Aquat. Living Resour. 17, 297–302 (2004)

DOI: 10.1051/alr:2004035
www.edpsciences.org/alr

Aquatic
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NOTE

Monitoring of immunotoxic responses in oysters reared in areas contaminated by the "*Erika*" oil spill

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Received 10 February 2004; Accepted 30 July 2004

Abstract – Immunological alterations may be used as ecotoxicological biomarkers to detect and monitor biological effects of chemical contaminants in polluted environments. This study reports on a survey of Pacific oysters from the Atlantic coast of Britanny (France) after the "*Erika*" oil spill. Reared stocks were sampled twice a year from November 2001 to March 2003 at three impacted sites and at an additional site outside the spill area. A multiparametric diagnosis of the immune system was performed on individual hemolymph samples to identify structural, immunopathological alterations and functional impairment of immunocompetent cells. One year after the spill, severe immunological alterations were observed in a site heavily impacted by oil. Since the oysters there had higher contents of PAH compared to the other sites, it was suggested that chronic contamination, possibly generated by oil trapped in the sediments, had induced immunotoxicity. Moreover, moderate variations in some hemolymph parameters observed at the non-impacted area strongly suggested that natural environmental factors may have generated physiological stress. An immunotoxicological index applied here in order to integrate all measurements of defense-related functions appeared to be an efficient tool to identify samples suffering immunological stress.

Key words: Immunotoxicology / Bivalve molluscs / Monitoring / Environmental stress / PAH

1 Introduction

Environmental surveys of chemical contamination rely on methodological procedures where biological responses to anthropogenic signals may be quantified and used as tools for risk assessment. In marine organisms, several measurable, biological and physiological changes may now be considered as biomarkers and their application in monitoring programmes has entered an operational phase (Depledge 1994; Forbes and Forbes 1994). Among benthic invertebrates, several species of bivalve molluscs such as mussels and oysters have been introduced as biological models for research in ecotoxicology. As sedentary filter-feeders, bivalves may bioaccumulate environmental pollutants to very high tissue concentrations (Zatta et al. 1992; Phillips 1995). Under normal conditions the immune system of molluscs maintains efficient protection against most microbial or parasitic attacks. However, many chemical contaminants may act as immunosuppressors and reduce immune efficiency, even at low concentration (see reviews by Anderson 1993; Pipe and Coles 1995). Among those, polycyclic aromatic hydrocarbons found in oil, have been demonstrated to alter many immune parameters in marine invertebrates (Coles et al. 1994; Gomez-Mendikute et al. 2002). Moreover, observations from experimental exposures suggested a species-dependent sensitivity to these contaminants (Wootton et al. 2003). Recent advances in the knowledge of how chemicals target immune mechanisms in aquatic organisms have allowed immunotoxicology to evolve as an area of primary importance for detecting early signals of physiological alterations in contaminated biota (Zeeman 1996; Auffret 2004). Field-collected resident oysters have been used to explore relationship between chemical contamination and defense-related measurements (Fisher et al. 2000; Oliver et al. 2001). Results from a two-year survey of immune characteristics in oysters reared in intertidal areas impacted by the *Erika* oil spill (December 1999) are presented here.

2 Material and methods

2.1 Animals

Pacific oysters, *Crassostrea gigas*, from aquaculture stocks were collected ($n = 15$) from November 2001 to March 2003

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Table 1. Sampling protocol applied to oysters for the survey. Tagged cells identify collection dates in the sites (S-year: spring, A-year: autumn). N99, N00, N01, N02: spat settled respectively in 1999, 2000, 2001, 2002 in the sites.

Sites	Oyster stocks	Sampling dates			
		A-2001	S-2002	A-2002	S-2003
Pen Bé	N99	X			
	N00	X	X	X	
	N01			X	X
Le Croisic	N99	X			
	N00	X	X	X	
	N01			X	X
	N02				X
Les Moutiers en-Retz	N99	X			
	N00	X	X	X	
	N01			X	X
	N02				X
Rade de Brest	N00	X	X	X	
	N01			X	

at three locations in the Loire-Atlantique (France): Pen-Bé, Le Croisic, Les Moutiers-en Retz. The oysters were reared according to standard farming protocols from spat settled each year from 1999 to 2002. In the present paper, the stocks were identified according to spat year-of-birth. They were sampled in spring and autumn from the year after settling and thereafter, the year corresponding to a commercial size (Table 1). All of this area had been severely contaminated by hydrocarbons during the winter 1999-2000 and thereafter, following winter storms disturbing buried oil slicks. In addition, oysters were sampled from the Rade de Brest (Finistère), an area not exposed to oil contamination. There were no reports of abnormal mortalities, pathogenic attack or specific disesase in any of these stocks during the survey. Growth was considered as normal by the farmers. After sampling, the individuals were transported to the laboratory and maintained 24 h, until processing in 30 L-tanks containing aerated sea-water.

