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DNA integrity assessment in hemocytes of soft-shell clams (*Mya arenaria*) in the Saguenay Fjord (Québec, Canada)

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Abstract The purpose of this study was to examine the effects of pollution on DNA integrity in the feral soft-shell clam (*Mya arenaria*) in the Saguenay Fjord. Intertidal clams were collected downstream and upstream of the fjord at sites under anthropogenic pollution. DNA integrity was assessed by following changes in single- and double-stranded breaks, variation in DNA content and micro-nuclei (MN) incidence in hemocytes. The results revealed that clams collected at polluted sites had reduced DNA strand breaks (lower DNA repair activity), increased DNA content variation and MN frequency in hemocytes. The data revealed that DNA content variation was closely related to MN frequency and negatively with DNA strand breaks formation. Water conductivity was also related to reduced MN frequency and DNA content variation, indicating that, in addition to the effects of pollution, the gradual dilution of saltwater could compromise mussel health.

Keywords DNA strand breaks · DNA content variation · Micro-nuclei · Hemocytes · Flow cytometry

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Introduction

The Saguenay Fjord is an important tributary of the St. Lawrence Estuary in Quebec, Canada. This fjord is under the influence of important commercial (industrial and touristic) boat traffic as a link between the St. Lawrence River and the upstream populated area of the city of Saguenay (estimated population of 160 000). This brackish environment is affected by numerous contaminations linked to industrial (discharges from aluminum and pulp and paper plants), domestic (sewage effluents from surrounding municipalities) and portuary activities (pleasure and commercial boat traffic) (Fortin and Pelletier 1995; Pelletier and Canuel 1988; Gagné et al., 2009). The usual toxic organic and inorganic compounds (heavy metals, aromatic hydrocarbons, organotin compounds) were detected in sediments and waters sampled along the fjord (Gagné et al. 2003; Roy et al. 2000; Viglino et al. 2004, 2006).

The impacts of pollution on local populations of the endobenthic clam *Mya arenaria* have been the subject of intense investigations over the last 15 years (Gagné et al. 2008a, b; Pellerin et al. 2009). These studies determined that the clams were under many pathophysiological stresses, especially those located in the vicinity of marinas and urban areas: increased heavy metal detoxication activity (metallothioneins), biotransformation of organic compounds (mixed function oxidase, glutathione *S*-transferase activities), municipal-effluent-induced estrogenicity (increase in vitellogenin), masculinization effects by organotins (decreased production of vitellogenin and increased number of males), immunocompetence (altered phagocytosis activity at polluted sites), oxidative damage (lipid peroxidation) and changes in DNA strand breaks. Moreover, clams are well known to accumulate many harmful environmental pollutants, such as organotin compounds, arsenic, polyaromatic hydrocarbons

and polychlorinated biphenyls from water and sediments (Blaise et al. 2002; Gagnon et al. 2004; Ma et al. 2009; White et al. 1997; Yang et al. 2006). This accumulation could even render clam tissue extracts genotoxic in bacterial-based genotoxicity tests (White et al. 1997; Coughlan et al. 2002). Genotoxicity could be assessed by different means, which can give insights on the severity of genotoxic effects or damage. DNA strands breaks assays are based on the selective isolation of single and double DNA strand breaks from protein-bound genomic DNA in cells (Olive 1988). The amount of DNA strand breaks was shown to be a function of the formation of DNA adducts, or modifications such as the increase in the number of alkali-labile sites (abasic sites) and the rate of DNA repair activity which consists of the excision of large DNA strands and the replacement of nucleic bases on the genomic DNA backbone. In organisms exposed to genotoxic compounds, the levels of DNA strand breaks could increase initially as the number of strand breaks are formed during repair, but this response could be followed by a gradual decrease in DNA strand breaks as the repair mechanism becomes saturated and depleted. In previous studies, increases in the DNA strand breaks were sometimes observed in clams collected at polluted sites in the St. Lawrence Estuary, but DNA strand breaks were decreased at more heavily impacted sites (Gagné et al. 2006). These sites were large marinas with a history of organotin contamination which induced oxidative stress in clams. Oxidative stress represents another pathway leading to tissue damage such as lipid peroxidation (LPO) and oxidative DNA adducts (Lesser 2006). At the genome level, variation in DNA content could result when there is loss of chromosomal fragments and aberrations (Bihari et al. 2003; Neuparth et al. 2006). The loss of large DNA fragments or parts of chromosomes will result in cells having less DNA content and more DNA content in diploid cells, hence increased variation of the DNA content in cells during analysis by flow cytometry. At the histological level, very large DNA chromosomal fragments could form visible micro-nuclei (MN) that are distinct from the cell's nuclei and represent a severe form of DNA damage or genotoxicity. It is hypothesized that in cells with compromised DNA repair activity, an increase in DNA content variation and the formation of MN would be observed. It was also proposed that variation in DNA content could serve as an indirect (alternative) measure of MN formation, which depends on microscopic observation that can be labour-intensive and time-consuming.

