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Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada)

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Abstract

Five biomarkers (MT: metallothionein-like proteins, EROD: ethoxyresorufin ortho-dééthylase, DNA strand breaks, LPO: peroxidation of lipids, VG: vitellogenin-like protiens) were measured in the soft tissues of zebra mussels (*Dreissena polymorpha*) in order to assess the spatial variation of exposure to contaminants along the St Lawrence River (Canada). Fifteen mussels > 25 mm shell length were analyzed from each of the 13 sampling sites. Significant differences between sites were noted for all biomarkers, but the general level of variability was low. Three biomarkers (DNA, LPO and VG) exhibited a similar pattern of spatial variation while MT and EROD had distinct and specific patterns. MT had the strongest discriminating power and EROD showed the largest range of variation among sites. Highest biomarker responses were measured in specimens from local contaminated sites such as harbors and industrial sectors. A positive relationship was found between MT and copper (Cu), but no significant correlation was observed between other biomarker responses and the levels of ten trace metals bioaccumulating in the zebra mussels tissues. Results indicate that the measurement of biomarker responses is technically feasible. The performance of each biomarker is assessed in the context of the role and advantages of selecting a battery of biomarkers for detecting contamination problems. The use of zebra mussels as a sentinel species for biomonitoring potential toxic effects in situ is discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Biomarkers; Zebra mussel (*Dreissena polymorpha*); Freshwater ecosystems

1. Introduction

Biomarkers consist of biochemical and/or physiological changes in organisms exposed to contaminants, and thus represent initial responses to

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environmental perturbations and contamination (McCarthy and Shugart, 1990; Bengtson and Henshel, 1996; Roy et al., 1996). They are generally taken as more sensitive than bioindicators at higher levels of the biological hierarchy, such as the organ, the individual or the population (Mayer et al., 1992; Stegeman et al., 1992). In contrast to the simple measurement of contaminants accumulating in body tissues, biomarkers can offer a more complete and biologically more relevant information on the potential impact of toxic pollutants on the health of organisms (Van der Oost et al., 1996).

Various biomarkers have been measured in different groups of aquatic organisms, particularly in fish and some bivalve mollusks. Commonly distributed in Europe and recently introduced in North America, the zebra mussel (*Dreissena polymorpha*) is a freshwater bivalve whose attributes would make it a useful bioindicator in surveillance programs for freshwater ecosystems (Hellawell, 1986; Lower and Kendall, 1990; Mayer et al., 1992; Phillips and Rainbow, 1992). Zebra mussels are sedentary, commonly distributed in a variety of habitats, easy to sample, sufficiently long-lived to allow the sampling of distinct annual cohorts, and, more importantly, have a high filtration rate which favors the uptake and bioconcentration of toxic chemicals (Reeders et al., 1989; Fisher et al., 1993; Bruner et al., 1994; Gossiaux et al., 1998). Consequently, the species is frequently used in Europe as well as in North America for the biomonitoring of aquatic contaminants, particularly trace metals (Kraak et al., 1991; Mersch et al., 1992; Mersch and Johansson, 1993; Secor et al., 1993; Johns and Timmerman, 1998). There are however, few reports on biochemical responses of zebra mussels to exposure of contaminants. Although contaminants alter the behavior, notably by changing the valve movement (Borcherding, 1992; Borcherding and Jantz, 1997) and reducing the filtration rate (Kraak et al., 1994, 1997), biomarkers response has, however, neither been tested nor validated for zebra mussels.

Kwan et al. (2000) recently reported significant variation in the concentrations of trace metals in the soft tissues of zebra mussels from 14 sites in the St Lawrence River (SLR). Spatial differences have been also previously observed for various contaminants and for different river ecosystem components (water: Pham et al., 1993; Quémerais et al., 1994; Cossa et al., 1998 sediments: Champoux and Sloterdijk, 1988; Lorrain et al., 1993 biota: Metcalfe and Charlton, 1990; Ion et al., 1997; Ion and de Lafontaine, 1998). These differences were in general associated with point source loadings of contaminants into the river. This spatial variability is nevertheless ‘buffered’ by the large size and the hydrological dynamic of the river system (mean annual flow rate = 12 600 m^3 s^{-1}, current speed > 1 m s^{-1} in the fluvial corridor, current reversal due to strong tidal effect near Québec City). With the exception of local areas of concern, such as harbors, industrial discharge zones and tributary outlets, the SLR could be considered relatively ‘homogeneous’, because of the important dilution process, and be comparable to weakly or moderately contaminated aquatic systems. In comparison to other large river systems in the world, the levels of trace metals and PCBs recently measured in the main water mass of the SLR are one to two orders of magnitude lower than in contaminated rivers, whereas PAHs and atrazine concentrations correspond to intermediate levels of pollution (Cossa et al., 1998).

The present study is complementary to that of Kwan et al. (2000) and aims to characterize the biochemical responses to spatial variation of exposure to contaminants in zebra mussels of the SLR and, by inference, to assess its water quality. A battery of five biomarkers was used: metallothionein-like proteins (MT); mixed function oxidase (EROD); DNA strand breaks (DNA); peroxidation of lipids (LPO) and vitellogenin-like proteins (VG). To our knowledge, this is the first in-situ study reporting on biomarker response in zebra mussels. We tested the null hypothesis that spatial variability in biomarker response would not reflect any significant trend of contamination impact along the river. In addition, we examined the relationships between these five biomarkers in the context of the role and advantages of using a battery of biomarkers to increase our capacity to detect contamination problem in-situ. Finally, a

critical analysis on the choice of biomarkers and the selection of the zebra mussel as a biomonitor is presented as well as future implications for biomonitoring the aquatic ecosystems.

1.1. Types and use of biomarkers

Our study includes biomarkers widely different in their significance and terminology (i.e. biomarkers of exposure, of effects, of stress, of alteration, of susceptibility). It is generally admitted that MT-like proteins and EROD respond to specific classes of contaminants: the first to trace metals and the second to coplanar organic chemicals, particularly PAHs and PCBs. The three other biomarkers (DNA, LPO and VG) are less specific and can therefore respond to several chemical cues. DNA strand breaks are indicative of genetic damage potentially leading to mutation, cancer, or cell death (Vukmirovic et al., 1994; Black et al., 1996). LPO is associated with oxidative perturbations, particularly within the polyunsaturated lipid-rich cell membranes (Chan, 1987; Stegeman et al., 1992). VG-like proteins are precursors of vitellin synthesis in vertebrates and some invertebrates, including bivalve mollusks (Pipe, 1987; Suzuki et al., 1992). They are indicative of an exposure to a substance that promotes the synthesis of vitellogenin and that could perturb this endocrine function (see Palmer and Selcer (1996) for a list of pesticides and various industrial chemicals having some estrogenic action).

