

Seasonal variation of pollution biomarkers to assess the impact on the health status of juvenile Pacific oysters *Crassostrea gigas* exposed in situ

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Abstract

Background, aim, and scope In this study, a suite of sublethal stress biomarkers were analyzed in juveniles of the sentinel species, the Pacific oyster *Crassostrea gigas*, with a view to using them as pollution monitoring tools. The aim of this work was (1) to study baseline seasonal variations of biomarkers in different body compartments of *C. gigas* in the reference site and, after selecting biomarkers presenting no seasonal variations, (2) to compare responses of these biomarkers between contaminated and reference sites.

Materials and methods Juvenile oysters were transplanted from Bouin (France), a reference site, to three different sites in Marennes-Oleron Bay (France), located in another water body and next to different contamination sources. Animals were exposed in situ for 3 months in summer, autumn, and winter. The following biomarkers were measured: superoxide dismutase (SOD) and glutathione peroxidase (GPx) in gills and digestive gland and lysozyme and phenoloxidase (PO) in plasma.

Results No significant seasonal variations for SOD in gills and digestive gland, GPx in gills, and PO in plasma were observed in the reference site. Significant differences in enzyme activity were observed between contaminated and reference sites for SOD in gills and digestive gland and PO in plasma, depending on the body compartment, the season, and/or the site.

Conclusions In conclusion, these data suggest the potential application of these biomarkers in *C. gigas* to provide ecologically relevant information and, therefore, to be used as biomarkers in coastal pollution monitoring.

Keywords Biomarkers · Pollution · Monitoring · Antioxidant enzymes · Immune defenses · *Crassostrea gigas*

1 Background, aim, and scope

Sessile and filter feeder organisms inhabiting coastal waters and estuaries, such as the Pacific oyster *Crassostrea gigas* (Thunberg 1753), are constantly in contact with various chemical products. Therefore, networks such as the National Monitoring Network (RNO) in France use this organism as a sentinel species to assess the presence of pollutants on coasts and estuaries. However, massive summer mortalities of this ecologically and economically important organism, and particularly of juvenile populations, have become a widespread concern in the world in recent decades (Cheney et al. 2000; Garnier et al. 2007; Perdue et al. 1981). Marennes-Oleron Bay is the first production site of this species in France (Soletchnik et al. 1999), but this area is also subjected to many recurring pollution by heavy metals, pesticides, and polycyclic aromatic hydrocarbons (PAHs), brought mainly by the Charente River and to a less extent by the Seudre and the Gironde rivers (Miramand et al. 2003; Munaron 2004; Munaron et al. 2006). Nevertheless, environmental stress from pollutants seems to be an important determining factor signaled by the occurrence of or by the increase of diseases (Lacoste et al. 2001).

To cope with this type of challenges, bivalve molluscs possess the two types of innate responses: cellular, i.e.,

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phagocytosis and encapsulation, and humoral, i.e., lectins, cytokines, heat shock proteins, nitric oxide synthases, proteases, antimicrobial peptides, phenoloxidas (PO) and hydrolytic enzymes such as lysozymes (Tryphonas et al. 2005). Moreover, during phagocytosis, reactive oxygen species (ROS), such as O_2^- and H_2O_2 , play an important role in protection against pathogens. The content of ROS in the organism is regulated by antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx; Neumann et al. 2001). These antioxidant enzyme activities may be modulated by the presence of contaminants (Cavaletto et al. 2002; Cheung et al. 2004; Verlecar et al. 2007). Other immune parameters such as lysozyme and PO activities may also be modulated by contaminants. High concentrations of zinc (Zn) have shown to inhibit lysozyme activity in the sea star (*Marthasterias glacialis*) mucus (Stabili and Pagliara 2009). In addition to that, a 48-h exposure of a soluble fraction of heavy fuel oil has shown to inhibit PO activity in plasma of *C. gigas* (Bado-Nilles et al. 2008), and the presence of trichlorfon has been shown to inhibit PO activity in the giant freshwater prawn (*Macrobrachium rosenbergii*; Chang et al. 2006). Biomarkers are defined as measurements on body fluids, cells, tissues, or on whole animals that indicate the presence of contaminants in biochemical, cellular, physiological, behavioral, or energetic terms (Bodin et al. 2004; Livingstone 2003; Magni et al. 2006; Ross et al. 2002). Therefore, these immune parameters may be used as effect biomarkers, i.e., to detect sensitive signals for deleterious effects of environmental contamination (Bernier et al. 1995; Luster et al. 1989). Some effect biomarkers, such as antioxidant enzymes, may also be used as exposure biomarkers, i.e., to detect if the organism has been exposed to pollutants (Winston and Di Giulio 1991). An increasing use of both types of biomarkers has appeared in recent years in programs such as the North Sea Task Force Monitoring Master Plan and the National Oceanic and Atmospheric Administration's National Status and Trends Program (Cajaraville et al. 2000).