In this context, a spatial survey was carried out at one reference (clean) site and four polluted sites in the Saguenay Fjord, to examine the severity of DNA damage in local clam populations. The severity of DNA damage was determined by following changes in single- and double-stranded DNA strands based on alkaline fractionation of genomic DNA, DNA content variation (determined by flow cytometry), the number of MN in hemocytes and oxidative stress (LPO) in soft-shell clams. Relationships between these three levels of DNA alterations were examined to predict the severity of pollution-induced DNA damage in wildlife.

Materials and methods

Intertidal clam collection

Soft-shell clams were collected (n=12 individuals per site) by hand in mud flats during the morning low tide from five sites in the Saguenay Fjord (Fig. 1). The clams were collected in two consecutive days. The first site, Baie Éternité (BE), was located 50 km upstream of the estuary. It supports a moderate-size marina (capacity of ten to 20 ships of<10 m in length) for pleasure and tourist boating activities. Anse de Saint-Étienne (ASE) was the reference site, located at the south shore 20 km upstream of the estuary. Anse-aux-Érables (AE) was the farthest upstream site in the fjord (70 km upstream of the estuary), located downstream of aluminum smelters and pulp and paper mills, and of four cities that discharge municipal wastewaters. Anse Saint-Jean (ASJ) was the site exposed to an urban wastewater effluent, with minimal treatment (sieving), from about 2000 residents, located 40 km upstream of the estuary. Anse du Petit Saguenay (PS) was located 32 km upstream on the south shore of the estuary and harbours (marinas used for seasonal and pleasure boating activities).

General clam health status assessments

Age was estimated by counting the number of major grooves (annual rings) on shells. The clam weight and longitudinal shell length were also measured. A 1-mL fraction of the hemolymph was collected with aid of a syringe at the anterior adductor muscle, for cytogenetic and DNA content variation evaluations as described in the "DNA content variation assessment" section. The weights of clams and soft tissues and the shell length were also determined in the laboratory, to determine soft tissue weights for the gonado-somatic index (wet weight gonad/wet weight soft tissues), and condition factor (clam weight/shell length). Lipid peroxidation (LPO) was determined in gonad homogenates by the thiobarbituric acid method, which measures the production of malonaldehyde (Wills 1987). Gonad tissues were homogenized in 140 mM NaCl containing 1 mM EDTA, 1 mM dithiothreitol and 25 mM Hepes–NaOH, pH 7.4, at 4°C, using a Teflon pestle tissue grinder (five passes). A 25-μL volume of the homogenate was collected, mixed with 75 μL of water, 50 μL of thiobarbituric acid (0.7 % in water) and 100 μL of 1 mM $FeCl_3$ in 10 % trichloroacetic acid. The mixture was heated at 75°C for 15 min and cooled