From a semantic point of view, a biomarker is often labeled by the terms 'exposure' or 'effect' to typify its biochemical response. This terminology is somewhat misleading because it may erroneously suggest some relationship between biomarkers of 'exposure' and those of 'effects'. Although a causal relationship must exist between exposure to contaminants and biological effects, such a causal link does not necessarily hold between the two types of biomarkers, except if biomarkers share a common metabolic pathway (e.g. proteins metabolism, see Stegeman et al., 1992). In the context of biomonitoring and in order to categorize biomarkers according to their type of response, we find more appropriate to refer to them as biomarkers of 'defense' (e.g. the induction of MT or EROD is indeed a defense reaction by an organism exposed to some contaminants) or as biomarker of 'damage' (e.g. DNA strand breaks or lipid peroxidation products are direct alterations caused by toxic contaminants to the organisms). Although not all biomarkers may fall in these two categories, the five biomarkers used in this study were considered under this rationale.

2. Materials and methods

2.1. Sampling

Zebra mussels (*D. polymorpha* Pallas) were collected at 13 sites between Cornwall and Ile d'Orléans (a distance of ≈ 350 Km) between June 11–23, 1996 as part of an abundance survey in the SLR (Fig. 1). Sampling sites were therefore not necessarily selected on the basis of specific sources of pollution, but rather represent an exploratory investigation of the large-scale variability in the levels of contaminants and biomarkers responses along the river. Stations 1, 6, 8, 11 and 12 were located in harbor zones with variable degree of ship activity and level of organic and metallic pollution. Station 11 (Bassin Louise) is considered one of the most contaminated aquatic sites in the SLR (Fortin and Pelletier, 1995). As a result of more than a century of intense harbor activity in this area, the proximity of a local pulp and paper mill effluent, the presence of small contaminated tributaries and other diffuse sources of pollution, a total of 309 000 m^3 of sediments are contaminated by metals (mostly Cd, Cu, Pb and Zn), PAHs, PCBs and other organic contaminants (HCB, chlorophenols). Organotin (TBT) has also been recently measured at that station (Regoli et al., 1999). Similarly, sediments of the station 8 harbor site, adjacent of a very important industrial complex including aluminum smelters (considered to be one of the main sources of PAHs in Quebec), are contaminated by trace metals, PCBs and PAHs (Pelletier and Fortin, 1998). Station 2 was located in the southern sector of Lake St Francis where in the past, bottom

sediments have been contaminated by PCBs from upstream sources near Massena (Lorrain et al., 1993). Station 3 was in the vicinity of an important industrial complex having in the past discharged PAHs, Hg and trace metals directly in the nearshore environment (Fortin et al., 1994). Station 10 was at the mouth of the Chaudière River, a tributary of the SLR draining a large agricultural basin. Stations 4, 5 and 13 were not directly associated with punctual sources of pollution but were located near Montreal and Quebec City, the two largest urban communities on the SLR. In contrast, stations 7 and 9 were relatively far from known local sources of industrial and/or urban pollution.

Collections were made by SCUBA divers at a sampling depth of 3–5 m. Mussels were brought to the surface and rapidly sorted to select individuals with a shell length >25 mm. These could correspond to 3-year old mussels and older. Because large mussels were scarce at the two upstream sites (Cornwall and South Lancaster), selected specimens ranged between 18 and 25 mm (mostly at age 2). Approximately 50 live mussels from each site were placed onto a plastic screen suspended at mid-depth into a 20 L container filled with aerated river water ($\approx$ 18–23°C) from the sampling site for 24 h to allow gastric purgation. After that period, mussels were shipped within 6 h to our laboratory where they were frozen at -85°C for several months awaiting for biochemical analyses. Samples for the analysis of trace metal residues in zebra mussels undertaken by Kwan et al. (2000) were collected at the same locations, depth and time.

2.2. Biochemical analyses

Fifteen mussels of very similar size from each sample were selected for biochemical analyses. Samples were thawed on ice for approximately 10–15 min when valves could be opened easily.

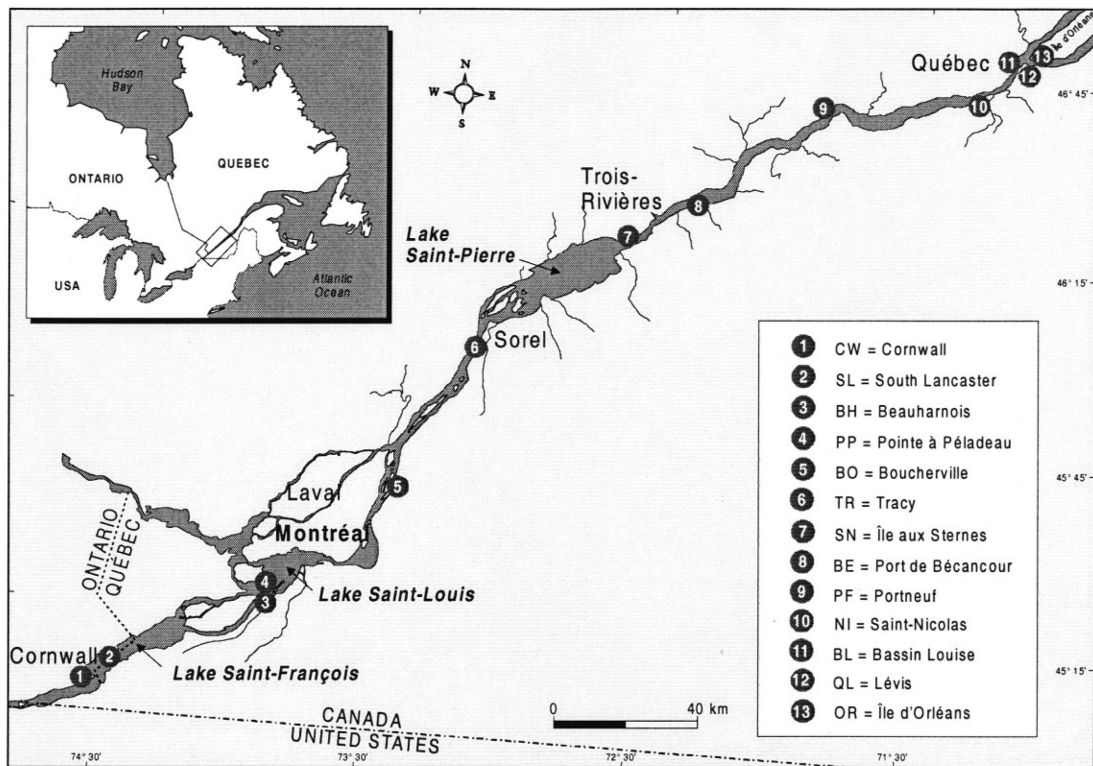

Fig. 1. Sampling sites for zebra mussel collection in the St Lawrence River during June 1996.

After rapid dissection, soft tissue was placed in a Tris-acetate buffer (25 mM, pH 7.6, containing 250 mM sucrose and 10 mM dithiothreitol 1:10 weight:volume, 2–4°C) and maintained on ice until homogenization in a glass tube with a Teflon pestle. Homogenization (50–60 s duration) was done in ice bath and samples were maintained on ice until centrifugation. Aliquots of each homogenate (10, 100, 50 and 100 μl, respectively) were sampled for total protein (Bradford 1976), lipid peroxidation (LPO), DNA damage (DNA) and vitellogenin (VG) determinations. Concentrations of these three biomarkers were normalized with the protein concentrations of the homogenate. The remaining homogenate was centrifuged at $10\ 000 \times g$ for 30 min at 2°C. The supernatant was then separated and measured for metallothionein (MT) and mixed function oxidase activity (EROD) concentrations. These two biomarkers were normalized with protein concentrations of the supernatant.

