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Development of a biomarker-based index for assessing the ecotoxic potential of aquatic sites

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The use of biochemical or physiological measurements as indicators of ecotoxicity is under constant development and has the advantage of delineating effects before the appearance of disease. However, these biomarkers are often part of a battery of tests, and it is difficult to integrate them together to gain an overall view of an organism's health. The aim of this study was to develop an index that could integrate the data derived from a battery of biomarkers for application to both spatial and temporal studies. *Mya arenaria* clams were collected at different sites along the Saguenay Fjord (Quebec, Canada). Six biomarkers were measured: metallothioneins, DNA strand breakage, lipid peroxidation, vitellin-like proteins, phagocytosis, and non-specific esterase activity in haemocytes. A biomarker index was obtained by summing the biomarker values expressed in term of classes. Classes were determined by a distribution-free approach derived from the theory of rough sets. The results of the spatial study show that the index values discriminated well between contaminated and uncontaminated sites. The highly polluted sites had the highest index values (18 compared with a reference value of 14). In the temporal study, the index was also able to highlight possible contamination-induced alterations, even though the interpretation of temporal variation is complicated by natural variations occurring throughout the year. A control chart approach is proposed for determining contaminated sites in both spatial and temporal surveys.

Keywords: index, ecotoxicity, aquatic sites, biomarkers.

Introduction

Studies on the early biological effects of contaminated sites on aquatic organisms such as fish, bivalves and invertebrates have gained attention over more recent years (Etxeberria *et al*. 1995, Black *et al.* 1996, Cosson 2000). Biomarkers offer some insight into the physiological status of organisms under stress resulting from human-source or natural contamination, and make it possible to identify sites that could threaten the long-term survival of natural populations. Such studies, however, usually employ a battery of biomarker tests, along with tissue/chemical data, and are designed to examine early effects such as DNA integrity, the redox state of cells, membrane integrity, and the induction of defence mechanisms such as metallothionein synthesis in target tissues in response to exposure to heavy metals. While these endpoints, taken individually, can indicate the presence of a potentially deleterious effect at the molecular and cellular levels, it is often difficult to combine these effects into an assessment of the overall status of an organism. One approach is to develop an index that integrates biomarker values

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and that can serve as a tool for environmental managers to evaluate the relative environmental hazard at various aquatic sites. Index values have proven their usefulness in evaluating sediment toxicity (Bombardier and Bermingham 1999) and in assessing the toxic potential of industrial effluents (Costan *et al*. 1993). Narbonne *et al.* (1999) recently developed a similar approach using a battery of biomarkers to evaluate marine environmental quality.

In this study, a biomarker approach was used to assess the physiological status of *Mya arenaria* clams in the fjord of the Saguenay River (Quebec, Canada) (Blaise *et al.* 2002a). The battery of tests was designed to evaluate the effects of contaminants on selected target tissues in these organisms. Lipophosphoprotein levels were measured in the haemolymph to determine their reproductive status (Li *et al.* 1998, Blaise *et al.* 1999). The major lipophosphoproteins are related to vitellins in females, which are under oestrogenic control in these bivalves. Metallothioneins were determined in the digestive gland to assess heavy metal stress (Cosson 2000). Oxidative tissue damage was also determined in the digestive gland by measuring lipid peroxidation (Gutteridge 1995). DNA damage was determined in peripheral haemocytes by the alkaline precipitation assay (Olive 1988). Immune function was evaluated by the capacity of the haemocytes to ingest fluorescently labelled bacteria and by cell viability (Blaise *et al*. 2002b). These biochemical endpoints represent a comprehensive view of the health of the bivalves and have been shown in many other field studies to respond to environmental changes.

In the first part of this study, an index was developed from the spatial data on biomarkers. The aim was to examine the discriminative potential of the index value and its possible use as an indicator of contaminated sites. The second part of the study concerned data from a temporal study. As biomarker values vary naturally over time, the identification of contaminant-related stress in temporal studies is more difficult than in spatial studies. An index could therefore be helpful in identifying problematic sites in such studies.