Laboratory studies may help to identify biomarkers of interest. However, a lack of validation may occur when using these biomarkers in the field with organisms from contaminated and reference sites, e.g., immune parameters may vary depending on the season (Auffret and Oubella 1994; Oliver and Fisher 1995) and/or biomarker responses may vary depending on the body compartment of the organism that is analyzed (Amiard et al. 2006; Geffard et al. 2001). Therefore, it is important to know baseline seasonal variations of biomarkers, especially when they are used to monitor the presence and/or effect of contaminants during in situ studies. The aims of the present study were (1) to assess baseline seasonal variations of different biomarkers

in different body compartments of *C. gigas* collected from a reference site and, after selecting biomarkers presenting no seasonal variations, (2) to compare the responses of these biomarkers between contaminated and reference sites. To this end, juvenile Pacific oysters *C. gigas* were transplanted from Bouin (France), a reference site located far from pollution sources, to three different sites in Marennes-Oleron Bay, located in another water body and next to different contamination sources, for 3 months in summer, autumn, and winter. Juveniles were chosen among other developmental stages since they have shown to be more sensitive to summer mortality events (Perdue et al. 1981). Biomarker analyses were carried out by measuring SOD and GPx levels in gills and digestive gland and lysozyme and PO levels in plasma.

2 Materials and methods

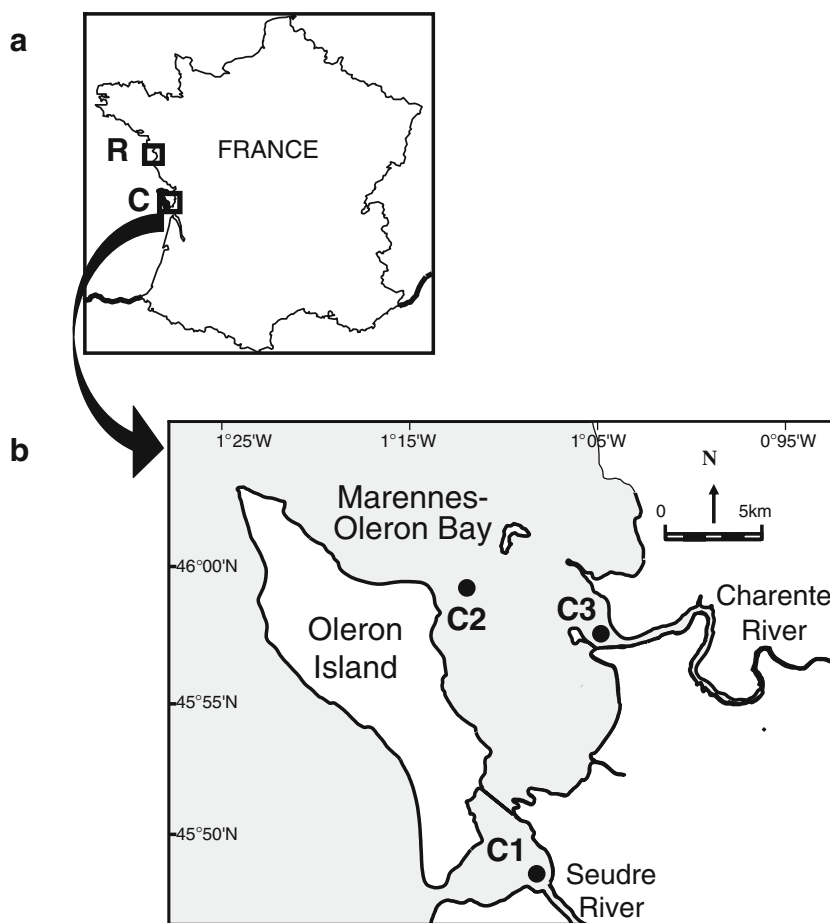
2.1 Study area and transplant design

Juvenile Pacific oysters *C. gigas* (3–4 cm in height) were purchased from the oyster hatchery France Naissain, located at Bouin in Vendée, France (46° 58' 28" North, 2° 00' 02" West), considered as a reference site (Fig. 1a; Geffard et al. 2002). All animals were issued from the same cohort in order to reduce genotype variability. Juveniles were transplanted from Bouin to three "contaminated" sites that were far from the reference site, in the Marennes-Oleron Bay, C1, C2, and C3, i.e., C1: Mus du Loup (45° 46' 10" North, 1° 08' 30" West), mildly influenced by the Seudre River; C2: Boyard (45° 58' 0" North, 1° 15' 0" West), mildly influenced by the Gironde and the Charente rivers; C3: Les Palles (45° 58' 0" North, 1° 08' 0" West), highly influenced by the Charente River (Fig. 1b). Three months after transplantation, nine oysters from each site were collected and were processed immediately after their arrival in the laboratory. Experiments were carried out in summer, autumn, and winter 2008.