2.2 Hemolymph analysis

Approximately 1 ml of hemolymph was withdrawn from the adductor muscle sinus of each individual as previously described by Auffret and Oubella (1995). Because only microassays were applied, a full set of immunological measurements could be performed on individual samples. Most parameters were determined using a flow cytometer (FACSCalibur™, Becton Dickinson) as described by Auffret et al. (2002). Briefly, the flow rate was measured to allow absolute cell counts. Cell mortality was measured using the propidium iodide-exclusion method (final concentration = 10 $\mu g\,L^{-1}$). Phagocytic activity was measured *in vitro* by incubating hemolymph cells with 2.2 μm latex beads at a 1:30 cell-to-bead ratio. A phagocytic index (PI expressed in % positive cells) was calculated as the percent of cells that had engulfed three beads and more, a means to consider only the most active hemocytes. An overall phagocytic capacity (PC expressed in arbitrary units) of the hemocyte population was calculated by the CellQuest software™ from the histogram of relative fluorescence intensity distribution, as the geometric mean of values provided by positive cells. The microbicidal capacity of hemocytes was assessed by measuring the production of reactive oxygen species (ROS) using the fluorescent probe DHR 123 (final concentration = 50 $\mu g\,L^{-1}$). The oxidative burst of phagocytic cells was triggered by the addition of phorbol myristate acetate (PMA) (final concentration = 100 nM). The specific, lipophilic probe $DIOC_6$ (final concentration = 1.25 μM) was used to detect possible alterations of the mitochondrial membrane potential $\Delta\Psi_m$. To detect lysosomal destabilization and cell lysis, acid phosphatase (ACP) release from lysosomes was measured in serum by microtitration using p-nitrophenyl phosphate as a substrate. A specific activity has been calculated after total serum protein (PRTS) determination. The level of metallothioneins was assayed in hemolymph cell lysates by ELISA according to Boutet et al. (2002).

2.3 Contaminant analysis

The concentration of polyaromatic hydrocarbons (PAHs) in oyster soft tissues was determined by gas chromatography/mass spectroscopy in either lyophilized or raw material. The values presented are those for the 16 compounds selected by US-EPA. Trace metal burdens were measured by atomic absorption.

2.4 Statistical analysis

Normal distribution of the data and homoscedasticity were examined using Kolmogorov-Smirnov and Levene tests, respectively. Where necessary, data were transformed to allow variance analysis. The difference among sites for oysters reared from year-2000 spat (N00) was tested with an ANOVA. When significant differences ($\alpha = 0.05$) were observed, Tukey's test was used for pairwise comparisons.

2.5 Data management

To increase the ecotoxicological relevance of the multiparametric study, a "site immunotoxicological index" has been calculated from the set of biological characteristics measured in each oyster sample. The principle was to cumulate, for each sample collected, the variation of each parameter from a reference value. Considering that no immunological data were available for this species in the area impacted by the spill and that oysters reared in the additional site (Rade de Brest) were not from the same stocks as in the impacted sites, reference values were defined *a posteriori* as each parameter average from all sites and sampling dates (Table 2). First, $\log_{10}$-transformed values were calculated from the mean value of each biological measurement. The formulation was adapted to arbitrarily affect the unit value when the parameter was either doubled or reduced by half. The calculation for a parameter p_i in a sample was as following:

$$\text{Transformed } p_i = (\log_{10} \text{ mean value } p_i - \log_{10} \text{ "reference" value}) / \log_{10} 2.$$

Table 2. Immunological parameters measured in oysters with their reference value (mean and standard error) defined from individuals collected from all sites/campaigns and corresponding ecophysiological significance factors (EPSF). Groups refer to clusters of parameter related either to structural changes in the hemolymph compartment (immunopathology) or to functional alterations in hemocytes (immunocompetence).