The shell length of each mussel was measured (± 0.1 mm) and sex was determined following the procedure of Neumann et al. (1993). Briefly, a smear was prepared on a microscope slide from the visceral mass of the soft tissue when dissected out. Large oocytes seen at a 400 x magnification indicated females, while spermatozoa (observed as small triangular-shaped bodies with flagellae) indicated males. Mussels where neither oocytes nor spermatozoa were observed were taken (by default) to be males.

2.3. Metallothionein-like proteins (MT)

The level of MT-like proteins was measured by the silver saturation assay (Scheuhammer and Cherian, 1986; Gagné and Blaise, 1993). A 50 μL sub-sample of the supernatant was mixed with an equal volume of 25 mg/L^{-1} Ag^{1+} in 100 mM glycine (pH 8.5) for 5 min. After adjusting volume to 500 μL with glycine buffer, 50 μL of 2.5% hemoglobin (Hb) was added and allowed to stand at room temperature for 5 min. Removal of hemoglobin-bound silver was undertaken by heating at 100°C for 2.5 min and by centrifuging at $10\ 000 \times g$. The Hb addition/heat denaturation steps were repeated once more and the remaining silver in the supernatant was determined by atomic absorption spectrophotometry equipped with Zeeman effect background correction. A ratio of 12 moles of bound Ag to 1 mole of MT was assumed (Kille et al., 1994). Results were expressed as nmoles of MT mg^{-1} of proteins.

Metals bound to MT were characterized by HPLC-ICP-MS method as described in High et al. (1997). Briefly, 97 μL of supernatant of mussel homogenate was injected onto a gel filtration column (Progel-TSK-G3000PWXL) and eluted with a Tris/HCl buffer (30 mM, pH 8.6) at 0.8 mL min^{-1}. Metal isotopes (Ni60, Ni61, Cu63, Zn68, Ag107, Cd111, Hg203) were monitored with an ICP-MS (Elan 5000 PE-SCIEX).

2.4. Mixed function oxidase assay (EROD)

Mixed function oxidase activity was measured using the EROD assay (Gagné and Blaise, 1993). The reaction mixture contained 50 μL supernatant, 10 μM 7-ethoxyresorufin and 10 mM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was initiated by the addition of NADPH, allowed to proceed for 60 min at 30°C, and stopped by the addition of 100 μL of 0.1 M NaOH. The 7-hydroxyresorufin was measured fluorometrically using 520 nm (excitation) and 590 nm (emission) filters. Blanks corresponded to $t = 0$ min and quantification was achieved with standard additions of 7-hydroxyresorufin. Enzyme activity (EROD) was expressed as pmoles of resorufin min^{-1} mg^{-1} of proteins.

2.5. DNA damage

DNA strand breaks were quantified using an alkaline precipitation assay (Olive, 1988; Gagné and Blaise, 1995). A 500 μL of a SDS solution (2%) containing 50 mM NaOH, 10 mM Tris and 10 mM EDTA was added to 50 μL of each soft tissue homogenate. Afterwards, 500 μL of 0.12 M KCl were added and the combined mixture was placed in a 60°C water bath for 10 min, followed by cooling at 4°C for 15 min, to precipitate SDS-associated nucleoproteins and genomic DNA. The mixture was further centrifuged at $8000 \times g$ for 4 min to enhance the precipitation process. The

levels of (single and double-stranded) DNA remaining in the supernatant were assessed by mixing 50 μL of the supernatant with 200 μL of hoescht dye at a concentration of 1 μg mL^{-1} in a 100 mM phosphate buffer, pH 7.0. Fluorescence was then measured using 360 nm (excitation) and 450 nm (emission) filters. Blanks contained identical constituents without homogenate and salmon sperm DNA standards were added for DNA quantification. Results were expressed as μg of supernatant DNA mg^{-1} of homogenate proteins.

2.6. Lipid peroxidation (LPO)

The level of thiobarbituric reactive substances (TBARS) resulting from lipid peroxide breakdown was evaluated with the thiobarbituric method (Wills, 1987). Briefly, 50 μL of the soft tissue homogenate were diluted to 1 mL with distilled water and mixed with 500 μL of 20% trichloroacetic acid solution containing 1 mM $FeSO_4$, 1 mL of 0.67% thiobarbituric acid was added and the mixture was allowed to stand in a hot water bath (90°C) for 10 min. Blanks and standards of tetramethoxypropane (stabilized form of malonaldehyde) were prepared in the presence of the homogenization buffer. All assay tubes were allowed to stand at room temperature for 15 min to allow sedimentation of denatured proteins. Afterwards, a 1-mL aliquot was withdrawn and mixed with 2 mL of distilled water. Fluorometric analysis was measured at 532 nm (excitation) and 556 nm (emission). Results were expressed as μg of TBARS/mg^{-1} of homogenate protein.

2.7. Vitellogenin-like proteins (VG)

Levels of vitellogenin-like proteins in mussel soft tissue were determined using the alkali-labile phosphate (ALP) measurement procedure (Campbell and Illenye, 1980; Gagné and Blaise, 1998; Blaise et al., 1999). This indirect assay for vitellogenins is based on the fact that such lipophosphoproteins contain alkali labile phosphates that can be easily quantified. Briefly, 100 μL of tissue homogenate were extracted in 500 μL of t-butyl methyl ether for 30 min at room temperature. Then, 400 μL of the ether phase were withdrawn and mixed with 100 μL of 1 M NaOH for 1 h. The NaOH phase was removed and used to quantify the levels of free phosphate according to a colorimetric phosphomolybdenum method (Stanton, 1968). Vitellogenin-like proteins levels were expressed as μg of alkali labile phosphate (ALP) mg^{-1} of homogenate protein.

3. Statistical treatment

The data distribution of each biomarker being not normal (as verified by the Kolmogorov-Smirnov and the χ^2 goodness-of-fit tests), a logarithmic (log10) transformation was applied to achieve normality prior to statistical analysis. Between sites variability was assessed for each biomarker by one-way ANOVA followed by a posteriori multiple comparison Tukey test (Zar, 1984). Homogeneity of variances was verified by visual inspection of ANOVA residuals. The relationship between biomarkers was evaluated by simple linear Pearson correlation analysis of the ANOVA residuals. This minimizes the effect of between sites correlations and highlights within site relationships for easier interpretation in terms of biochemical responses.

The effect of mussel size on biomarker response was first investigated by simple linear Pearson correlation analysis by using all data available (all sites confounded) and then by looking at each site separately. The effect of sex on VG was tested using a 2-way ANOVA (sites and sex as main factors). Because the male/female ratio in our samples varied between sites, the results from 10 sites having a sex ratio close to one were retained for statistical analysis and four mussels of each sex were randomly chosen from each site to ensure a balanced and orthogonal analytical design (Winer, 1971; Underwood, 1981).