Materials and methods

Data set

The data used in this study were measured in *Mya arenaria* clams collected in the Saguenay Fjord (Quebec, Canada) during extensive spatial and temporal surveys (Blaise *et al.* 2002a). For the spatial study, seven sites were selected along the Saguenay Fjord (Figure 1). Three of these sites have no direct source of contamination – Anse de Saint-Étienne (ASE), Anse à la Barque (Barq) and Baie-du-Moulin à Baude (Baude), and four sites are located near a source of pollution – Anse aux Érables (Era; an industrial area), Baie Éternité (BE; a marina harbour), Anse Saint-Jean (ASJ; a municipal effluent plume) and Petit Saguenay (PS; a marina harbour). The following six biomarkers were measured in clams in the spatial study: metallothionein-like proteins (MT), which are considered to be defence biomarkers, and DNA strand breakage (DNA), lipid peroxidation (LPO), vitellin-like proteins (Vn), a phagocytosis assay (PHAG) and cell viability (non-specific esterase activity in haemocytes, NspE), which are considered to be biomarkers of effects (Blaise *et al.* 2002a). Fifteen clams were sampled per site, resulting in 105 individuals in which the six biomarkers were measured. Aberrant values were identified using the following test: x, the tested value, is aberrant if $|(x-\text{mean})/\text{median}| > 6$. Six aberrant values were determined and removed from the analysis. The data set was then composed of 99 individuals in which six biomarkers were measured.

In the temporal study, the three sites ASE, Baude and BE were selected. The six biomarkers (MT, DNA, Vn, PHAG, NspE and LPO) were measured in 15 clams at each site for 6 months (May to October 2001), which encompasses the spawning period of clams.

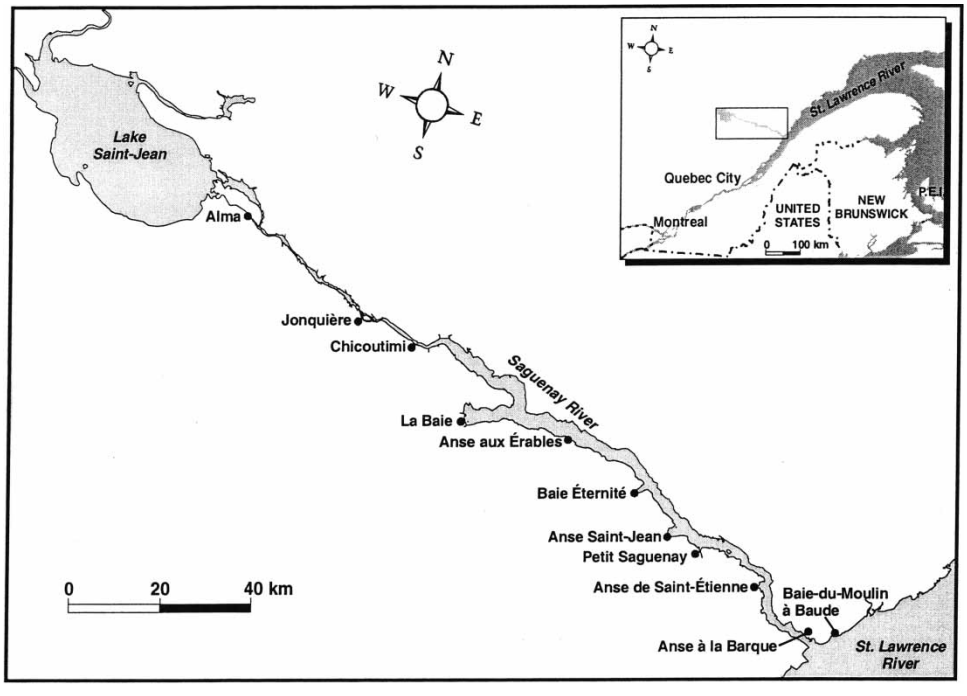

Figure 1. Selected sites along the Saguenay River. Three sites have no direct source of contamination: Anse de Saint-Étienne (ASE), Anse à la Barque (Barq) and Baie-du-Moulin à Baude (Baude). Four sites are near a source of pollution: Anse aux Érables (Era; an industrial area), Baie Éternité (BE; a marina harbour), Anse Saint-Jean (ASJ; a municipal effluent plume) and Petit Saguenay (PS; a marina harbour).

Data analysis

Data were analysed by rough sets analysis (Pawlak 1982), as described elsewhere (Chèvre *et al*. 2003). This non-parametric approach is independent of data distribution type – an advantage in this study because the data did not always follow a normal pattern of distribution at each site or sampling time. Rough sets analysis has been successfully used to discriminate between polluted sites along the Saguenay Fjord, comparing well with discriminant analysis. It has also been successfully applied in clinical settings to diagnose patients presenting with different symptoms (Fibrak *et al.* 1986, Pawlak *et al.* 1986, Slowinski *et al.* 1989). Prior to the analysis by rough sets, a local discretization, performed with an algorithm developed by Fayyad and Irani (1993), was applied to the data to form classes of biomarker values. It has been shown that this process does not decrease the quality of classification, although it is based on a non-parametric approach.