Group	Immunological parameter	Reference value	EPSF
Immunopathology	Total hemocyte counts	2.1 (1.3)	2
	Serum acid phosphase	48.1 (14.3)	0.5
	Cell mortality	1.05 (0.46)	1
	Granulocyte $\Delta\Psi_m$	127 (106)	1
	Hemocyte metallothioneins	0.90 (0.85)	0.25
Immunocompetence	Phagocytic index	39 (10)	2
	Phagocytic capacity	1243 (340)	2
	Hemocyte microbicidal capacity	75 (26)	1

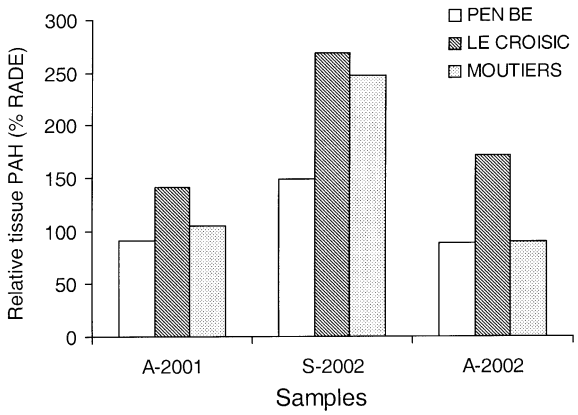

Fig. 1. Monitoring of PAH contents (sum of 16 compounds according to US-Environmental Protection Agency) in soft tissues from oysters settled in year 2000 in three rearing sites. Relative values were calculated as the percent of levels measured at the non-impacted site (Rade de Brest).

In a second step, to increase the discriminating power of highly stress-responsive parameters, an "ecophysiological significance factor" (EPSF) was applied to these values (Table 2). Establishing this factor was based on previous experimental results obtained through single contaminant assays run in the laboratory with so-called "sentinel-bivalve species", especially mussels and oysters. It was arbitrarily high for hemolymph parameters observed to consistently vary with most contaminants. Indeed, experimental exposure to heavy metals or organics induced a conspicuous rise of circulating hemocyte numbers either in mussels (Coles et al. 1995; Pipe et al. 1999) or in oysters (Auffret et al. 2002). In all bivalve mollusc species studied yet, phagocytosis was found to be altered by various xenobiotics and has become a very sensitive tool for immunotoxicologists when hemocyte competence in non-specific immunity has to be assessed (Auffret 2004). Other measurements as cell viability, which appear more responsive when hemocytes are exposed *in vitro* to contaminants (unpublished data) were attributed here a low EPSF value. In a third step, the transformed parameters were cumulated into two groups defined as i) parameters related to structural changes in the hemolymph compartment (immunopathology) ii) parameters related to functional alterations in hemocytes (immunocompetence) (Table 2).

3 Results and discussion

The present study was performed on several series of adult oysters raised from spat settled either just before or after the spill depending on the site. Twenty-three months after the spill, the PAH content had decreased to reach background levels on these coasts (around 200–300 $\mu g\,kg^{-1}$ DW). However, values 2.5 fold higher than in the non-impacted site were measured during March 2002 at two severely impacted sites, Moutiers and Le Croisic (Fig. 1). The levels at Le Croisic remained high until the following autumn. Even if they were much below those measured in the months following the spill (over 1500 $\mu g\,kg^{-1}$ DW in Le Croisic, RNO 2002), the variations observed there may have resulted from local harbour activities but disturbance of oil in the sediments in the vicinity of the oyster growing site was also suspected. At the Le Croisic site, contamination by PAHs could therefore be considered as chronic. Other contaminants known to generate biological responses, namely heavy metals, were also measured in oysters. During this survey, the tissue concentration of cadmium throughout the sites remained within the range of expected values on these coasts (1.5–2.5 $mg\,kg^{-1}$ DW, RNO 2004). Copper levels were also found around normal at 100–150 $mg\,kg^{-1}$ DW, except at the site Moutiers where contents 2.5 to 3 fold higher were regularly found. The source, not related to the spill, has not been identified yet.