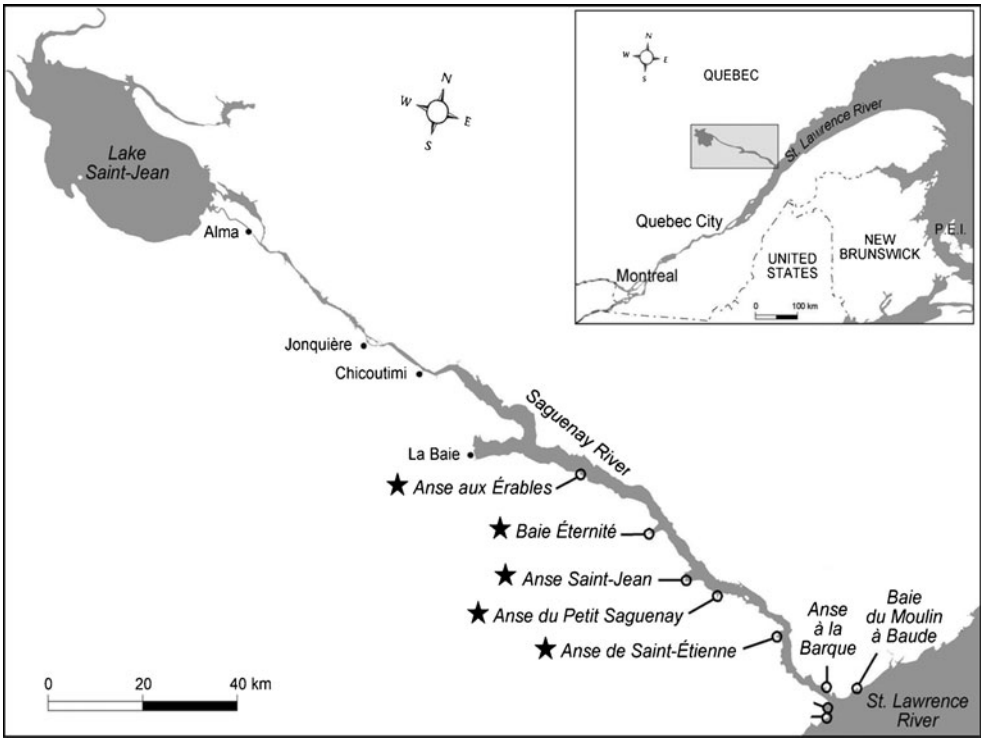

Fig. 1 Map of the study area. Clams (*Mya arenaria*) sampled in five different sites following a downstream–upstream distribution: Anse-aux-Érables (ASE), Baie Éternité, Anse Saint-Jean, Anse du Petit Saguenay, Anse Saint-Étienne in the Saguenay Fjord. ASE is considered the reference site with no direct input of pollution and is the most upstream site (closest to the estuary). The stars indicate the sites where clams were collected

at room temperature before fluorescence analysis. Blanks and external standards of tetramethoxypropane were prepared in the presence of the homogenization buffer for calibration. Fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence microplate reader (Biotek, Winooski VT, USA). Because the reagent could react with other aldehydes, results were expressed as micrograms of thiobarbituric acid reactants (TBARS)×milligram^{-1} of total protein. Total proteins in the homogenate were determined using the principle of protein–dye binding (Bradford 1976).

DNA strand break concentration assessment

DNA strand break concentrations were determined by the alkaline precipitation assay developed by Olive (1988) with fluorescence quantization of DNA strand breaks in the presence of detergents and alkaline pH (Bester et al. 1994). The assay principle is based on the potassium-dodecylsulphate precipitation of protein-bound genomic DNA, which leaves protein-free DNA strand breaks in the supernatant. A volume of 250 μL of the hemolymph was centrifuged at 1000×*g* for 5 min, and the cell pellet was resuspended in 100 μL phosphate buffered saline (140 mM NaCl, 5 mM KH_2PO_4, pH 74). Cell density was measured by the absorbance at 600 nm. A 50-μL volume of the cell suspension was mixed with 250 μL of 1 % SDS containing 40 mM NaOH and 10 mM EDTA (pH 11–12) and 250 μL 0.1 M KCl. The mixture was incubated at 60°C for 15 min, cooled on ice for 5 min and centrifuged at 8000×*g* for 5 min at 2°C. DNA (strands) in the supernatant was measured using the Hoescht dye (100 ng/mL) in the conditions described by Bester et al. (1994) to compensate for interference by the detergent SDS. Fluorescence was measured at 350 nm excitation and 450 nm emission in a microplate fluorescence reader (Fluorite 1000, Dynatech Laboratories, VA, USA). The results were expressed as micrograms of DNA/cell density. Calibration was achieved with salmon sperm DNA (Sigma-Aldrich, USA).

DNA content variation assessment

The variation in DNA content was determined by flow cytometry using propidium iodide for staining DNA (Ciudad et al. 2002). A volume of 250 μL of the hemolymph from each individual was mixed with 750 μL of 3 % formaldehyde containing 25 g NaCl/L, 10 mM EDTA and 10 mM Hepes, pH 7.5, and stored at 4°C for transport to the laboratory. The fixed cell suspension was then centrifuged at 1000×*g* for 5 min and re-suspended in phosphate-buffered saline containing 25 μg/mL propidium iodide and 20 μg/mL ribonuclease A and analyzed by flow cytometry after standing for 60 min at room temperature to allow for DNA staining (and digestion of RNA). Using an argon-laser flow cytometer (Becton, Dickinson and Company, Franklin Lakes NJ, USA), 20 000 events (cells) were collected for analysis. The cells were first gated using the forward-light scatter (cell size) and orthogonal side scatter (cell granulosity or intracellular complexity index). A second gate was performed with cell size as the ordinate axis and the DNA

content as the *x* axis. The mean DNA levels of diploid and tetraploid cells were determined where the coefficient of variation of DNA for diploid cells was calculated as a measure of genomic DNA integrity. The mitotic index was estimated by determining the number of tetraploid cell (4 N) over the number of diploid (2 N) cells: 4 N cell number/2 N cell number. We found no evidence of either aneuploid or tetraploid dividing (leukemic cells) cells in the hemocytes (no traces of 8 N cells).