Index development

To standardize the biomarker data (i.e. to make values comparable), continuous data were discretized into classes using an algorithm based on entropy calculation, as described above. These classes were evaluated and validated by expert judgment (Chèvre 2001). The index is constructed as the sum of the classes of the different biomarkers for each clam. As effects are measured in the same individual, the discrete data are added to reflect the notion that bivalves support all the effects. No synergistic or antagonistic assumptions were incorporated into the index. Narbonne *et al*. (1999) used the same construction for their index. In contrast, the PEEP (Costan *et al*. 1993) and SED-TOX (Bombardier and Bermingham 1999) indexes used the mean responses of toxicity tests performed on different trophic levels.

Biomarker values were not weighted prior to summation. Although we might suppose that some biomarkers are more important than others, it is difficult to justify the selection of weight values. It would be preferable to over- or underestimate certain effects than to introduce unsuitable weights leading to incoherent results. Thus, the index is calculated as follows:

$$\text{index} = \text{MT} + \text{DNA} + \text{LPO} + \text{Vn} + \text{PHAG} + \text{NspE}$$

where MT, DNA, LPO, Vn, PHAG and NspE represent the class value of each biomarker at each site or for each sampling time (note that the temporal index is calculated without the LPO, for which no seasonal measurements were available, when index was developed). The index has no units.

Results

Spatial and temporal studies

The spatial survey of biomarkers was performed using clams collected in June, which is the month preceding spawning (Table 1). The biomarker values in this study were generally low at sites downstream of the Saguenay Fjord, whereas sites closest to the more populated areas (i.e. upstream of the river) displayed significant differences. PHAG activity varied less across the sites, while MT, LPO and NspE responded most of the time. The sites upstream of the Saguenay Fjord had a higher number of biomarkers with a higher response than those downstream. High levels of MT were found at the Era, BE, ASJ, PS and ASE sites. The amount of free DNA strands (not cross-linked to proteins) was high at the BE, ASE and Baude sites, while the degree of LPO was greatest at Era. The PS site had less LPO than the Baude or Barq sites. Clams from the ASJ site were collected in the area of a

Table 1. Spatial and temporal variations in biomarkers at seven selected sites (mean of 15 clams ± SE).

Month	Site	MT[a]	DNA[b]	LPO[c]	Vn[d]	PHAG[e]	NspE[f]
May	ASE	2.86 ± 0.43	297 ± 19	0.25 ± 0.05	71 ± 16	0.40 ± 0.10	1.71 ± 0.27
	BE	1.72 ± 0.11	555 ± 75	1 ± 0.09	8 ± 4	0.98 ± 0.14	3.75 ± 0.54
	Baude	0.37 ± 0.03	1258 ± 124	0.4 ± 0.02	85 ± 33	0.32 ± 0.03	0.37 ± 0.03
June	ASE	1.56 ± 0.12	1299 ± 41	6.0 ± 0.2	292 ± 13	0.65 ± 0.07	1.89 ± 0.12
	BE	0.85 ± 0.07	1185 ± 70	7.0 ± 0.5	253 ± 46	2.55 ± 0.70	4.58 ± 1.01
	Baude	0.61 ± 0.04	1258 ± 124	11.0 ± 0.6	288 ± 24	0.73 ± 0.06	2.34 ± 0.18
	ASJ	1.82 ± 0.07	331 ± 20	10.1 ± 0.9	761 ± 80	1.30 ± 0.10	15.00 ± 1.00
	Era	2.13 ± 0.08	633 ± 33	56.0 ± 5.0	380 ± 82	1.30 ± 0.08	7.80 ± 0.60
	Barq	0.58 ± 0.06	636 ± 172	9.5 ± 0.7	250 ± 17	0.70 ± 0.08	4.50 ± 0.30
	PS	1.36 ± 0.07	308 ± 24	3.6 ± 0.3	219 ± 79	0.90 ± 0.10	4.50 ± 0.30
July	ASE	2.17 ± 0.10	839 ± 136	8.3 ± 0.3	21 ± 8	0.35 ± 0.03	0.73 ± 0.04
	BE	2.71 ± 0.18	731 ± 52	12 ± 1	9 ± 2	0.89 ± 0.08	7.66 ± 0.88
	Baude	2.13 ± 0.14	1158 ± 66	51 ± 5	43 ± 5	1.21 ± 0.12	0.57 ± 0.05
August	ASE	2.05 ± 0.11	810 ± 34	21 ± 3	28 ± 3	1.22 ± 0.10	2.07 ± 0.17
	BE	1.88 ± 0.09	315 ± 26	9 ± 0.5	8 ± 1	1.52 ± 0.13	3.13 ± 0.37
	Baude	0.74 ± 0.04	469 ± 54	23 ± 3	29 ± 4	0.86 ± 0.10	1.69 ± 0.08
September	ASE	0.88 ± 0.13	1006 ± 92	10 ± 1	23 ± 4	0.45 ± 0.04	1.37 ± 0.17
	BE	2.46 ± 0.13	605 ± 46	40 ± 4	11 ± 2	0.65 ± 0.10	0.97 ± 0.12
	Baude	1.60 ± 0.09	344 ± 33	31 ± 2	12 ± 1	0.37 ± 0.04	0.46 ± 0.03
November	ASE	0.66 ± 0.05	549 ± 44	1.9 ± 0.2	23 ± 2	0.93 ± 0.09	0.39 ± 0.04
	BE	0.54 ± 0.03	699 ± 65	4.7 ± 0.6	14 ± 2	1.49 ± 0.20	0.52 ± 0.09
	Baude	1.09 ± 0.07	948 ± 71	16 ± 0.9	14 ± 2	0.40 ± 0.05	0.40 ± 0.05