2.2 Sample procedure

Pooled gills, digestive glands, and plasma of three oysters were used for each replicate sample, and three replicates were prepared per season and per site. After opening the oyster shells by cutting off the adductor muscle, a quantity of hemolymph (~0.3–0.5 ml for the three oysters) was withdrawn and centrifuged (260×g, 10 min, 4°C) to separate the cellular fraction from the plasma. Gills and digestive gland were removed from oysters. For a pool of three oysters, about 0.7±0.1 g of gills and 4.2±1.2 g of digestive gland were homogenized at 4°C in 0.1 M Tris-HCl buffer pH 7.0 (0.45 M NaCl, 26 mM MgCl₂, 10 mM CaCl₂; 0.5 ml of buffer g⁻¹ of fresh weight for the gills and

Fig. 1 Estuarine sampling areas in French Atlantic coast: **a** localization of reference (*R*) and contaminated (*C*) sites in France and **b** localization of contaminated sites Mus du Loup (*C1*), Boyard (*C2*), and Les Palles (*C3*) in Marennes-Oleron Bay



1 ml g⁻¹ of fresh weight for the digestive gland), using an Ultra Turrax (T25 basic, IKA-WERKE) and a Thomas-Potter homogenizer (IKA-Labortechnik RW 20.n, size 0.13–0.18 mm, BB). The homogenate was centrifuged at 10,000×g for 10 min at 4°C. The resulting supernatant was collected for enzymatic studies.

2.3 Biochemical analysis

Superoxide dismutase assay SOD was determined by an indirect method (Therond et al. 1996), based on competition of SOD with iodonitrotetrazolium (INT) for dismutation of the superoxide anion (O₂⁻). In the presence of O₂⁻, INT is reduced into a red formazan dye that can be measured at 505 nm at 25°C (kit Ransod SD 125, Randox, France). One unit of SOD is defined as the amount of enzyme that promotes a 50% decrease in the rate of INT reduction.

Glutathione peroxidase assay GPx activity was determined according to the method of Paglia and Valentine (1967). In the presence of glutathione reductase and substrates (i.e., reduced glutathione and cumene hydroperoxide), the decrease of absorbance at 340 nm is proportional to the reduction of the oxidized glutathione by NADPH, H⁺ (Kit

Ransel RS 504, Randox, France). One unit of GPx oxidizes 1 μmol of NADPH (ε_{NADPH}=6.22 mM⁻¹ cm⁻¹) per minute.

Lysozyme assay Lysozyme assay was done in triplicate for each sample and compared against hen egg white lysozyme standards (0.4–40 μg ml⁻¹) in the presence of *Micrococcus lysodeikticus* (Sigma-Aldrich, France). One unit of lysozyme corresponds to the amount of enzyme that diminishes absorbance at 450 nm of 0.001 per minute at pH 7.0, at 25°C (Soudant et al. 2004).