Micro-nuclei (MN) abundance assessment

A 300-μL volume of the hemolymph was smeared on poly-L-lysine-treated microscope glass slides, incubated for 1 h in a saturated humidity atmosphere. After this time, the supernatant was removed, and 300 μL of fixative solution was added (3 % formaldehyde in 25 g NaCl/L containing 10 mM EDTA and 10 mM Hepes, pH 7.5). The fixative layer was removed, and 300 μL of 90 % methanol was added and incubated on ice for 30 min. Afterwards, the excess methanol was removed and the slides were dried, and ten drops of Giemsa staining media (Sigma Chemical Company, USA) were added for 30 min. The slides were then washed in distilled water and were ready for microscopical examination at 400× enlargement. The number of cells with MN in the vicinity of the nuclei was counted. In parallel, the number of tetraploid cells was counted (considered an index of cell mitosis). The observation revealed that the number of hemocytes undergoing mitosis was relatively constant, ranging between 0.01 and 0.2 % throughout the study sites.

Data analysis

At each site, 12 clams were collected. A descriptive analysis of the data was used to calculate the mean value and the standard error for the different parameters. The normality of the data distribution was verified using a Levene's test. The data were log-transformed when significant deviation from normality occurred. Results were statistically analyzed by ANOVA followed by a Student *t* test to compare with the reference site (ASE). In some instances, analysis of covariance was performed in order to determine some interaction between sites and biomarkers that revealed significant correlations between each other. Pearson correlation analysis was also performed between the variables: age, condition factor, DNA stand breaks, DNA content variation, incidence of micronuclei, gonad LPO, mitotic index, hemocyte density, water conductivity and distance from the shore. Significance was set at $p<0.05$, and all tests were performed using the Statistica software package (version 8.0, France).

Results

The pH and the conductivity of the water were measured at low tide during the collection of the clams (Table 1). The mean conductivity of water samples was 11.47±3.2 mS cm^{-1}, indicating that conductivity varies in the range of 30 %. ASJ and ASE had the lowest (8.1) and highest (16.46) conductivity values, respectively. The water pH was more constant (less than 10 % variation), with a mean pH value of 8.3±0.62. BE and ASE had the highest (9.04) and lowest (7.48) pH values, respectively. Conductivity and pH values were not correlated with each other, but water pH was negatively correlated with the distance from the shore ($r=-0.97$; $p=0.004$). The mean age of the sampled clams differed significantly between sites with clams at BE significantly ($p<0.01$) younger (7.5 years) than those from the reference site (9 years). A negative correlation was found between the mean age value of the clams with water pH ($r=-0.90$; $p=0.04$) and a positive correlation of mean age values with clam bed distances from the shore ($r=0.89$; $p=0.04$). Water conductivity decreased with the upstream distance in the fjord ($r=0.67$; $p<0.001$; see Table 2). Clam beds that were farther from the shoreline tended to have

Table 1 Physico-chemical and clam characteristics assessed in five different sites (AE: Anse-aux-Érables; BE: Baie Éternité; ASJ: Anse Saint-Jean; PS: Anse du Petit Saguenay; ASE: Anse Saint-Étienne) of Saguenay Fjord

Sites	Conductivity (mS cm^{-1})	pH	Condition factor (g/cm)	Age (years)	Hemocyte density	Gonad LPO (μg TBA/mg proteins)
ASE	16.5	7.5	0.105±0.002[a]	9.6±0.4	0.39±0.02	6.1±0.2
PS	11.5	8	0.11±0.002	8.3±0.3[b]	0.27±0.01[b]	3.6±0.3
ASJ	8.1	8.1	0.104±0.003	8.5±0.3[b]	0.34±0.04	10±1[b]
BE	11.9	9	0.094±0.002[b]	7.2±0.2[b]	0.22±0.03[b]	7.4±0.6
AE	9.5	8.7	0.109±0.002	7.8±0.2[b]	0.33±0.02	46±3[b]