[a] MT: nmol MT mg^{-1} protein.
[b] DNA: μg DNA in supernatant mg^{-1} protein.
[c] LPO: μg thiobarbituric acid reactants mg^{-1} protein.
[d] Vn: μg phosphate in inorganic phase mg^{-1} protein.
[e] PHAG: μg fluorescein (ingested bacteria) mg^{-1} protein.
[f] NspE: μg fluorescein min^{-1} mg^{-1} protein.

municipal effluent dispersion plume and displayed maximum NspE activity and Vn in the haemolymph. Similar to the DNA levels, Vn was lower at the PS site relative to the other sites. PHAG activity increased at sites upstream of the Saguenay River (i.e. Era, BE and ASJ). NspE activity in clam haemocytes was increased at the Era, BE, ASJ and Barq sites, while lower activity was observed at the PS site. Rough sets analysis showed that the battery of biomarkers used allows for site discrimination with an accuracy of 91% (Table 2).

In the temporal study, the biomarkers were examined in clams collected over a 6 month period (May to October 2001) (Table 1). The level of MT was significantly higher at each sampling period relative to October at the BE and ASE sites. MT levels at the Baude site were generally lower than at the other sites, except in July and October. DNA strand breakage was high at the Baude site from May to July, at ASE and BE in June, and at ASE in August and September. Conversely, DNA values were lower at ASE in May, and at Baude in August and September. LPO was generally higher in June, July and August, corresponding to the clam spawning period (June to July). PHAG activity was found to be generally higher in the summer months. Relative to the Baude site, PHAG was higher at BE in May, at BE and ASE in August, and at BE in September. However, PHAG was lower at BE and ASE in July. NspE activity was also generally higher in the summer months, whereas it was generally higher at the ASE and BE sites than at the Baude site in every month but June. The relative levels of Vn rose in May, peaked in June, and returned to baseline values thereafter. The increase in Vn follows the development of oocytes (spawning) in female clams (Blaise *et al.* 1999). Vn levels at the BE site in May and July were significantly lower than at the Baude site. The data also showed that the BE site tended to exhibit greater effects than the ASE and Baude sites within each sampling month, the most striking difference being observed in July, coinciding with the post-spawning period.

Our series of biomarkers discriminated well between sites for each month (Table 3). The mean accuracy was 95%, except in October, when it was 80%. October is thought to correspond to low variations in biomarkers due to the lower temperature of the water and the metabolic dormancy of the clams. Overall, Vn levels were found to be redundant from June to September, showing no difference in or delaying of the spawning cycle throughout the sites, even though Vn levels were lower at the BE site.

Table 2. Classification matrix obtained with the rough sets analysis based on a 'leaving-one-out' test.

	ASE	BE	Baude	ASJ	Era	Barq	PS	None	% correct
ASE	14	0	0	0	0	0	0	1	93
BE	2	10	0	0	0	1	0	1	71
Baude	0	0	14	0	0	0	0	0	100
ASJ	0	0	0	14	0	0	0	1	93
Era	0	0	0	0	15	0	0	0	100
Barq	0	0	2	0	0	10	0	1	77
PS	0	0	0	0	0	0	13	0	100

The 'None' column shows the number of objects that could not be classified.
Total accuracy: 90.5%.