Phenoloxidase assay PO levels in plasma have been reported to be higher than PO levels in hemocytes of *C. gigas* (Hellio et al. 2007). Therefore, PO assay was measured in plasma and was carried out in triplicate for each sample in the presence of 100 mM dopamine and 0.1 M Tris-HCl buffer, pH 7.0 (0.45 M NaCl, 26 mM MgCl₂, 10 mM CaCl₂; Sigma-Aldrich, France). PO activity was followed by monitoring the increase of absorbance at 490 nm for 4 h. Nonenzymatic oxidation by the substrate was followed in wells without plasma and subtracted to oxidation of the substrate with plasma. One unit of PO specific activity corresponds to the amount of enzyme that catalyzes the production of 1 μmol of dopachrome

($\varepsilon_{\text{Dopachrome}}=3,300 \text{ M}^{-1} \text{ cm}^{-1}$) per minute (Pomerantz 1963; Fenoll et al. 2002).

Protein assay All activities were expressed in relation to protein concentration measured according to the Bradford method with slight modifications, by using bicinchoninic acid and copper sulfate 4% (Smith et al. 1985). Serum albumin was used as standard (Sigma-Aldrich, France).

Statistical analysis All results were expressed as mean \pm standard deviation (SD). Statistical analysis was carried out with the SYSTAT 11.0 software. Differences among seasons were checked for the reference site. For the same season, biomarkers responses were compared between contaminated and reference sites. Data were tested for normal distribution (Shapiro test) and homogeneity of variances (Bartlett test). Student's *t* test was performed to analyze results. Kruskal–Wallis nonparametric test, followed by Dunn's post hoc test, was performed when data were not normally distributed or when they exhibited heterogeneous variances (Zar 1984). Statistical significance was defined as being at the level of $p < 0.05$ or $p < 0.01$.

3 Results

3.1 Seasonal variations of biomarkers in the reference site

The results of SOD and GPx levels in gills and digestive gland measured in oysters collected from the reference site in summer, autumn, and winter are summarized in Fig. 2. There were no statistically relevant seasonal differences ($p > 0.05$) in SOD levels in gills (Fig. 2a) and in digestive gland (Fig. 2b). However, GPx baseline seasonal variations differed between gills and digestive gland: while no significant differences ($p > 0.05$) were observed in GPx levels in gills in summer, autumn, and winter (24.83 ± 7.44 , 16.58 ± 4.38 , and $16.03 \pm 8.90 \text{ U GPx mg}^{-1} \text{ protein}$, respectively; Fig. 2c), significant differences ($p < 0.01$) were observed in the digestive gland (Fig. 2d). Indeed, GPx levels in the digestive gland in summer were about twofold lower than GPx levels in autumn and winter (38.29 ± 8.68 , 64.55 ± 11.15 , and $77.51 \pm 5.37 \text{ U GPx mg}^{-1} \text{ protein}$, respectively; Fig. 2d).

Studies on lysozyme levels were carried out in plasma of *C. gigas*. Significant differences in plasmatic lysozyme levels ($p < 0.01$) were observed in oysters collected at the three different seasons (Fig. 3a). Lowest lysozyme levels were observed in winter, when compared to summer and autumn (42.90 ± 4.07 , 51.94 ± 3.27 , and $64.44 \pm 3.93 \text{ U lysozyme mg}^{-1} \text{ protein}$, respectively). No statistically relevant seasonal differences ($p > 0.05$) were observed in

plasmatic PO levels, i.e., in summer, autumn, and winter (53.90 ± 9.77 , 51.81 ± 9.32 , and $52.45 \pm 7.99 \text{ U PO mg}^{-1} \text{ protein}$, respectively; Fig. 3b). Therefore, since no significant seasonal differences were observed in SOD in gills and digestive gland, GPx in gills, and PO in plasma, these biomarkers were selected for further studies.

3.2 Biomarker responses in contaminated sites

The results of SOD levels in gills and digestive gland, GPx levels in gills, and PO levels in plasma collected from the three contaminated sites, i.e., C1 (Mus du Loup), C2 (Boyard), and C3 (Les Palles) and from the reference site, R (Bouin), are summarized in Fig. 4. In the present comparison, responses of biomarkers in C3 were measured only in summer and winter.