[a] The data are expressed as the mean with standard error

[b] Statistically significant from the downstream reference site ASE

Table 2 Correlation analysis of the various parameters

	DNA breaks	MN	DNA variation	Mitotic activity[a]	Condition factor	Hemocyte density	Age	LPO	Shoreline distance	Water cond.	Distance in the fjord
DNA breaks	1	**−0.54, $p<0.001$**	**−0.79, $p<0.001$**	−0.25, $p>0.05$	**−0.37, $p<0.01$**	−0.04, $p>0.05$	0.03, $p>0.05$	−0.16, $p>0.05$	**0.45, $p<0.001$**	**0.73, $p<0.001$**	−0.15, $p>0.05$
MN		1	**0.51, $p<0.001$**	0.24, $p=0.08$	**0.24, $p=0.05$**	−0.13, $p>0.05$	−0.02, $p>0.5$	**0.40, $p<0.001$**	**−0.41, $p<0.001$**	**−0.46, $p<0.001$**	**0.36, $p<0.01$**
DNA variation			1	0.27, $p=0.06$	**0.32, $p<0.05$**	−0.19, $p>0.05$	0.07, $p>0.05$	0.10, $p>0.05$	**−0.32, $p<0.01$**	**−0.66, $p<0.001$**	0.12, $p>0.05$
Mitotic activity[a]				1	0.01, $p>0.05$	−0.03, $p>0.05$	0.12, $p>0.05$	−0.06, $p>0.05$	−0.13, $p>0.05$	−0.14, $p>0.05$	−0.06, $p>0.05$
Condition factor					1	0.22, $p>0.05$	0.23, $p>0.05$	0.14, $p>0.05$	0.1, $p>0.05$	−0.07, $p>0.05$	−0.15, $p>0.05$
Hemocyte density						1	**0.38, $p<0.05$**	0.1, $p>0.05$	**0.38, $p<0.01$**	0.12, $p>0.05$	−0.21, $p>0.05$
Age							1	−0.18, $p>0.05$	**0.56, $p<0.001$**	**0.35, $p<0.01$**	**−0.51, $p<0.001$**
LPO								1	**−0.24, $p<0.05$**	**−0.36, $p<0.01$**	**0.67, $p<0.001$**
Shoreline distance									1	**0.74, $p<0.001$**	**−0.71, $p<0.001$**
Water cond.										1	**−0.67, $p<0.001$**

The significant correlations are highlighted in bold

[a] Mitotic activity was determined by the amount of tetraploid/diploid DNA in hemocytes

higher water conductivities ($r=0.74$; $p<0.001$) than those closer to the shoreline. The distances of the clam beds were also closer to the shoreline as the upstream distance in the fjord increased ($r=-0.71$; $p<0.001$). This suggests that clams that spend more time under water (i.e., farther from the shoreline) are more exposed to sea water (high conductivity).

First, the health status of the clams was determined by following changes in condition factor, mean age, hemocyte density (absorbance at 600 nm) and lipid peroxidation (LPO) (Table 1). The condition factor was significantly lower only at the BE site. The clam age was significantly decreased for all study sites. Correlation analysis revealed that clam age was significantly correlated with shoreline distance ($r=0.56$; $p<0.001$), conductivity ($r=0.35$; $p<0.01$) and upstream distance from ASE ($r=-0.51$; $p<0.001$). Hemocyte density (absorbance at 600 nm) was significantly reduced at the PS and BE sites. Hemocyte density was significantly correlated with age ($r=0.38$; $p<0.05$) and shoreline distance ($r=0.38$; $p<0.01$). LPO was significantly induced at the ASJ and AE sites and was significantly correlated with shoreline distance ($r=-0.24$; $p<0.05$), water conductivity ($r=-0.36$; $p<0.01$) and upstream distance from ASE ($r=0.67$; $p<0.001$).

DNA status was measured by following changes in DNA strand breaks, variation in DNA content in diploid hemocytes, mitotic index and incidence of MN. DNA strand breaks were significantly reduced at all polluted sites downstream of the reference ASE, with the exception of BE (Fig. 2). Correlation analysis revealed that DNA strand breaks were significantly correlated with shoreline distance ($r=0.45$; $p<0.001$) and water conductivity ($r=0.73$; $p<0.001$), suggesting that pollution was not the only contributor to reduced DNA strand breaks. Nevertheless, analysis of covariance revealed that site-specific effects remained significant when shoreline distance and conductivity were included as covariates. Variations in DNA content in diploid hemocytes were also determined (Fig. 3). DNA content variation was significantly induced at the upstream polluted sites in respect to ASE, except for BE. Correlation analysis revealed that DNA content variation was significantly correlated with DNA strand breaks ($r=-0.79$; $p<0.001$), condition factor ($r=0.32$; $p<0.05$), shoreline distance ($r=-0.32$; $p<0.05$) and water conductivity ($r=-0.66$; $p<0.001$). The amount of MN and the proportion of tetraploid cells were determined (Fig. 4a, b). The proportion of micro-nucleated cells was significantly increased at the polluted site, with the exception of BE. The proportion of tetraploid cells was measured as a measure of cell division, which could influence the incidence of MN in dividing cells. There were no significant changes in the proportion of tetraploid cells. Correlation analysis revealed that the proportion of micronucleated cells was significantly correlated with DNA

