites, indicating that pollution has "warming" effects but not the reverse (i.e., no cooling effects).

**Discussion**

Increased MET activity leads to increased activity in the malate shunt pathway, where malate is transferred to the cytosol and converted back to oxaloacetate by a cytosolic MDH. The process forms NADH, which can be used to support lipid synthesis. Indeed, a multiple regression analysis revealed that lipid gonad levels were positively related with MDH activity but temperature dependence was negatively correlated with gonad lipid levels. In fact, lower-temperature dependence (from cold-adapted organisms) of MDH, but with increased temperature sensitivity for MET, was related to increased lipid stores in gonad tissues. This suggests that increased mitochondrial energy production with a more temperature invariant MDH could favor the accumulation of lipids in gonad tissues. In turn, lipid synthesis calls for temperature-independent mechanisms (hence, the production of lipid precursors is maintained at cold temperatures). MDH activity was shown to be less sensitive to high temperature changes in cold-adapted bivalves (Fields

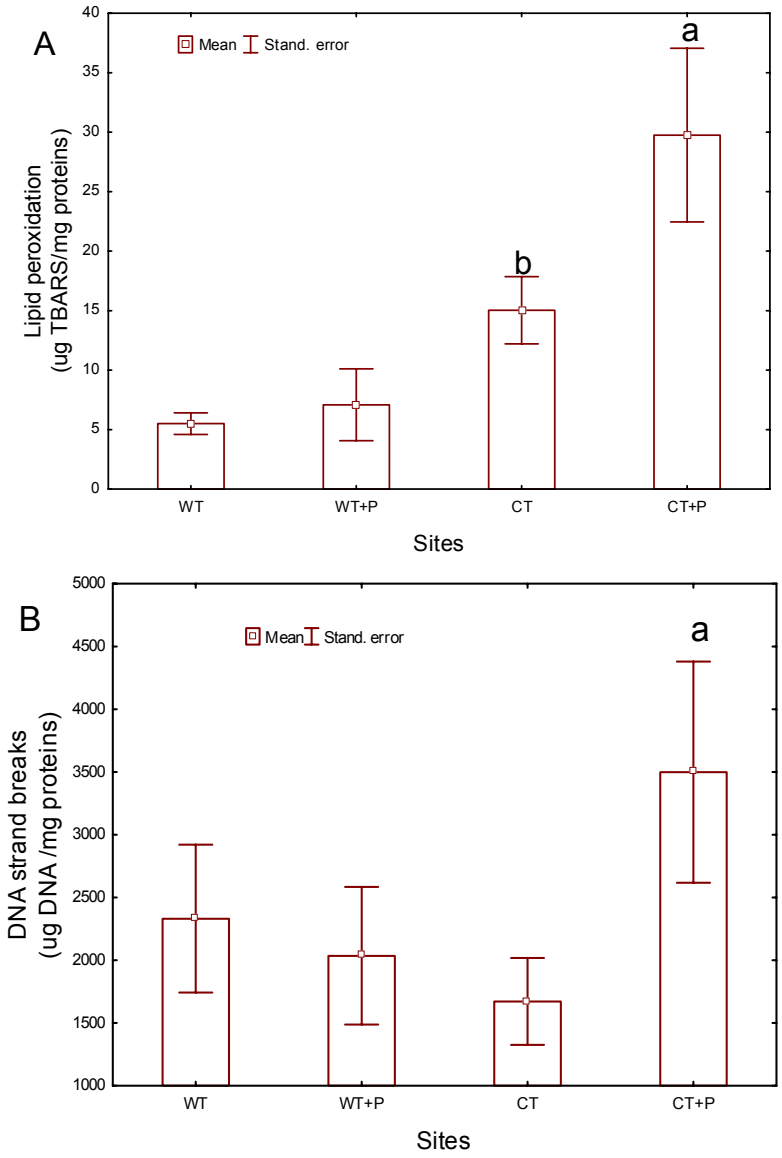


**Fig. 3** Tissue damage in *M. arenaria* clams collected at polluted sites. The levels of LPO (A) and DNA damage (B) were measured in the gonad. The letter 'a' indicates significance at each reference site (ASE or BAU).

*et al.*, 2006). The present study corroborated the pollution-induced increase in temperature sensitivity at the warmer site (WT+P) than at the colder site (CT+P). Increased sensitivity to temperature for MDH was associated with lower gonad lipids. It is noteworthy that these clams were fully ripped with no clear indication that spawning occurred. At the polluted cold-water site (CT+P), MET activity was more sensitive to temperature than MDH activity. The relative distance from the shore differed from the various study sites indicating that the clams were exposed to different emersion times. However, the relative distance from the shore was not significantly related with neither MDH activity (25 $^{o}$C) nor with $MDH_T$. However, the influence from the distance from the shore should be considered since it was shown to have an important influence on oxidative stress in clams (Gagné *et al.*, 2009).