There were statistically significant differences in SOD levels in gills for the three contaminated sites C1, C2, and C3 in summer (Fig. 4a). Indeed, SOD levels in gills were lower in contaminated sites compared to the reference site. In autumn, no significant differences were observed. In winter, SOD contents in gills were lower only in C2 when compared to the reference site.

In the digestive gland, statistically significant differences in SOD levels were observed depending on the season and/or the site (Fig. 4b). Indeed, while higher SOD contents were observed in C1 and C3 in summer, no significant differences were observed in autumn for C1 and C2. In winter, higher contents of SOD were observed in C1 and C2 and lower contents were observed in C3.

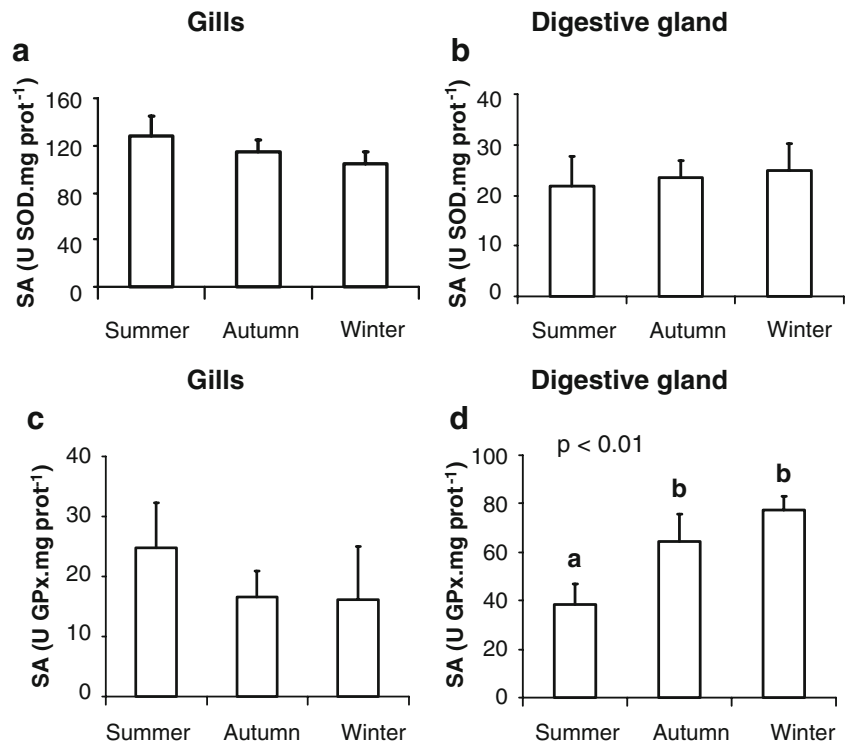
The results of GPx levels in gills in contaminated and reference sites are summarized in Fig. 4c. No significant differences were observed in summer for C1, C2, and C3, in autumn for C1, and in winter for C3. Low GPx levels were measured in autumn in C2 and winter in C3, and high GPx levels were measured in winter in C1.

Results on plasmatic PO levels revealed significantly lower levels in summer in C1, C2, and C3 and in autumn in C1 and C2, but higher levels in winter in C1 and C2, when compared to the reference site (Fig. 4d).

4 Discussion

Specific biomarkers are often used for studies in laboratory-controlled conditions (e.g., Boutet et al. 2002; Guilhermino et al. 1996). However, in the field, environmental conditions may modulate responses of biomarkers (Matsunaga et al. 1989; Monserrat et al. 2007; Neumann and Peter 1987; Sepcic et al. 1998). Moreover, compensatory adaptive mechanisms between different biomarkers may occur as well (Regoli and Principato 1995) and biomarker responses may differ depending on the body compartment of the

Fig. 2 Seasonal variations on SOD (**a** and **b**; in units of SOD per minute per milligram of protein) and GPx (**c** and **d**; in units of GPx per minute per milligram of protein) in gills and digestive gland of *C. gigas* collected from the reference site (Bouin). Letters indicate significant differences between seasons ($p < 0.01$). Data are expressed as mean \pm SD



organism that is analyzed (Amiard et al. 2006; Geffard et al. 2001). In the green-lipped mussel, *Perna viridis*, seasonal SOD variations observed in gills and digestive gland were likely to be due, in part, to variations of temperature in the water column (Verlecar et al. 2008). In the case of the present study, no seasonal variations were observed in SOD in gills and digestive gland in the reference site. This suggests that antioxidant modulations differ between species and that is necessary to study baseline levels on the organism of interest before comparing biomarker responses between contaminated and reference sites.