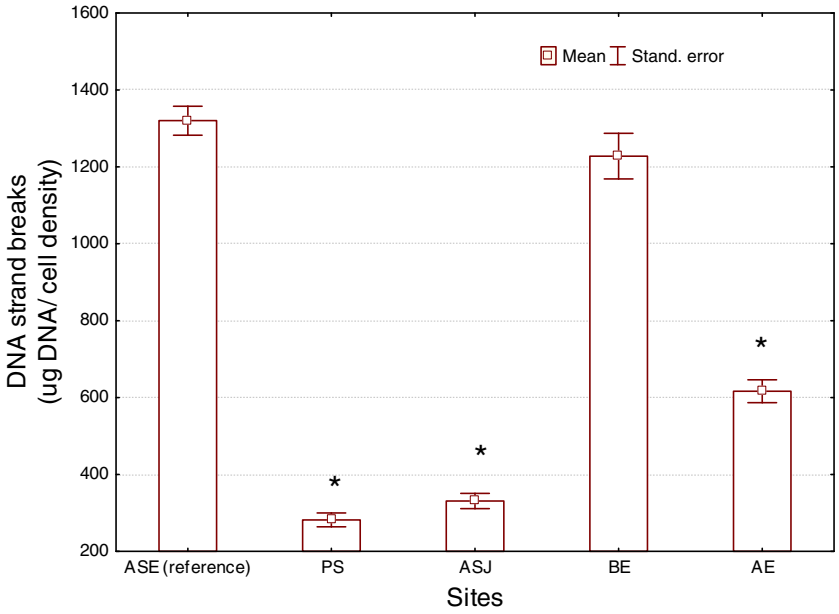

Fig. 2 DNA strand break concentrations in hemocyte solution from soft-shell clams (*Mya arenaria*) sampled in five different sites (*AE* Anse-aux-Érables, *BE* Baie Éternité, *ASJ* Anse Saint-Jean, *PS* Anse du Petit Saguenay, *ASE* Anse Saint-Étienne) of Saguenay Fjord. * indicates significant difference from the reference site ASE

strand breaks (r=−0.54; p<0.001), DNA content variation (r=0.51; p<0.001), condition factor (r=0.24; p<0.05), LPO (r=0.40; p<0.001), shoreline distance (r=−0.41; p<0.001), water conductivity (r=−046; p<0.001) and distance in the fjord (r=0.36; p<0.01). Analysis of covariance revealed that site-specific effects were the most significant variable when compared with shoreline distance and water conductivity metrics.

Discussion

The downstream reference site ASE revealed increased levels in DNA strand breaks in respect to the polluted upstream sites in the Saguenay Fjord. This was also observed in a previous study with soft-shell clams in the same area (Blaise et al. 2002). Water conductivity was somewhat higher than the downstream reference site (16–17 mS cm^{-1} compared to circa 10 mS cm^{-1} at the polluted and upstream sites) being closer to the estuary. This suggests that this variable has an influence on the baseline levels of DNA strand breaks data. Water conductivity was positively correlated with DNA strand breaks, mean age of clams and shoreline distance, but negatively correlated with MN frequency, DNA content variation and LPO. The increase in DNA strand breaks with conductivity suggests that higher salinity increases the number of alkali-labile sites in DNA. In a previous study with *Mytilus galloprovincialis*, the basal levels of DNA strand

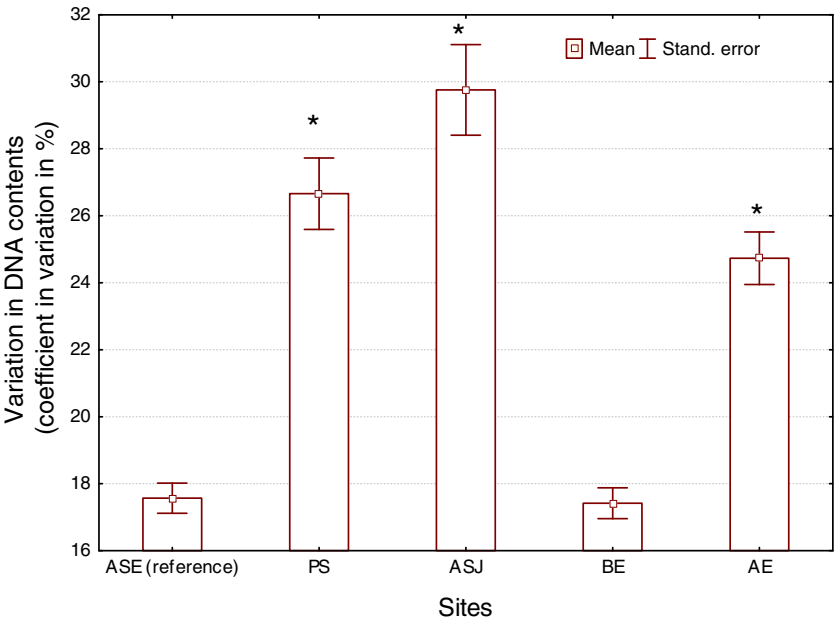

Fig. 3 Change in DNA content variation assessed in hemocyte solution from soft-shell clams (*Mya arenaria*) sampled in five different sites (*AE* Anse-aux-Érables, *BE* Baie Éternité, *ASJ* Anse Saint-Jean, *PS* Anse du Petit Saguenay, *ASE* Anse Saint-Étienne) of Saguenay Fjord. * indicates significant difference from the reference site ASE