Table 3. Discrimination between sites in the temporal study (rough sets analysis).

Month	Site	Accuracy[a] (%)	Redundant[b]
May	ASE	93.33	
	BE	93.33	
	Baude	100.00	
	Overall	95.56	MT, NspE
June	ASE	86.67	
	BE	100.00	
	Baude	100.00	
	Overall	95.56	DNA, Vn
July	ASE	100.00	
	BE	93.33	
	Baude	93.33	
	Overall	95.56	MT, DNA, Vn
August	ASE	100.00	
	BE	93.33	
	Baude	100.00	
	Overall	97.78	Vn, PHAG
September	ASE	93.33	
	BE	86.67	
	Baude	100.00	
	Overall	93.33	Vn, PHAG
October	ASE	80.00	
	BE	66.67	
	Baude	93.33	
	Overall	80.00	NspE

[a] The accuracy of prediction was calculated using a 'leaving-one-out' test.
[b] Biomarkers that could be removed from the data set without changing the results.

Biomarker index from the spatial survey

The classes used to calculate the index values are presented in Table 4. Figure 2a shows the index value at each site in June (spatial study). Values were between 13 and 14 at sites with no direct contamination (ASE, Baude and Barq). The index values of the upstream sites were 18 for ASJ and Era, 10 for PS and 14 for BE.

Table 4. Classification of values of biomarkers established for both spatial and temporal studies; for example, when the MT level is between 0.4 and 0.8 nmol MT mg^{-1} protein, the class number attributed to this biomarker is 1.

Class	MT[a]	DNA[b]	LPO[c]	Vn[d]	PHAG[e]	NspE[f]
1	0.4–0.8	100–500	2.2–4.4	0–100	<0.7	<0.6
2	0.8–1.1	500–900	4.4–7	100–350	>0.7	0.6–2.6
3	1.1–1.7	900–1700	7–20	350–400		2.6–4
4	1.7–2.4		20–100	400–550		4–10
5				550–1250		10–24

[a] MT: nmol MT mg^{-1} protein.
[b] DNA: µg DNA in supernatant mg^{-1} protein.
[c] LPO: µg thiobarbituric acid reactants mg^{-1} protein.
[d] Vn: µg phosphate in inorganic phase mg^{-1} protein.
[e] PHAG: µg fluorescein (ingested bacteria) mg^{-1} protein.
[f] NspE: µg fluorescein min^{-1} mg^{-1} protein.

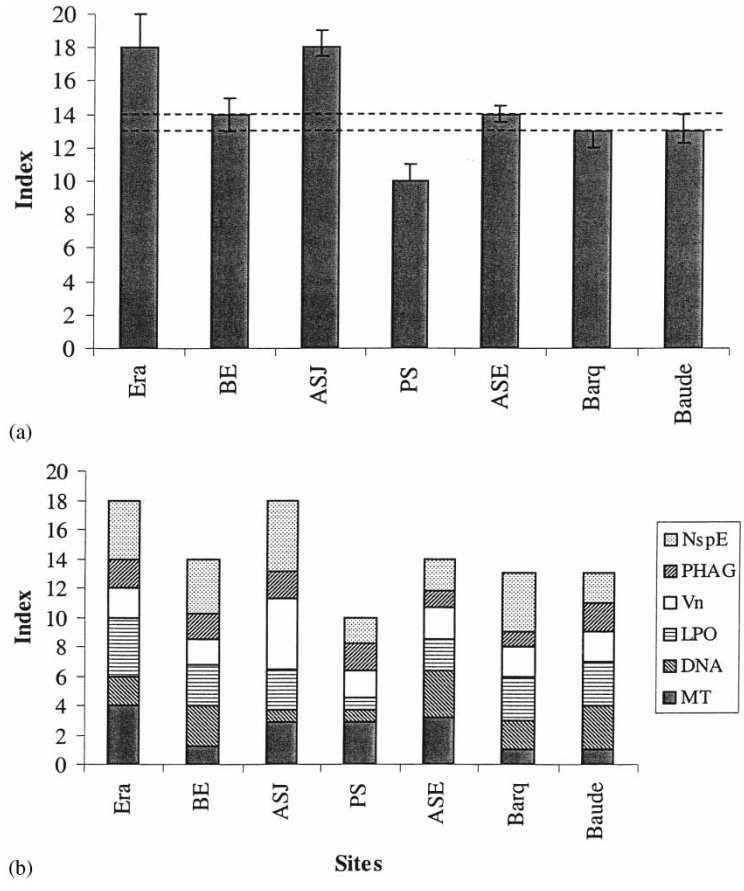

Figure 2. Spatial variations in the biomarker index. (a) Index value for each site in the spatial study (median ± first and third quartile). (b) Relative contribution of each biomarker to this index value.