When the immunological parameters of oysters reared from year-1999 spat were compared among sites, conspicuous immunopathological alterations were observed in November 2001 at Le Croisic (Table 3). In these oysters, the mitochondrial function was impaired in hemocytes as indicated by low membrane potential, suggesting depressed cell viability. All parameters used to assess the phagocytic function, as the phagocytic index and the phagocytic capacity, were reduced by 50 percent compared to expected values and reflected impaired hemocyte immunocompetence. By this time, total hemocyte counts were high. After data processing, a high immunotoxicological index (value > 10) was established in this site compared with normal values expected in the range of 2–4 units (Fig. 2b). This elevated index value summarized variations of several elementary, immunological parameters from their reference value. All these features considered together strongly suggest that immunosuppression occurred in these oysters reared from year-1999 spat and settled there in spring 2000. Induction of immunotoxic responses in molluscs collected from contaminated stocks is well documented (Auffret 2004). Structural alterations in hemolymph affecting cell numbers and viability have been regularly reported (Pipe and Coles 1995) and do reflect systemic, deleterious effects during the toxicokinetic phase of contamination. Moreover, any depression of phagocytosis appears as a direct evidence of impaired immune function in individuals (Fournier et al. 2000, 2001).

Table 3. Average values for hemolymph parameters measured in oyster samples collected at the four sites during the *Erika* survey. Station labels and abbreviations are those used in the text. Stock labels refer to spat from which oysters were reared in each site as described in materials and methods section. Results are expressed as the mean ± SEM (*n*: number of individual). Nd: no data. Site comparison at each sampling date (A-2001, S-2002 or A-2002) was made for N00. Values that share a same letter for the same sampling date were not statistically different.