In the present study, significant seasonal differences were observed in GPx levels in digestive gland in the reference site, with lower levels observed in summer. These

results were in agreement with other studies that revealed similar seasonal variations of GPx in *P. viridis* from the Arabian Sea (Verlecar et al. 2008). Winter is a period where oxidative stress is known to be high in bivalves (Manduzio et al. 2004). Therefore, high GPx activities in the digestive gland in autumn and winter may be a consequence of high oxidative stress. It is important to notice that no seasonal variations were observed in GPx levels in gills, making studies on pollution monitoring more interesting in this body compartment. Moreover, these results are in agreement with Manduzio et al. (2004) who suggested that biomonitoring studies dealing with oxidative stress in the blue mussel, *Mytilus edulis*, should preferentially be carried out in the gills rather than in the digestive gland.

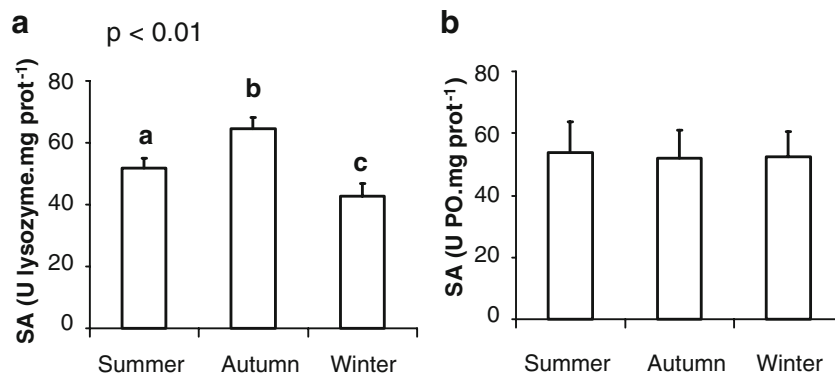


Fig. 3 Seasonal variations on **a** lysozyme (in units of lysozyme per minute per milligram of protein) and **b** PO (in units of PO per minute per milligram of protein) in plasma of *C. gigas* collected from the

reference site (Bouin). Letters indicate significant differences between seasons ($p < 0.01$). Data are expressed as mean \pm SD