Figure 2b shows the relative contribution of the different biomarkers to the index value. For the downstream sites, Vn values are the same, but the values for PHAG, LPO, DNA and especially MT and NspE are different. The BE site is comparable to the downstream sites for the different biomarker values. The PS site is lower than the other sites, mainly due to the low levels of DNA and LPO. Era has a high index, with a high level of MT and LPO, as does ASJ (located near a municipal effluent outfall), which also has high levels of Vn and NspE.

Temporal variation in the index

Figure 3a shows the temporal variations in the index value. Overall, the value increases in June (month 6) (just before spawning) and decreases afterward, reaching its lowest level in October. It is worth noting that the BE site seems to have a higher index value over time. The difference between the highest value in June and the lowest value in October (or May) was 6 for BE and 4.5 for ASE and Baude. The BE site is influenced by a small marina, whereas the ASE and Baude sites have no direct source of contamination. Thus, at sites influenced by contaminants,

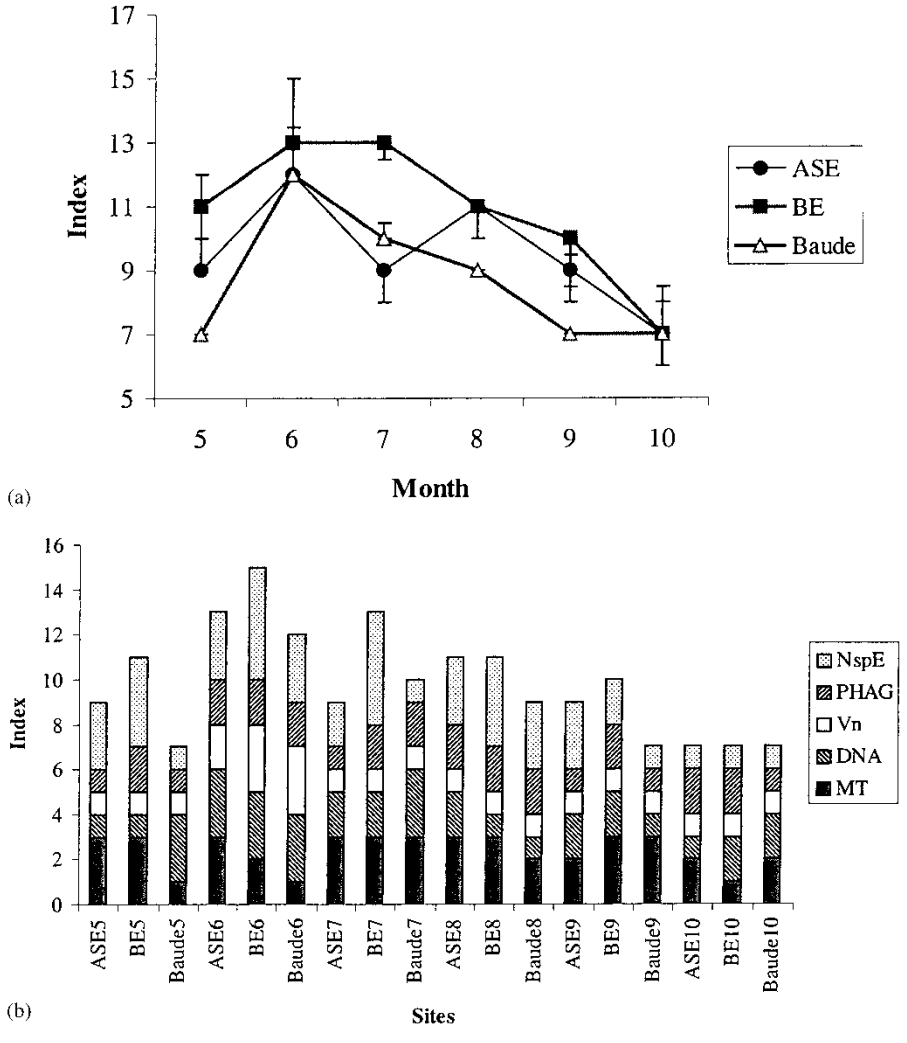

Figure 3. Temporal variations in the biomarker index. (a) Variation in the biomarker index at three sites over a 6 month period from May to October (months 5 to 10; median ± first and third quartile). (b) Relative contribution of each biomarker to this index value.

biomarker indexes show generally higher values, with a more pronounced difference between the highest and lowest values in a temporal setting.