In this study, increased MET activity and lipid stores in gonad were observed at the CT+P site. In intertidal clams subject to large variations in temperature (i.e., eurythermal), oxygen radical formation was positively related with temperature-controlled respiration rates (state 3 and 4) and negatively correlated with mitochondrial coupling (Abele *et al.*, 2002). Thus, wide temperature variations in clams from cold-adapted sites could lead to the formation of reactive oxygen species and LPO. However, no significant correlations were obtained with LPO in gonads and MET activity at low and high temperatures, suggesting that antioxidant mechanisms such as superoxide dismutase, glutathione peroxidase and catalase were able to scavenge the oxygen radicals resulting from mitochondrial uncoupling. Mitochondrial uncoupling from an increased temperature could be

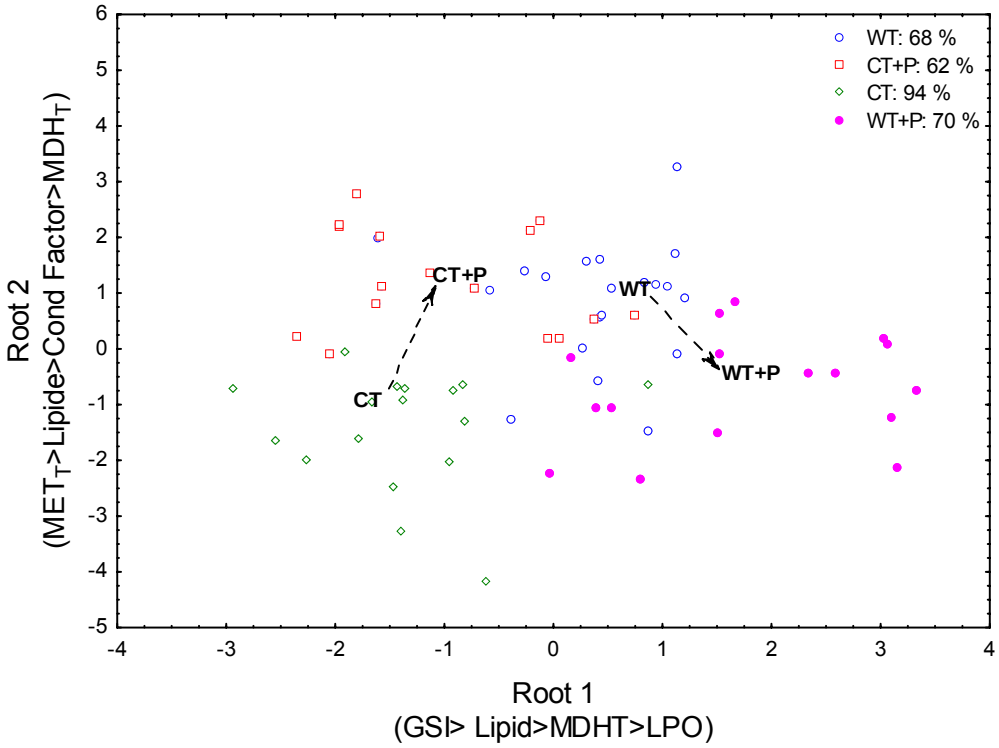


**Fig. 4** Discriminate function analysis of clam morphological status, energy status and tissue damage. A discriminate function analysis revealed that the sites were correctly assigned at > 62 %. The site names shown in bold correspond to the centre of gravity of the discriminate function. The dotted arrows indicate departures from the reference sites to the corresponding polluted sites (within the cold-water or polluted-water sites.

the result of changes in protein phosphorylation states in mitochondria of the clam *Mercenaria mercenaria* (Ulrich and Marsh, 2009). The investigators identified three proteins that followed temperature-specific phosphorylation patterns and suggested that a suite of protein kinases and phosphatases regulate mitochondrial physiology in response to temperature increases. Changes in protein phosphorylation in mitochondria show promise for use in investigating the interaction of temperature with pollution in the production of reactive oxygen species and oxidative stress.