The relationship between biomarker responses and levels of trace metals measured in zebra mussels (results from Kwan et al., 2000) was examined by principal component analysis (PCA) using a data matrix of 15 descriptors (ten trace metals and five biomarkers) and 13 objects (sampling sites). Due to scale differences between variables,

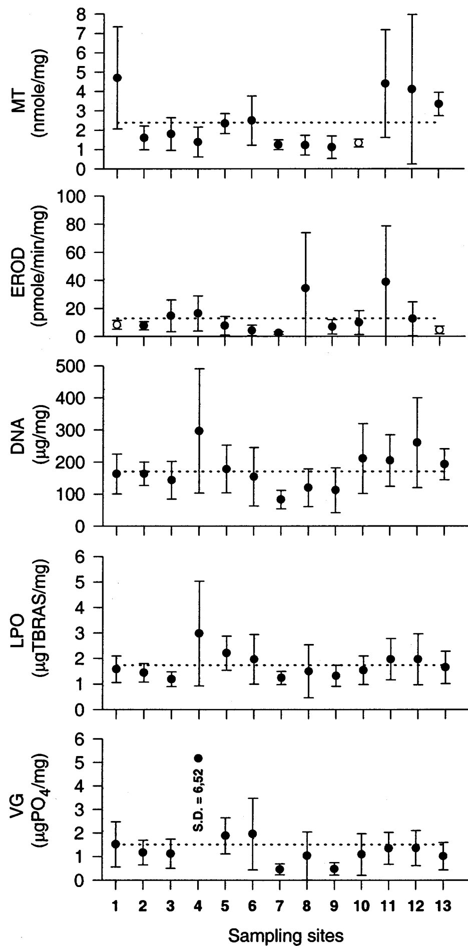

Fig. 2. Mean concentration and SD of five biomarkers at each site identified as in Fig. 1. Dotted line indicates mean of all sites.

the analysis was based on standardized residuals (Legendre and Legendre, 1984). We then performed a grouping of sampling sites using a hierarchical centroid clustering method (Milligan, 1980; Legendre and Legendre, 1984) to get a first approximation of the spatial variability defined by the contaminant levels and biomarker responses. Finally, in the light of the grouping results, we verified the hypothesis that stations grouping would correspond to the distribution of the main water masses of the SLR (see Rondeau, 1993, for chemical characteristics of these water masses) using a discriminant analysis based on a matrix of five biomarkers and 13 sites. Due to insufficient data for MT at station 10, concentrations of MT measured in mussels collected in November 1996 at the same site were used for the discriminant analysis. This was justified since there was no significant difference in other biomarkers between June and November samples (de Lafontaine, unpublished data). All statistical tests were undertaken with Statgraphics software, STSC version 5.0.

4. Results

4.1. Between sites variability

Differences between sites were significant for each biomarker (ANOVA F-test, $P < 0.05$) although some biomarkers exhibited high variability at some sites (Table 1, Fig. 2). Overall, the range of the mean values among sites was relatively small (2.5–4.2 times) for LPO, DNA and MT but reached 11.2 and 15.9 times for VG and EROD, respectively. MT was the most efficient biomarker to discriminate between sites where 32 out of the 66 paired-sites comparisons were significant, corresponding to a discriminating efficiency of 48.5% (Table 2). The efficiencies of other biomarkers were 26.9% for VG, 19.2% for DNA, 18.2% for EROD, and only 7.7% for LPO.

The patterns of spatial variability for biomarkers of damage (DNA, LPO and VG) appeared quite similar but distinct from those of defense (MT or EROD). Responses of the biomarkers of damage were significantly higher at stations 4, 11, 12 and 5 and lower at stations 7 and 9 (Table 2). There was no significant difference between sites within the upstream (1, 2, 3) or the downstream (10, 11, 12, 13) sectors (Tukey test for paired comparison, $P > 0.05$, Table 2). In contrast, MT concentrations were highest at stations 1, 11, 12 and 13 while EROD values were highest at the two harbor sites 8 and 11. Stations 7 and 9 were again either low or intermediate (as found for the biomarkers of damage) and station 4 yielded the third lowest value for MT (Table 2). Contrary to DNA, LPO and VG, both biomarkers of defense showed significant differences among stations

Table 1
Arithmetic mean and minimum and maximum values (in parentheses) of each biomarker for each of the 13 sites

Sites	Biomarkers					Length (mm) (n = 195)	
	MT (nmole mg^{-1}) ($n = 178$)	EROD (pmole min mg^{-1}) ($n = 134$)	DNA (µg DNA/mg^{-1}) ($n = 195$)	LPO (µg MDA mg^{-1}) ($n = 194$)	VG (µg PO_4 mg^{-1}) ($n = 180$)	Mean[c]	SD[c]
1	4.70 (1.14–8.25)	–[a]	162.5 (89.8–317.3)	1.58 (1.02–3.18)	1.52 (0.53–4.67)	17.1	1.8
2	1.59 (0.95–3.18)	7.66 (2.96–11.77)	163.1 (108.7–222.8)	1.44 (1.00–2.25)	1.17 (0.71–2.29)	18.1	1.0
3	1.80 (0.44–3.51)	14.68 (0.49–38.24)	142.9 (51.1–249.5)	1.19 (0.76–1.87)	1.12 (0.27–2.83)	29.5	2.3
4	1.38 (0.39–2.83)	16.42 (2.32–35.21)	296.6 (40.8–675.3)	2.98 (0.71–7.50)	5.17 (0.78–23.12)	27.7	1.4
5	2.34 (1.49–3.28)	7.64 (0.67–21.56)	177.9 (97.3–380.2)	2.21 (1.33–3.42)	1.88 (0.31–3.03)	27.4	1.1
6	2.49 (0.48–4.80)	4.20 (1.13–10.54)	153.9 (53.1–400.0)	1.97 (0.82–3.94)	1.96 (0.69–5.52)	27.7	1.8
7	1.24 (0.81–1.76)	2.44 (0.82–3.67)	82.7 (54.9–235.5)	1.24 (0.80–1.76)	0.46 (0.30–1.17)	27.9	1.7
8	1.22 (0.50–1.93)	34.38 (1.83–130.5)	119.6 (50.4–235.5)	1.50 (0.75–4.45)	1.03 (0.15–4.14)	29.1	1.8
9	1.11 (0.56–2.65)	6.82 (1.65–20.56)	112.0 (47.0–304.6)	1.32 (0.83–2.34)	0.48 (0.20–1.27)	27.9	1.9
10	–[a]	9.87 (0.87–24.32)	210.5 (107.5–387.8)	1.54 (0.15–2.80)	1.09 (0.13–2.90)	27.0	1.7
11	4.40 (2.26–13.4)	38.81 (4.29–158.3)	204.5 (100.8–346.6)	1.97 (0.91–3.56)	1.35 (0.39–2.74)	26.9	1.6
12	4.10 (2.04–17.0)	12.53 (1.02–37.56)	259.9 (68.1–479.9)	1.97 (0.88–3.60)	1.36 (0.31–3.03)	30.1	1.7
13	(2.45–4.25)	–[a]	192.8 (128.5–326.8)	1.65 (0.74–2.87)	1.02 (0.06–2.34)	27.2	1.6
Anova *F*-value	16.79***	5.93***	6.49***	3.46***	9.43***	86.42***	
Mean ratio[b]	4.20	15.90	3.58	2.50	11.23		

[a] Sample size for MT at site 10 and for EROD at sites 1 and 13 were too low ($n = 3$) and were not used in ANOVA.
[b] Mean ratio is the ratio of maximum to minimum mean site values and corresponds to the amplitude of variation between sites.
[c] Mean and standard deviation (SD) of shell length of mussels at each site.
*** *F*-values and level of significance (*** = $P < 0.001$) of ANOVA test are indicated.