Figure 3b presents the amount of each biomarker used to calculate the temporal index. In spring, the Baude site had a lower index due to the low level of MT and NspE compared with the other sites. The BE site had a higher index due to the particularly high level of NspE. Indexes increased at all sites in June due to an increase in Vn. The index value for site BE stayed high in July due to a high level of NspE.

Figure 4 presents the biomarker data (temporal and spatial studies) for each site in the form of a control chart. The median value at the ASE site, used as a reference, is given with the 25% and 75% quartiles. The biomarker index for the upstream and more impacted sites (BE, Era, ASJ and PS) are well outside the third

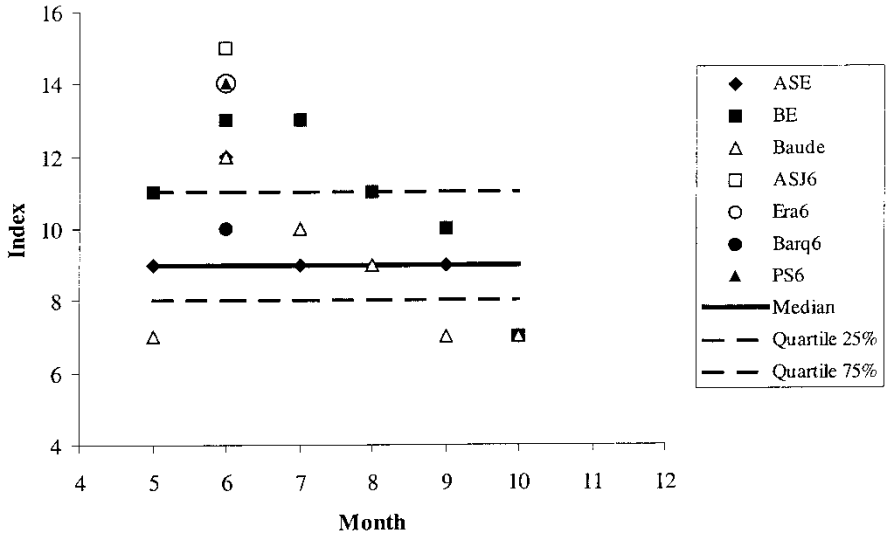

Figure 4. Control chart based on the temporal index values from the uncontaminated site ASE. The median (solid line) and the first and third quartile values (dashed lines) were calculated using all the index values from May to October from the ASE site. The index values of the other sites were compared with this reference site. Note that the index value of site PS has been rendered positive as described in the Discussion.

quartile in June and July, which is the spawning period. As with Figure 3b, BE index values tended to be higher than values at the 'cleaner' sites (i.e. ASE and Baude).

Discussion

The aim of this study was to develop a biomarker index for use in either spatial or temporal studies. A more detailed discussion on the physiological meaning is given elsewhere (Blaise *et al*. 2002a). The use of a biomarker index as part of a spatial survey was quite effective, facilitating the comparison of different sites and the identification of contaminated sites against a reference site. The biomarkers were transformed according to their respective ranges of response, and no consideration of site type (impacted or reference) influenced the discretization (or ranking) of the biomarker responses. The index values were shown to be quite similar for sites downstream, which represent sites with no direct source of contamination. The values from these sites (between 13 and 14) can be considered as a baseline and compared with the index values of the other sites. The index values from three of the upstream sites were clearly and significantly different from those of the reference site: two were greater (Era and ASJ) and one was lower (PS). The Era site is located in an industrial area. Figure 2b indicates that biomarkers MT and LPO are higher for this site than for the others, contributing to the higher index value. The Era site is in the most contaminated area, which may explain the high level of LPO, as this is considered to be one of the first signs of cellular damage due to miscellaneous pollutants. The ASJ site is highly contaminated and is located near a small but untreated municipal effluent plume. The high index value is due to

higher levels of Vn and NspE, which is consistent with this type of contamination (Blaise *et al.* 1999). Wastewater is suspected of containing pollutants that act as endocrine disruptors and can disrupt reproductive mechanisms, such as increasing Vn levels in fish (Sumpter 1995) and bivalves (Blaise *et al.* 1999). Municipal wastewaters are known to contain faecal bacteria, which may explain the increased esterase activity (Cheng and Yoshino 1976) in haemocytes and the phagocytic activity (Table 1).