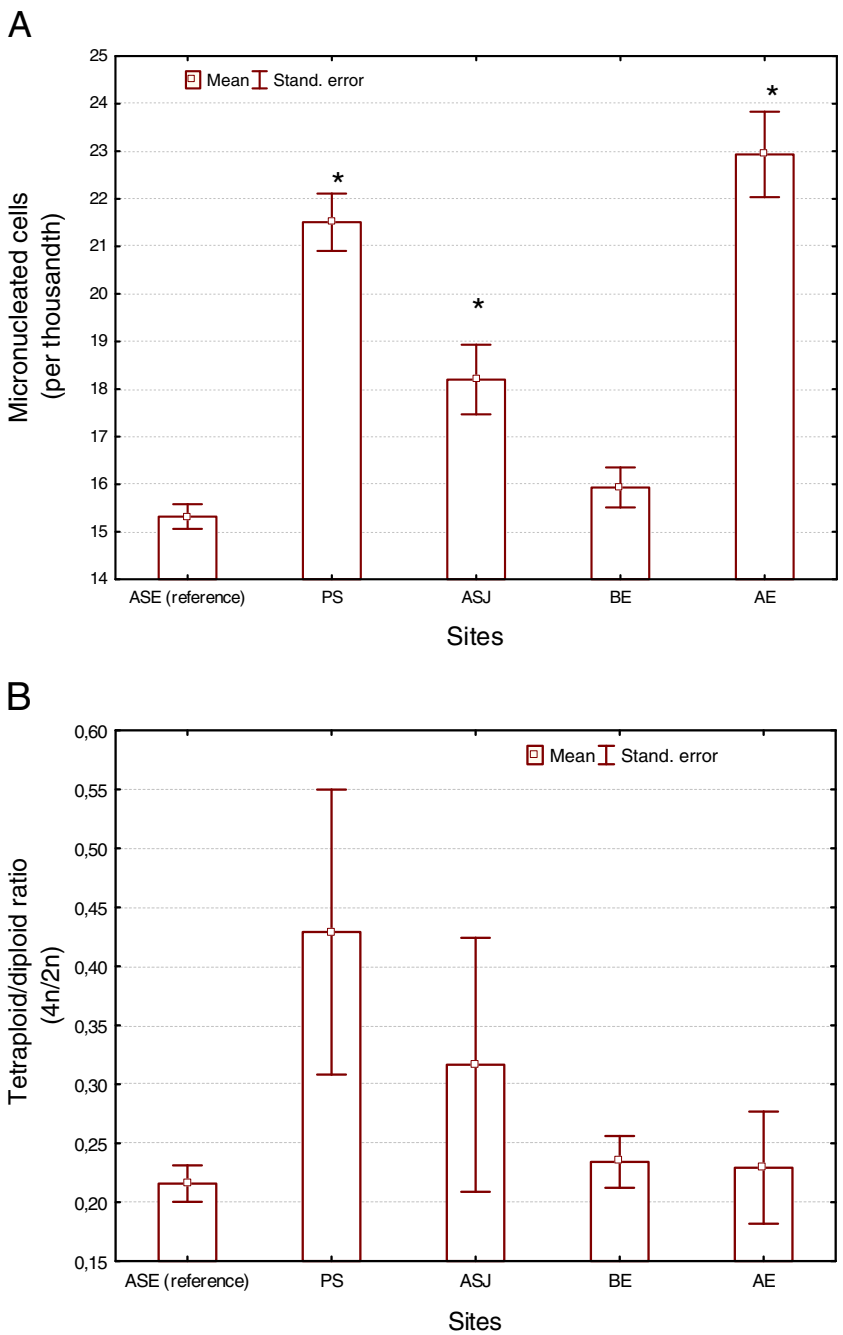

Fig. 4 Relative levels of hemocytes with micro-nuclei from soft-shell clams (*Mya arenaria*) sampled in five different sites (*AE* Anse-aux-Érables, *BE* Baie Éternité, *ASJ* Anse Saint-Jean, *PS* Anse du Petit Saguenay, *ASE* Anse Saint-Étienne) of Saguenay Fjord. * indicates significant difference from the reference site ASE

breaks were attributed to a larger number of alkali-labile sites from more condensed DNA in higher salinities (Machella et al. 2006). In a previous study in the Baltic and North Sea, coastal salinity variations were not significantly related with the incidence of MN in mussels (ICES 2011). Thus, clams living closer to the shoreline are exposed longer to air temperatures and lower conductivity which can stress DNA integrity especially in summer times. In the attempt to tease out whether conductivity could account of increased DNA damage in clams found at distance close to the shoreline, analysis of covariance revealed that conductivity (F=80; p<0.001) was more important than shoreline (F=19; p<0.001) and site location (F=20; p<0.001) although all these factors remained significant. This suggests that conductivity changes would be one of drivers of change in DNA status in clams. However, in an estuary where many inputs of soft water could lead to more important changes in water conductivity (i.e., 8.1–16.5 mS cm^{-1}), effects on DNA condensation could be observed. In the current study, the previously described effect of temperature on the proportion of MN (Burgeot et al. 1996) is not relevant, because no significant changes of the water temperature were observed at the sampling sites. Although the mean clam age population was associated with water conductivity, the mean age