Table 2
Results of the Tukey's Studentized range test for each biomarker

MT		EROD		DNA		LPO		VG	
Sites	Groups	Sites	Groups	Sites	groups	Sites	Groups	Sites	Groups
9	a	7	a	7	a	3	a	7	a
8	a	6	a b	9	a b	7	a	9	a
4	a	5	a b c	8	a b c	9	a b	8	a b
7	a b	9	a b c	3	a b c d	8	a b	10	a b
2	a b	10	a b c	6	a b c d	10	a b c	13	a b
3	a b c	2	a b c	1	b c d	2	a b c	3	a b
6	b c d	12	a b c d	2	b c d	1	a b c	2	b
5	c d	3	b c d	5	b c d	13	a b c	11	b
13	c d e	4	b c d	10	c d	12	a b c	12	b
12	d e	8	c d	13	c d	6	a b c	1	b c
1	d e	11	d	11	c d	11	a b c	6	b c
11	e			12	d	5	b c	5	b c
				4	d	4	c	4	c
66[†]		55[†]		78[†]		78[†]		78[†]	
32 (48.5)[††]		10 (18.2)[††]		15 (19.2)[††]		6 (7.7)[††]		21 (26.9)[††]	

[†] Total number of paired comparisons.
[††] Number and relative percentage (in parentheses) of significant comparisons ($P<0.05$).

within each of the upstream and downstream sectors (Fig. 2). In the case of MT-like proteins, mussels from station 1 had significantly higher concentrations than those collected from the two closest sites (2, 3). Similarly, EROD levels in mussels from station 11 were much higher than those from nearby sites 10, 12, and 13. These results therefore indicate that stations potentially impacted by contaminants were not identified simultaneously by all biomarkers.

Most biomarkers were significantly correlated to each other, but the correlation coefficients were generally low (Table 3) and would not yield strong predictive power when using linear regression models. Correlation coefficients were high between DNA, LPO and VG, which corroborates the above observation of a common spatial pattern of variability for the three biomarkers of damage. MT was also significantly correlated to DNA and LPO but not to VG. Finally, EROD was strongly correlated to MT, but less so to the biomarkers of damage (DNA, VG, LPO). These results are consistent with the distinct patterns of spatial variation among biomarkers inferred from Fig. 2.

4.2. Effect of biological variables

A weak negative correlation ($r = -0.19$, $P = 0.02$, $n = 177$) was found between MT-like proteins and size of mussels when using data pooled from all sites. Site-by-site analysis did not show any significant correlation ($P > 0.05$), though the negative coefficients at all but two sites would tend to confirm the inverse relationship.

Given the significant difference in the size of mussels between sites ($F = 86.42$, $P < 0.001$, Table

Table 3
Pearson linear correlation coefficients between biomarkers, calculated from ANOVA residuals (see text for explanation)

	MT	EROD	DNA	LPO	VG
MT	–				
EROD	0.583***	–			
DNA	0.338***	0.388***	–		
LPO	0.240**	−0.024	0.468***	–	
VG	0.132	0.177*	0.384***	0.583***	–

* $P<0.05$
** $P<0.01$
*** $P<0.001$

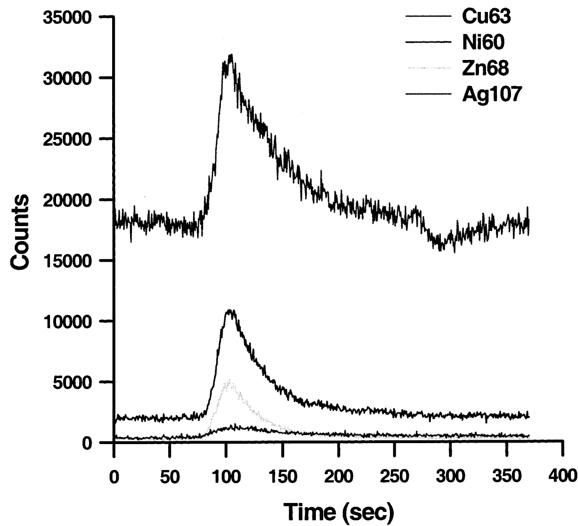

Fig. 3. Representative chromatogram of the gel-filtration HPLC-ICP-MS analysis of zebra mussel extract from station 11.

1), we verified if the spatial variability observed for MT (Fig. 2) could be partially due to variation in mussel size. Results of ANOVA incorporating shell length as a co-variate showed the effect of mussel size to be significant ($F = 5.61$, $P = 0.019$) but the 'site' effect became more significant and explained most of the MT variance ($F = 16.39$, $P < 0.001$). No significant correlation was found between the size of mussels and any other biomarker.

The effect of sex on VG-like proteins levels was not significant (Stations: $F = 5.03$, $P < 0.001$; sex: $F = 2.34$, $P = 0.13$; stations $*$ sex: $F = 1.66$, $P = 0.12$) and had therefore no influence on the spatial variability of this biomarker.

4.3. Spatial structure and relationship between contaminants and biomarkers

The range of the mean concentrations among sites was relatively small (1.8–5.9 times) for most trace metals, except for Cr (20.5 times) (Table 4). MT exhibited some correlation with Cu and Se and EROD was correlated with As. Other biomarkers were not correlated to trace metals (Table 5). The MT peak was eluted at 104 s. Only Cu, Ni, and Zn were found to be associated with MT in mussel extracts from all sites (Fig. 3). Cu and Zn were major metals found in mussels at all sites (Table 4). Results from PCA using both biomarkers (Table 1) and trace metals (Table 4) data revealed that the first axis (34.2% of the overall variance) was mainly formed by trace metals and MT (Fig. 4A). This axis appeared associated with a spatial gradient in concentrations of trace metals in mussels, since the three upstream sites were separated from the remaining downstream sites (Fig. 4B). LPO, DNA and VG mainly composed the second axis which (19.6% of the variance) with little contribution from EROD. These biomarkers response did not appear to be directly related to the levels of metallic contaminants in the soft tissues of zebra mussels. This suggests that the second axis of the PCA may indeed reveal the relative specificity of the

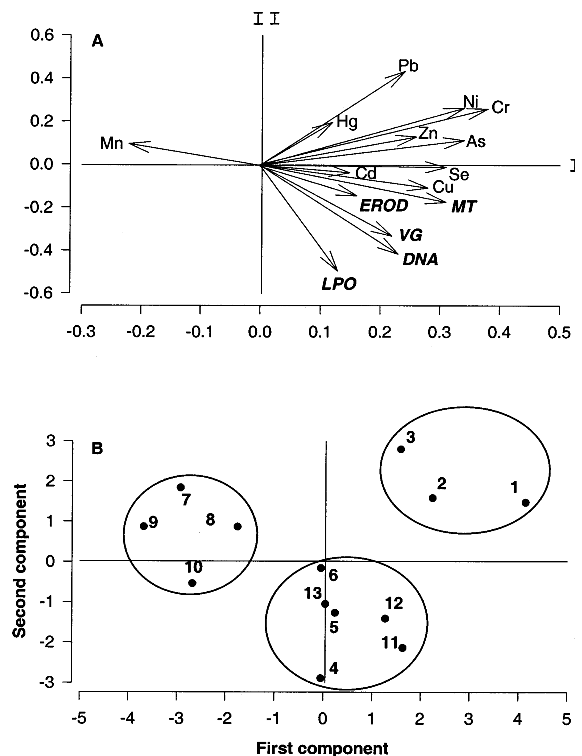

Fig. 4. Graphical representation of biochemical and chemical descriptors (A) and sampling sites (B) in the first two planes of the PCA and station clusters derived from centroid grouping analysis.