	Oysters		Total hemocyte counts	Serum ACP	Cell mortality	$\Delta\Psi_m$	Hemocyte metallothioneins	Phagocytic index	Phagocytic capacity	ROS level
Site	Stock	Sampling date	10^6 cells ml^{-1}	Units.mg^{-1} PRTS	%	Units	ng mg^{-1} protein	% positive cells	Units	Units
Pen-Bé	N99	A-2001	1.5±0.2 (15)	55 ± 2 (15)	0.7 ± 0.1 (15)	237±22 (10)	2.04±0.29 (15)	34±2 (10)	1165±85 (10)	121±8 (10)
	N00	A-2001	4.6±0.9 (15)^{ab}	57 ± 3 (15)	nd	165±32 (10)	2.94±0.41 (15)^{b}	23±3 (10)^{a}	790±65 (10)^{a}	100±11 (10)
		S-2002	2.7±0.4 (10)^{a}	63 ± 7 (10)^{ab}	0.9 ± 0.3 (10)^{b}	112±18(10)^{b}	0.60±0.07 (10)^{b}	51±2(10)^{a}	1520±95 (10)^{b}	52±5 (10)^{a}
		A-2002	0.8±0.1 (10)^{b}	26 ± 2 (10)^{b}	1.5 ± 0.2 (10)^{b}	57±5 (10)	0.15±0.05 (10)	42±3 (9)	1750±55 (9)^{b}	85±5 (10)^{b}
	N01	A-2002	1.4±0.3 (10)	28 ± 3 (10)	0.7 ± 0.2 (10)	72±5 (10)	0.38±0.12 (8)	44±4 (10)	1425±100 (10)	62±6 (10)
		S-2003	4.1±1.2 (10)	nd	1.0 ± 0.3 (10)	38±3 (10)	0.35±0.06 (10)	49±4 (10)	1500±115 (10)	134±25 (10)
Le Croisic	N99	A-2001	4.1±0.6 (14)	53 ± 3 (13)	0.2 ± 0.1 (14)	40±6 (10)	2.57±0.37 (13)	21±2 (10)	675±105 (10)	77±13 (10)
	N00	A-2001	2.7±0.3 (13)^{ab}	59 ± 3 (14)	0.2 ± 0.1 (15)^{a}	446±127 (10)	1.84±0.34 (13)^{ab}	34±2 (10)^{b}	780±115(10)^{a}	73±8 (10)
		S-2002	0.9±0.1 (10)^{b}	50 ± 2 (10)^{a}	1.6 ± 0.2 (10)^{a}	227±14 (10)^{a}	1.29±0.19 (10)^{a}	56±3 (10)^{a}	1860±85 (10)^{a}	46±3 (10)^{a}
		A-2002	1.2±0.2 (10)^{a}	37 ± 4 (10)^{a}	0.9 ± 0.2 (10)^{ab}	64±6 (10)	0.48±0.05 (10)	41±3 (10)	1055±85 (10)^{a}	47±4 (10)^{a}
	N01	A-2002	0.8±0.2 (10)	25 ± 2 (10)	1.9 ± 0.3(10)	104±11 (10)	0.46±0.08(10)	52±4 (10)	1785±120 (10)	84±10 (10)
		S-2003	2.1±0.4 (10)	nd	1.7 ± 0.4 (10)	44±6 (10)	0.44±0.08 (9)	38±3 (10)	1105±100 (10)	59±4 (10)
	N02	S-2003	2.9±0.6 (10)	nd	0.8 ± 0.2 (10)	31±3 (10)	0.34±0.09 (10)	43±4(10)	1230±65) (10	72±7 (10)
Moutiers	N99	A-2001	2.7±0.4 (15)	58 ± 6 (15)	1.1 ± 0.3 (14)	225±25 (10)	1.80±0.20 (15)	35±2 (10)	1080±160 (10)	71±5 (10)
	N00	A-2001	4.2±0.5 (20)^{a}	56 ± 1 (18)	0.6 ± 0.1 (20)^{b}	266±27 (10)	1.43±0.12 (20)^{a}	28±2 (14)^{ab}	1140±85 (14)^{b}	91±13 (9)
		S-2002	1.4±0.2 (10)^{b}	58 ± 4 (10)^{ab}	0.6 ± 0.1 (10)^{b}	218±44 (10)^{a}	0.50±0.09 (10)^{b}	47±4 (10)^{ab}	1495±80 (10)^{b}	42±5 (10)^{ab}
		A-2002	0.9±0.1 (10)^{ab}	31 ± 4 (10)^{ab}	0.7 ± 0.2 (10)^{a}	47±5 (10)	0.17±0.05 (8)	43±3 (10)	1330±75 (10)^{c}	53±6 (10)^{a}
	N01	A-2002	1.0±0.3 (10)	41 ± 3 (10)	1.2 ± 0.2 (10)	90±12 (10)	0.17±0.03 (10)	32±3 (10)	945±50 (10)	53±9 (10)
		S-2003	2.3±0.5 (10)	nd	1.2 ± 0.2 (10)	48±7 (10)	0.25±0.07 (10)	34±4 (10)	1025±60 (10)	69±5 (10)
	N02	S-2003	3.2±0.6 (10)	nd	0.9 ± 0.3 (10)	38±6 (10)	0.15±0.03 (10)	42±3 (10)	1010±60 (10)	94±13 (10)
Rade	N00	A-2001	2.1±0.3 (15)^{b}	60 ± 6 (15)	1.2 ± 0.3 (15)^{c}	217±30 (9)	1.42±0.24 (15)^{a}	21±3 (10)^{a}	820±60 (10)^{ab}	98±7 (10)
		S-2002	1.0±0.2 (10)^{b}	70 ± 4 (10)^{b}	1.5 ± 0.2 (10)^{a}	88±15 (10)^{b}	1.25±0.21 (6)^{a}	38±3 (10)^{b}	965±35 (10)^{c}	33±2 (10)^{b}
		A-2002	1.3±(0.2 (10)^{a}	48 ± 3 (10)^{c}	0.9 ± 0.2 (10)^{ab}	56±5 (10)	0.42±0.10 (10)	41±3 (10)	1290±65 (10)^{ac}	78±8 (10)^{b}
	N01	A-2002	1.0±0.2 (10)	40 ± 8 (10)	2.6 ± 0.3 (10)	109±12 (10)	0.19±0.02 (8)	45±3 (10)	2090±130 (10)	101±14 (10)