was not related to genotoxicity parameters in the present study. This suggests that other environmental factors prevailed given the age interval of the clams (between 6 and 12 years of age). Notwithstanding the above, clams from polluted sites had decreased DNA strand breaks with increased DNA content variation and MN frequency. Reduced DNA strand breaks could be the result of decreased DNA repair activity which can occur with age, which would lead to increased cytogenetic damage. This was corroborated in the present study by the significant and negative correlation between DNA strand breaks and MN frequency ($r=-0.54$; $p<0.001$). Because the basal levels of DNA strand breaks could be either induced or decreased by variation in the rate of DNA repair activity, careful interpretation of DNA strand breaks with habitat characteristics and pollution is warranted.

In cells, DNA may be affected by both natural and pollution-induced modifications, such as base changes, formation of alkali-sensitive abasic sites, DNA adduct induction and decreased repair efficiency (Favier 2003). As the frequency and number of DNA alterations increases in polluted environments, low DNA strand break levels could be related to either repair mechanism inhibition (Blaise et al. 2002; Zhitkovich and Costa 1992) or clastogenic effects. As with the comet assay test, the tail length moment, indicative of DNA breakage, depends on the balance between DNA damage and repair mechanisms (Bolognesi et al. 2004a). It was suggested that the flow cytometric determination of DNA content variation in diploid cells could serve as quick surrogate to the microscope counting of micro-nucleated cells. In the present study, the frequency in MN was moderately correlated with DNA content variation ($r=0.51$; $p<0.001$), which supports this contention—although the relationship explained 51 % of the MN variation. The variation in DNA content and the MN frequency were seemingly not affected by the proliferation status of the cells which was determined by the mitotic index. The change in DNA content variation could also be the result of other clastogenic effects, such as abnormal chromosome separation during division (aneugeny) and loss of DNA fragments during the formation of chromosome exchange aberrations (Custer et al. 2000; Neuparth et al. 2006). The high number of MN reported in hemocytes of clams collected in contaminated sites is related to an increase in the abundance of cells with abnormal DNA content caused by underlying genotoxic disturbances. This result is consistent with the work by Coughlan et al. (2002), who reported an increase in DNA damage in clams exposed to contaminated sediments. Štambuk et al. (2009) also noticed an increase in DNA damage in caged mussels placed in highly polluted rivers (Štambuk et al. 2009). In the present study, the increase in DNA content variation would support the hypothesis that both clastogenic and aneugenic effects were at play, because disturbance in the nuclei cytoskeleton (aneugenic effect) could also lead to the formation of micro-nucleated cells (Kirsch-Volders et al. 1997).

MN frequency in hemocytes was also related to increased LPO in tissues of clams ($r=0.50$; $p<0.001$), which suggests that oxidative stress was also at play. In previous studies with clams in the same sector, evidence of oxidative stress was found (i.e., increased LPO) which was associated to decreased phagocytosis activity in clam hemocytes at some of the upstream and polluted sites (Gagné et al. 2008a, b). This suggests that increased MN incidence in hemocytes from oxidative stress might have contributed to reduced capacity to defend against microorganisms in these clams. The reduction in phagocytosis and elevation of mincronuclei in immune cells were also observed in seal leucocytes exposed to arsenic, vanadium and selenium salts (Frouin et al. 2010), which suggests that DNA damage could reduce immunocompetence in animals. However, many pollutants are direct-acting clastogenic agents in mussels, such as benzo(*a*)pyrene (Banni et al. 2010), paracetamol from municipal effluents (Parolini et al. 2010), phenanthrene and numerous metals (Koukousika and Dimitriadis 2008). The presence of MN induced by both direct- and indirect-acting genotoxic compounds (methyl methane sulfonate and cyclophosphamide) was associated with decreased algae clearance in the blue mussels (Canty et al. 2009). This suggests that sustained incidence of MN could lead to decreased feeding behavior in bivalves, which could compromise the long-term sustainability of the population. In conclusion, this study revealed that hemocytes in clams shows signs of DNA damage as evidenced by altered DNA strand breaks levels, increased variation in DNA content in diploid cells and frequency in micronucleated cells. The frequency of micronuclei was significantly correlated with the variation in DNA content metric which could serve as automated measure of cytogenetic damage in aquatic organisms. We also found that genotoxicity was significantly affected by environmental variables such as shoreline distance and water conductivity which can increase variations in the responses.

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