Table 4
Sex ratio and mean values of trace metals (in μg g^{-1} d.w.) in body tissues of zebra mussels at each sampling site

	Sampling sites													Ratio[a]
Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	Max/min
Sex ratio	nd[b]	–	–	2.75	2.75	0.50	0.67	0.67	1.50	1.50	1.14	1.50	0.67	
As	6.40	8.23	7.03	5.03	5.20	3.64	4.27	5.22	3.44	3.31	7.32	5.05	5.01	2.5
Cd	3.08	3.01	3.67	2.33	2.28	3.01	3.93	1.80	2.37	1.41	7.43	2.34	2.55	5.3
Pb	1.51	1.27	1.78	0.52	0.85	1.07	0.89	0.89	0.36	0.50	0.31	0.68	0.53	4.8
Mn	41.30	39.04	67.09	35.17	63.18	93.32	44.24	55.86	78.39	96.08	41.94	35.03	49.26	2.7
Ni	52.59	40.42	34.88	14.60	25.49	13.84	9.56	19.02	8.84	19.17	13.61	21.64	15.72	5.9
Cr	9.45	8.94	6.54	1.75	2.76	3.97	1.90	2.27	0.46	2.32	4.72	3.01	2.99	20.5
Se	7.36	7.31	7.08	5.25	7.22	6.18	4.68	4.43	4.96	4.05	5.46	7.26	7.40	1.8
Zn	340.30	179.80	180.10	168.20	197.80	168.30	140.30	189.30	192.00	162.90	182.00	184.90	147.90	2.4
Cu	28.27	21.38	22.29	23.36	18.45	35.96	16.98	16.93	15.19	14.26	25.86	25.04	25.51	2.5
Hg	0.123	0.154	0.220	0.144	0.104	0.213	0.146	0.109	0.120	0.103	0.127	0.154	0.125	2.1

[a] The maximum/minimum ratio in mean levels of contaminants between sites is indicated (data from Kwan et al. 2000).
[b] Nd: not determined.

Table 5
Pearson correlation coefficients between trace metals and biomarkers

	As	Cd	Pb	Mn	Ni	Cr	Se	Zn	Cu	Hg	MT	EROD	DNA	LPO	VG
As	1.00														
Cd	0.51	1.00													
Pb	0.47	−0.05	1.00												
Mn	−0.59*	−0.31	−0.03	1.00											
Ni	0.64*	−0.06	0.77**	−0.25	1.00										
Cr	0.78**	0.28	0.74**	−0.29	0.89***	1.00									
Se	0.43	−0.09	0.37	−0.39	0.51	0.49	1.00								
Zn	0.29	−0.03	0.43	−0.19	0.74**	0.59*	0.26	1.00							
Cu	0.20	0.31	0.26	−0.10	0.19	0.42	0.50	0.24	1.00						
Hg	0.19	0.20	0.57*	0.13	0.11	0.31	0.29	−0.18	0.56*	1.00					
MT	0.35	0.43	0.05	−0.32	0.32	0.44	0.67*	0.40	0.64*	0.01	1.00				
EROD	0.55*	0.37	−0.09	−0.35	0.18	0.20	−0.09	0.24	0.02	−0.19	0.17	1.00			
DNA	0.24	0.00	−0.24	−0.26	0.18	0.16	0.44	0.05	0.34	−0.19	0.53	0.37	1.00		
LPO	0.00	0.07	−0.35	−0.24	−0.12	−0.12	0.23	0.04	0.42	−0.13	0.38	0.17	0.69**	1.00	
VG	0.17	−0.07	0.06	−0.08	0.26	0.22	0.21	0.18	0.46	0.11	0.28	0.28	0.77**	0.80***	1.00

* $P<0.05$
** $P<0.01$
*** $P<0.001$

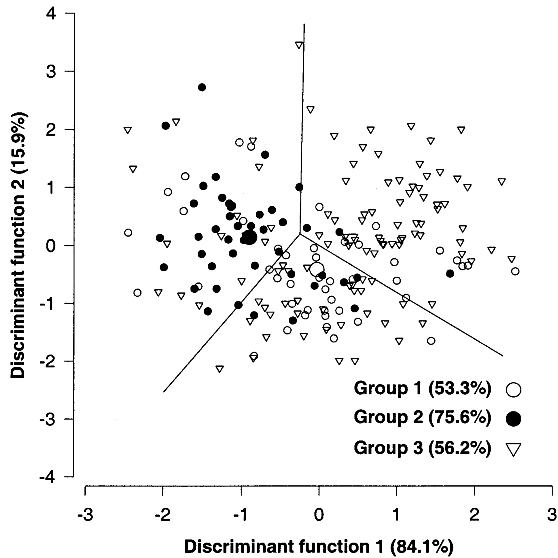

Fig. 5. Data distribution of biomarkers on the two principal axes of the discriminant analysis. Larger dots correspond to the centroid of each group.

biomarkers by distinguishing between the three biomarkers of damage (DNA, LPO, VG) which are less specific to a class of contaminants and the two biomarkers of defense (MT, EROD).

The hierarchical clustering of sampling sites yielded three groups of stations (Fig. 4B) that corresponded to the different water masses of the SLR. The three upstream sites (1, 2, and 3) formed a group associated with the 'green' and highly mineralized (conductivity > 285 μS cm^{-1}) waters coming from Lake Ontario. In contrast, stations 7, 9 and 10 belonged to a second group of sites under the influence of 'brown' or poorly mineralized (conductivity < 200 μS cm^{-1}) waters coming from some tributaries of the SLR. The third group included stations characterized by 'mixed' or intermediate waters (see Rondeau 1993 for detailed characteristics of these water masses).

Based on the hypothesis that the grouping of sites can be influenced by water masses, we performed a discriminant analysis with biomarker data only. The distribution of data and the position of the centroids in the first two discriminant functions ($\lambda_1 = 84.1\%$, $\lambda_2 = 15.9\%$) are shown in Fig. 5. The first axis is useful to distinguish stations characterized by 'brown' and poorly mineralized waters (group 2–75.6% of data were well classified) from the other stations. Standardized discriminant function coefficients (Table 6) show that this axis is primarily associated with MT, VG and DNA (in decreasing order). The negative side of the axis was associated with EROD suggesting that this biomarker would respond more strongly in 'brown' or low mineralized waters within the SLR. The two other groups were not well discriminated however, and the percentage of well-classified data in each group was rather low, being 53.3 and 56.2% for groups 1 and 3, respectively (Fig. 5).