The data in the present study show that pollution could contribute to temperature sensitivity in MDH activity, which is related to lower lipid content in the gonad and DNA strand breaks. This was consistent with discriminate function analysis of the biomarker data, which showed a shift in the centre of gravity of the discriminate functions of the polluted cold-water site toward the warmer sites (ASE and ASJ). It is noteworthy that the principal biomarkers with the highest factorial weights were temperature sensitivity in MDH and MET activities, gonad weight and lipid content. The physiological equivalency between low temperature dependence and cold adaptation observed here could perhaps favor the formation of DNA strand breaks in the gonad, rendering the organisms more susceptible to genotoxic compounds. This is consistent with the observation that DNA strand breaks were much higher at the polluted site in the cold-water sector (CT+P). However, this is difficult to ascertain at present since the genotoxicity could have been the result of relatively more contamination occurring at site CT+P relative to WT+P. In conclusion, clams from cold-water sites display less sensitivity to temperature but pollution increases temperature sensitivity in cytosolic MDH activity. Temperature-dependent MET at the polluted and cold-water site rises to levels identical to those of clams from the warmer sites, indicating that pollution alleviates cold-adaptation processes. Although clams had lower GSIs at the cold-water and polluted site, gonad lipid levels were higher at the polluted site than at the corresponding cold-water reference site indicating altered energy allocation towards gamete development. The impacts of pollution in clams from cold water site increased MDH activity at 25 $^{o}$C and MET temperature-dependence which would favor the formation of NADH in the cytosol for the production of lipids and steroidogenesis. The increase of MDH activity was related to a 2-fold increase of the enzyme's affinity constant towards malate at 25 $^{o}$C while the affinity at 6 $^{o}$C was somewhat lower. These changes were consistent with increased lipids stores at the CT-P site which were related with oxidative stress and genotoxicity. The impacts of pollution in clams from warm water sites seems to intervene rather at the mitochondria level (increased MET activity) which was related to decreased lipid stores in the gonad and this was independent from changes in GSI or

gonad maturity in the clams. However, temperature-dependent MDH activity was higher at the polluted site.

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**References**

Abele D, Heise K, Pörtner 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.

Bester MJ, Potgieter HC, Vermaak WJH. Cholate and pH reduce interference by SDS in the determination of DNA with Hoescht. Anal. Biochem. 223: 299-305, 1994.

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.

De Coen W, Janssen CR. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22: 1632-1641, 2003.

Dong Y, Somero GN. Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus *Lottia*): Differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions. J. Exp. Biol. 212: 169-177, 2009.

Fields PA, Rudomin EL, Somero GN. Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): Sequence-function linkages and correlations with biogeographic distribution. J. Exp. Biol. 209: 656-667, 2006.

Gagné F, Blaise C, Pellerin J, Fournier M, Gagnon C, Sherry J, Talbot A. Impacts of pollution in feral *Mya arenaria* populations: the effects of clam bed distance from the shore. Sci Total Environ. 407, 5844-5854, 2009.

Gagné F, Blaise C, André C, Pellerin J. Implication of site quality on mitochondrial electron transport activity and its interaction with temperature in feral *Mya arenaria* clams from the Saguenay Fjord. Environ. Res. 103: 238-246, 2007.

Gagné F, Blaise C, Pellerin J, Fournier M. Biomarker studies of the soft-shell clam (*Mya arenaria*) in the Saguenay Fjord: Research results (1997-2006). Revue Science de l'Eau, 22 : 253-259, 2008a.

Gagné F, Blaise C, Pellerin J, Fournier M, Durand MJ, Talbot A. Relationships between intertidal clam population and health status of the soft-shell clam *Mya arenaria* in the St. Lawrence Estuary and Saguenay Fjord (Québec, Canada). Environ. Internat. 34: 30-42, 2008b.

Gagnon F, Tremblay T, Rouette J, Cartier JF. Chemical risks associated with consumption of shellfish harvested on the north shore of the St. Lawrence River's lower estuary. Environ. Health Perspect. 112: 883-888, 2004.

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

Olive PL. DNA precipitation assay: A rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11: 487-495, 1988.

Pellerin J, Fournier M, Gauthier-Clerc S, Blaise C, Florent G, Amiard J-C, Gagné, F. How is the health status of *Mya arenaria* in the Saguenay fjord? A statetement from a 14-year survey**.** Rev. Sci. de l'Eau 22 : 271-289, 2009.

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

Stryer, L. Pentose phosphate pathway and gluconeogeneis. In: Biochemistry 4th edition, Chap. 22, edited by WH Freeman and Company, New York, USA, pp 559-579, 1995.

Ulrich PN, Marsh AG. Thermal sensitivity of mitochondrial respiration efficiency and protein phosphorylation in the clam *Mercenaria mercenaria*. Mar. Biotechnol. (NY) 11: 608-618, 2009.

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, USA, pp 127-150, 1987.

Yang R, Zhou Q, Jiang G. Butyltin accumulation in the marine clam *Mya arenaria*: An evaluation of its suitability for monitoring butyltin pollution. Chemosphere 63: 1-8, 2006.