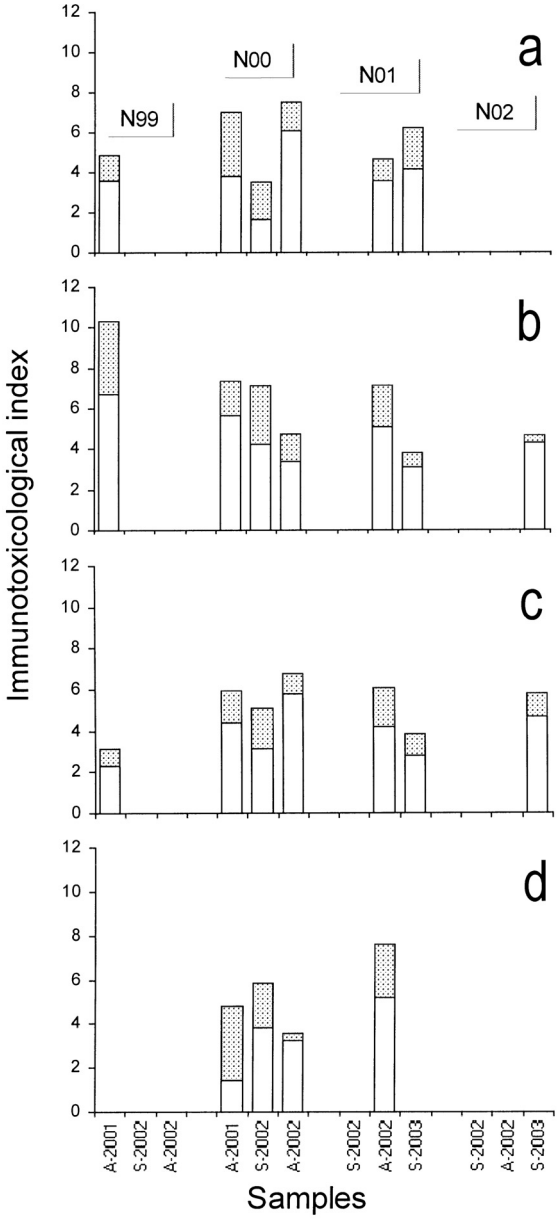

Fig. 2. Cumulative immunotoxicological index (white bar: immunopathology, grey bar: immunocompetence) calculated in oysters sampled from November 2001 to March 2002 at the three impacted sites (a: Pen Bé, b: le Croisic, c: Moutiers) and at the non-impacted area (d: Rade de Brest). The samples were grouped on the horizontal axis according to spat characteristics. Sample labels are those detailed in Table 1.

Stocks reared from year-2000 spat had been settled in impacted sites 10 to 15 months after the spill. At Le Croisic, very high hemocyte numbers were measured in November 2001 (Table 3). In this sample however, all phagocytosis parameters were abnormally low. Consequently, the immunotoxicological index reached nearly 8 units (Fig. 2b). The spring after, this high level was maintained. Persistent alterations of immune parameters at this site could be related to environmental stress generated by winter conditions and possibly, hydrocarbon release as mentioned above. At Pen Bé and Moutiers, high index values were calculated in autumn 2001 and 2002 (Figs. 2a,c), suggesting a seasonal variation pattern. Finally, the most severe immunotoxic alterations were observed at Le Croisic in the first samples of the survey, more than twenty months after the spill. This persistence of effects suggests that they rather resulted from chronic contamination than from the spill itself. All the sites appeared to have recovered two years later.

In the non-impacted area, immune parameters were occasionaly found altered, as a reduced phagocytic activity in November 2001 and low numbers of circulating cells in 2002 (Table 3). Consequently, the index was above expected values (Fig. 2d) but taken as a whole, this immunological condition most probably revealed temporary, environmental stress in this estuarine site. Indeed, physico-chemical factors such as salinity and temperature naturally varying in coastal waters may affect hemocyte activities (Fisher 1988; Anderson 1993; Auffret and Oubella 1994). Even if site-specific differences in hemocyte characteristics may occur in oysters (Oliver and Fisher 1995), the immunological changes revealed here strongly suggest that natural environmental factors may also generate physiological stress. Complementary investigations would be necessary to study its influence on immune parameters of benthic organisms and especially, possible synergistic effects with pollution.

The immunotoxicological index allowed here to identify samples suffering immunological stress, even if the reference value had been calculated from all sites and sampling dates. This calculation procedure was found reasonable here because the survey was run after the major episode of contamination in a wide geographic area. By including samples where immunological changes occurred, reference values were undoubtedly overestimated and slight alterations obscured. To increase the discriminating power of the method, it is recommanded that baseline values would be obtained for this species by a long-term survey in pristine sites and subsequently integrated in index calculation. The definition of quantitative, descriptive parameters deriving from multiparametric sets of data generally contributes to the development of physiological biomarkers to assess possible effects of environmental stress in contaminated ecosystems. For immunological alterations which appear as various structural and functional changes, the calculation of an immunotoxicological index may increase the ecotoxicological relevance of the observations.

Acknowledgements. The authors wish to thanks the French MEDD for funding this monitoring study and the oyster farmers P. Chellet, J. Garel and G. Le Gal who reared the stocks and kindly collaborated to sampling campaigns.

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