5. Discussion

This is the first study that provides the evidence that significant response of biomarkers can be detected and assessed in field-collected zebra mus-

Table 6
Standardized discriminant function coefficients calculated based on five biomarker descriptors

	Discriminant function	Eigenvalue	Relative percentage	Canonical correlation
	λ_1:	0.280	84.04	0.47***
	λ_2:	0.053	15.94	0.22*
Biomarkers		λ_1	λ_2	
MT		0.670	−0.015	
EROD		−0.105	0.010	
DNA		0.276	−0.547	
LPO		−0.085	1.216	
VG		0.520	−0.444	

sels. Having no 'reference point', the absolute values of each biomarker could not be compared with those of other studies. Although the responses were generally low, all biomarkers differed significantly between sites suggesting the presence of some perturbed site which merit further assessment in the future. Significantly higher concentrations of one or more biomarkers were measured in mussels from stations impacted by contaminants (see Section 2.1), notably in harbors (11, 1, 12, 8) and in industrial areas (6, 3). The high concentrations of some biomarkers at stations 4 and 13 are however difficult to interpret in the light of current knowledge, but may reflect their proximity to urban areas. On the other hand, the response of most biomarkers was minimal at stations 7 and 9, both of which were most distant from local sources of pollution.

Metallothionein-like proteins (MT) had the most discriminating power (48.5%) for site separation and EROD values exhibited the largest amplitude of variation (15.9 times) (Tables 1 and 2). The biomarkers of defense were thus more variable (and more sensitive) than biomarkers of damage in zebra mussels from the SLR. Given the lack of a 'reference' uncontaminated site in our study, it is difficult to determine to what extent the response of the five biomarkers may have resulted from stress conditions owing to contaminant exposure or to natural environmental variability. Our results indicate that the biomarker responses in zebra mussels may be influenced by the water mass characteristics. Within the SLR, the two main water masses (high-mineralized 'green' versus low mineralized 'brown' waters) seem to modify the capacity of some biomarkers to respond to environmental variations (Fig. 4,Table 6). Biomarkers of damage (DNA, LPO and VG) were more sensitive and more specific to 'green' or intermediate waters while EROD seemed to perform better in 'brown' waters. The reason for this variability remains unexplained, but the type of water mass was also shown to influence the contaminant levels in water (Cossa et al., 1998) and the bioconcentration of various trace metals in aquatic plants of the SLR (Hudon, 1998).

At the large scale of the SLR between Cornwall and Ile d'Orléans, our battery of biomarkers in zebra mussels showed a generally low spatial variation in biochemical effects, potentially resulting from contaminant exposure. The maximum/minimum ratio in the mean values of each biomarker (Table 1) was close or higher than that of the mean concentrations of most trace metals at each site (Table 4) and may reflect a lack of large gradient of contaminants among the sampling sites. This would thus support the hypothesis of a 'buffering effect' in contaminant levels due to the large size and the dynamic of the system. These results corroborate those reported by Ion et al. (1997) for bioaccumulated contaminants in fish (yellow perch *Perca flavescens*) and those by Hudon (1998) in macrophytes (American wildcelery *Vallisneria americana*) as both show low spatial variation in bioavailable contaminants within the SLR ecosystem. It also supports the observation that, except for localized conditions, the general level of contaminants in the SLR, in comparison to other large rivers in the world, is low or intermediate (Cossa et al., 1998; Hudon, 1998).

The lack of significant effect of mussel size or sex on biomarkers response in zebra mussels is noteworthy since it allows easier and more reliable interpretation of spatial variability in present or future studies.

5.1. Comparison of biomarker performance

MT is generally known to be a sensitive and specific biomarker of metallic contamination (Petering et al., 1990; Kägi, 1991; Stegeman et al., 1992) and its measurement is often suggested for aquatic pollution biomonitoring programs (Neff, 1985; Garvey, 1990). Our results showed that MT exhibited the highest correlation coefficients with trace metals (Table 5) which therefore confirmed its usefulness as a biomarker of metal uptake in zebra mussels.

The component formed by MT and trace metals in the PCA analysis suggests that MT reflect a spatial variability in metals bioavailability to zebra mussels along the river (Fig. 4). MT was only significantly ($P < 0.05$) correlated with

selenium (Se) and copper (Cu), but correlation coefficients with other metals (Cr, Cd, Zn) were close to significance levels (Table 5). The levels of Cu and Zn in SLR zebra mussel soft tissues (Table 4) were 3–5 times lower than those reported from contaminated sites (Kraak et al., 1991; Mersch and Pihan, 1993) whereas Cd concentrations ranged from low to moderate relative to those reported in other studies (Mersch et al., 1992; Secor et al., 1993; Johns and Timmerman, 1998), a condition that may not induce high expression of MT. The contaminant levels were much less than those measured in mussels when exposed to high concentrations of metals in laboratory conditions (Mersch et al., 1993). High et al. (1997) demonstrated that Zn and Cu are the major elements bound to MT-like proteins in zebra mussels and that Cd can displace Zn following an acute exposure to Cd in the laboratory. Yang and Thompson (1996) found similar results with a marine bivalve. Our results of metal speciation (Fig. 3) also showed that Cu, Zn and Ni were bound to MT-like proteins in field-collected zebra mussels. The association of Cu and Zn in invertebrates has been widely reported (Roesijadi, 1992). This is the first observation that Ni is associated with mussel MT in situ and it does support the recent laboratory observations for Ni uptake by zebra mussels (Klerks and Fraleigh, 1997). The absence of Cd and Hg in any of the MT extract may reflect the low level of exposure to these metals in the SLR.

The lack of significant correlation between MT-like proteins and trace metal levels in mussel tissues can be partially due to the low sample size ($n = 13$), the high between-site variability in biochemical response relative to the lesser variability observed in trace metal levels in mussels (Table 4), the relatively low levels of some bioaccumulated trace metals and/or the fact that MT-like proteins was measured in whole mussels rather than in the hepato-pancreas where specific induction of MT-like proteins takes place. Extracting proteins from the entire soft body tissue as opposed to specific tissues may have somewhat 'diluted' the response and resulted in low values for MT.

The correlation analysis between metals and MT does not necessarily allow us to conclude on the relative importance that various metals have on the induction of MT in zebra mussels. However, metals induced the variability (4.2 times) in mean MT concentrations among the 13 sites in the SLR was sufficiently large to assume that MT. Couillard et al. (1995a), Couillard et al., (1995b) reported that MT in the freshwater mussel *Pygan-odon grandis* varied by a factor of four between contaminated and uncontaminated (control) sites varying by a factor of ten for metal contaminant levels. Malley et al. (1993) reported a two to four fold increase of MT in the freshwater mussel *Anodonta grandis grandis* for two lakes having a 30-fold variation in contamination levels. Even after considering possible differences in MT inducing capacity between species (Langston et al., 1989), our results support the hypothesis that exposure to trace metals was indeed responsible of MT induction in zebra mussels from the SLR.