The index value of the PS site was clearly lower than the reference value, with very low levels of DNA and LPO. Although it is low relative to the reference sites, the index value highlights an effect. This site is influenced by a marina, which certainly contributes to increased contamination. To eliminate the possibility of a negative index, the value could be normalized to give a positive response (e.g. a decrease of 3 relative to the reference could be seen as an index value of 16).

Site BE is the only contaminated site for which biomarker values were not different enough to distinguish them from those of the uncontaminated sites (Figure 2b). This result was also observed on rough sets analysis (Table 2), for which site BE has the worst classification (accuracy 71%). This may be due to decreased reproductive activity (as shown by lower Vn values), in which case the impacts on this site would be more easily seen in a temporal survey. *Mya arenaria* from site BE have been shown to have lower vitellogenin-like properties and a significant delay in gonadal maturation (Blaise *et al.* 1999, Gauthier-Clerc *et al.* 2002). This could lower rank attribution and explain, in part at least, why the biomarker index for site BE was not very high in the spatial survey (Figure 2a). Therefore, not only can the natural reproductive period alter biomarker responses, but it may also alter the effects of contamination on reproductive activity (e.g. delayed gonadal maturation, oestrogen-induced vitellogenins and spawning activity).

In the temporal survey, the production of an index is complicated by the fact that the individual biomarkers vary naturally throughout the year (i.e. with temperature, reproduction cycle, nutrients and predation). Hence, a way must be found to discriminate between natural variation and contaminant-induced alterations. For example, acetylcholinesterase activity in clams and mussels has been shown to vary 1.2- to 1.5-fold throughout the year (Dellali *et al.* 2001). In another study, MT levels were found to have as much as a four-fold variation at the whole organism level, including the gonad (Baudrimont *et al.* 1997). In oysters, on the other hand, although MT concentrations were high in the digestive gland, the correlation between levels of metals and MT in tissue was lower during the pre-spawning period (Geffard *et al.* 2001). These studies demonstrate the inherent difficulty of integrating biomarker data over time, and care should be taken to characterize the natural variations in the biomarkers over at least one reproductive cycle. For example, in the work of Bresler *et al.* (1999), the total biomarker index of Narbonne *et al.* (1999) failed to reveal any consistent trends or discrimination between polluted and pristine sites, even if statistically significant results were obtained within the biomarkers. This suggests that the natural variation of the biomarker responses confounded the variation caused by contaminant exposure.

The variation in the biomarker index at two uncontaminated sites (Baude and ASE) showed the same pattern as the variation in the biomarker index over time: an increase in June and a slow decrease until October (Figure 3a). This is not due solely to the higher level of Vn in June, following reproduction; Figure 3b shows a change in the contribution of the other biomarkers. The same effect is seen when considering the curves representing temporal changes in the centres of gravity of the best discriminant function obtained during discriminant analysis (Blaise *et al.* 2002a). These curves appear to follow a sinusoidal trend, where the centres of gravity of the discriminant functions in October are similar to those in May (*t*-test, $p = 0.30$).

At the more contaminated BE site, however, the biomarker index tended to be higher than the index values at the reference site. As mentioned earlier, biomarkers at site BE show a disruption of the reproductive process. The production of a biomarker index therefore needs to model, in some way, the natural or background variation, especially in temporal surveys.

The biomarker index developed in this study allows for the identification of pollutant-induced change over time. This approach would also be of value in long-term biomonitoring studies. Moreover, Bresler *et al.* (1999) emphasized the problem of finding a reference to compare index values with within a temporal study. One strategy consists of characterizing the baseline or natural variation over time, as was done in this study (Figure 3a). We also examined the possibility of reporting the biomarker index in a control chart format, as depicted in Figure 4, using non-parametric descriptors (i.e. median and quartiles). Both approaches appeared to be equivalent in identifying the most impacted sites located upstream of the fjord. Such a chart does, however, allow for the most problematic sites, in both a spatial and a temporal framework, to be identified. In conclusion, then, the calculation of a biomarker index and its representation in the form of either a non-parametric control chart or a graph with time as the second dimension, are interesting tools that can be useful for detecting sites that have contamination problems, in both spatial and temporal surveys.

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