EROD is strongly and rapidly induced in fish and mammals exposed to PCBs and PAHs (Stegeman et al., 1992; Livingstone, 1996). In situ comparisons between contaminated and uncontaminated sites showed that EROD mean values may vary by a factor of eight to 14 times (Andersson et al., 1987; Jimenez et al., 1990; Livingstone et al., 1995). Under laboratory conditions, Gagné and Blaise (1993) reported that EROD activity in rainbow trout exposed to sub-lethal concentrations of pulp and paper mills effluents can be 1.3 to 9.4 times higher than that in control fish (see also Andersson et al., 1987; Lehtinen et al., 1990). Although studies on mollusks are rare, a six to 30 fold increase in EROD activity has been measured in clams exposed to chemical stress (McDonald et al., 1994). Levels of EROD activity in zebra mussels at stations 11 and 8 were 15.9 and 14.2 times higher than the mean lowest response (station 7) (Table 1). Such variability strongly suggests that EROD response resulted from exposure to numerous organic contaminants present at these two sites located in industrial harbor zones (Fortin and Pelletier, 1995; Pelletier and Fortin, 1998). Except for these two sites, the spatial variation in EROD activity in zebra mussels was relatively weak (2–6 times) and within the range of what could be attributed to natural environmental variability (background

levels) (Jimenez et al., 1990). Therefore, we cannot rule out the possibility that EROD concentrations in zebra mussels from low contaminated sites could result from natural variability.

The three biomarkers of damage (DNA, LPO and VG-like) measured in zebra mussels were characterized by a very similar pattern of variation between sites. These biomarkers are not specific to a particular group of contaminants but would represent an integrative response to the impact of multiple toxic and/or environmental factors (Mayer et al., 1992). This lack of specificity could explain the significant correlation between their response (Tables 3 and 5). For LPO and DNA, the amplitude of variation was relatively small (2.5 and 3.6 times, respectively) but comparable to that reported in studies with bivalves from various contaminated sites (e.g. a 4-fold variation for DNA damage in the mussel *Mytilus galloprovincialis* Vukmirovic et al., (1994); a 1.5-fold variation for LPO in the mussel *Pygan-odon grandis* Couillard et al. (1995b)). Mean concentrations of VG-like proteins in zebra mussels varied by a factor of 11.2, which is slightly higher than that observed (3.2 times) in marine clams *Mya arenaria* by Blaise et al. (1999). The range of variation drops to 3.3 times, if we exclude station 4 where values were highest but also most variables. The high response of these biomarkers of damage at some sites (4, 12, and 5) suggests that various metabolic functions in zebra mussels can be negatively perturbed. Some SLR sites therefore appear to be of 'lesser' quality than others, at least from a zebra mussel point of view. The sources of such perturbing stress remain unknown, but do not appear to be related to trace metal levels in the present case.

5.2. Biomonitoring choice

The concept of a bioindicator for monitoring environmental pollution includes that of sentinel species measuring changes at the organism level and that of the sentinel bioassay measuring changes in biological functions (Lower and Kendall, 1990). The common application of these concepts is the 'key-stone' for biological monitoring.

Recent advances in biochemical techniques have permitted the development of several biomarkers for aquatic pollution studies. The challenge is to select an adequate number and type of biomarkers in order to obtain the most complete and most reliable information at the lowest cost. The use of a battery of biomarkers is generally justified because each positive response adds on the 'weight of evidence' in identifying a poor quality ecosystem (Stegeman et al., 1992; Roy et al., 1996). The argument is based on an epidemiological approach where the cumulative evidence increases our confidence in the interpretation of results and of the possible causal relationship.

However, uncertainty can prevail concerning the biomarker responses, due to a lack of information and/or control on the numerous mechanisms possibly operating at the biochemical, cellular or physiological level. If one considers that several biomarkers can rely on similar metabolic pathways (Stegeman et al., 1992), a common response among several biomarkers might not constitute much stronger evidence, but rather redundant information resulting from direct or indirect metabolic links between biomarkers. The similar spatial pattern of variation and the significant within site correlation between DNA, LPO and VG-like proteins observed in our study suggest a metabolic link between these three biomarkers of damage in zebra mussels. Hence, the simultaneously high responses observed at site 4 might not necessarily represent cumulative evidence of an environmental problem at that station. On the other hand, stations impacted by specific contaminants (e.g. station 8 for EROD and station 1 for MT) were not identified necessarily by all biomarkers.

In the context of large-scale biomonitoring (as in the SLR) in which the cost-benefit ratio has to be maximized, specific and more discriminating biomarkers of defense, such as MT and/or EROD for which biochemical dynamics are relatively well known, should be used in combination with a non-specific biomarker of damage to eventually detect other potential problems. In the case of zebra mussels in the SLR, both MT and EROD appear to 'perform' better for biomonitoring pro-

grams. VG-like proteins would be the best candidates for a non-specific biomarker of damage because of its larger range of response and higher discriminating power (Table 1) relative to DNA and LPO. Further studies are required however to better understand and interpret the response of this biomarker in zebra mussels as well as in other bivalves (Blaise et al., 1999).

5.3. Is zebra mussel a good bioindicator for water quality assessment?

Our study demonstrates that zebra mussels in their natural environment can yield a significant level of variation in different biomarkers presumably as a result of variable levels of contamination. However, given that

1. this species recently introduced in North America comes from European aquatic systems being relatively more contaminated than the SLR (Cossa et al., 1998),
2. the populations in North America do not genetically differ from those in Europe (Marsden et al., 1995) and
3. presumably, these populations with a relatively fast turnover rate may have developed some resistance to contaminants as demonstrated in oligocheates by Klerks and Levinton (1988) and suggested for freshwater mussels by Couillard et al. (1995b),
4. the results of our study may not only reflect an ecological response to ambient levels of contaminants in the river, but may also represent an evolutionary response of zebra mussels to chronic exposure to contaminants.

The response levels of 'defense' or 'damage' biomarkers in zebra mussels may be more suggestive of the presence of bioavailable contaminants rather than pointing out a potential risk for the organism itself. In such a case, the role of a biomarker as a tool for detecting an environmental risk due to pollution is lost (Couillard et al., 1995b).

In the case of low or moderately contaminated aquatic systems, such as the SLR, biomarkers in zebra mussels may be more useful to monitor the bioavailability of contaminants and identify potentially perturbed or contaminated sites than to infer a level of risk to the organisms. Many questions need to be answered prior to the establishment of a biomonitoring program at large scale. For example, it would desirable to assess the power of biomarkers to discriminate between sites and also between natural environmental factors and contaminants and to elucidate the metabolic interactions among the various biomarkers in order to minimize redundancy of information. Future studies involving in situ exposures of caged bivalves (as done by Roper et al., 1996) will prove crucial in determining whether zebra mussels are capable of being a genuine bioindicator of environmental risk. Answers to these questions will certainly require considerable effort but are viewed essential for achieving efficient and adequate management of aquatic ecosystems.

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