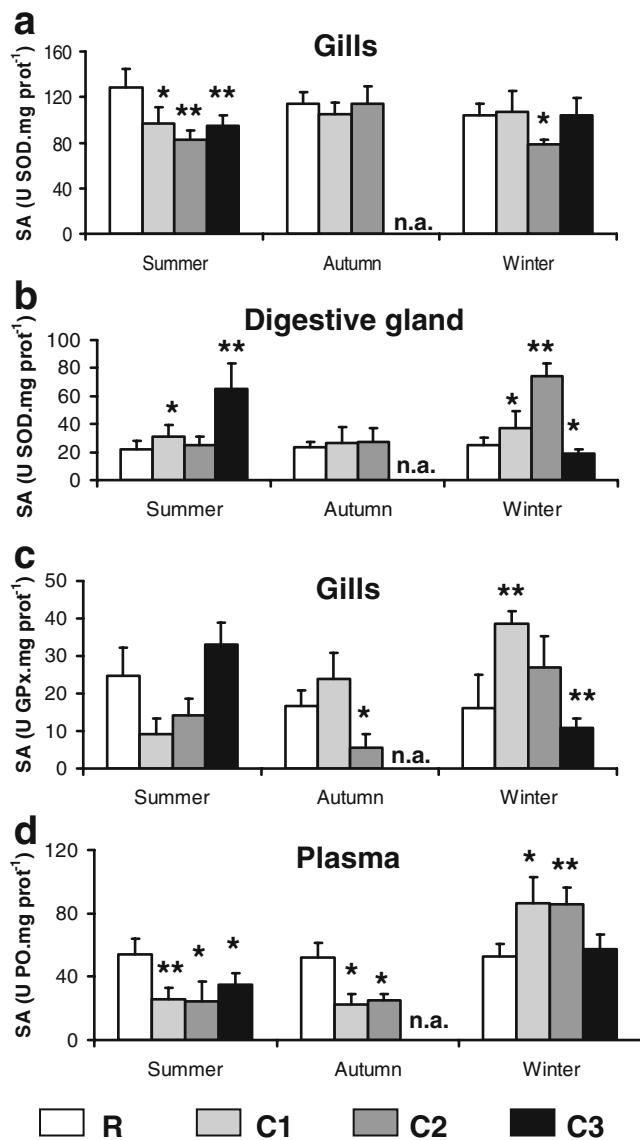


Fig. 4 SOD in gills and digestive gland, GPx in gills, and PO in plasma in *C. gigas* collected from contaminated and reference sites. R reference site (Bouin, white bars), C1 contaminated site 1 (Mus du Loup, light grey bars), C2 contaminated site 2 (Boyard, dark grey bars), C3 contaminated site 3 (Les Palles, black bars). Data are expressed as mean \pm SD. * $p < 0.05$; ** $p < 0.01$

In the present study, significant seasonal differences were observed in plasmatic lysozyme activity. Results revealed lower levels in winter and summer. Low lysozyme levels in summer were in agreement with results in other molluscan and nonmolluscan species (Chu et al. 1995; Hutchinson and Manning 1996). However, in the present study, low levels were also observed in winter. Even if generally low lysozyme levels are associated to the reproductive cycle of oysters in summer, low levels have also been found in the flat oyster *Ostrea edulis* in winter (Cronin et al. 2001). This suggests that low lysozyme levels

are not necessarily associated to the reproductive cycle of oysters, which starts at the end of spring (Fabioux et al. 2005). No significant seasonal differences were observed in PO levels in plasma. However, seasonal variations of PO activities have been reported in different species (Ferrer et al. 1989; Hauton et al. 1997; Travers et al. 2008). These seasonal variations have been attributed to bacterial population contents in the water column or to the reproductive state of the organism. Ferrer et al. (1989) observed seasonal variations in PO activity after treating with trypsin but not in endogenously active PO in the spiny lobster *Panulirus argus*. Therefore, results of the present study are in agreement with Ferrer et al. (1989) since, in the present study, only endogenously active PO levels were measured. No significant seasonal variations were observed in SOD in gills and digestive gland, GPx in gills, and PO in plasma in the reference site. Interestingly, these results suggest that differences in water temperature (18.39 ± 1.73 in summer, 15.33 ± 2.89 in autumn, and 8.83 ± 2.18 in winter; Table 1), in age (12 months in summer, 15 months in autumn, and 18 months in winter), and in condition index (31.85 ± 5.43 in summer, 23.90 ± 1.42 in autumn, and 17.15 ± 1.42 in winter; Table 1) between seasons do not modulate responses of these biomarkers. Consequently, SOD in gills and digestive gland, GPx in gills, and PO in plasma were measured in oysters transplanted to different contaminated sites in Marennes-Oleron Bay.

Comparisons of biomarker responses between reference and contaminated sites revealed that (1) SOD levels in gills were generally lower, (2) SOD levels in digestive gland were generally higher, (3) GPx levels in gills were not modulated in summer but were lower in autumn in one contaminated site and lower or higher in summer depending on the contaminated site, and (4) PO levels were generally lower in summer and autumn and higher in winter. Differences in pollutant contents between contaminated and reference sites could explain differences observed in biomarker responses, e.g., in the case of GPx in gills or SOD in the digestive gland, as it has been shown by other studies carried out in different contaminated sites (Bodin et al. 2004; Valdez Domingos et al. 2007). Marennes-Oleron Bay is an area subjected to many recurring pollution, brought mainly by the Charente River (i.e., contaminated site C3) and to a less extent by the Gironde (i.e., C2) and the Seudre rivers (i.e., C1). The recrudescence use of herbicides in agriculture implies the transfer towards the aquatic environment of new pollutants in estuarine areas (Munaron 2004; Munaron et al. 2006). In 2001, the Charente River discharged approximately 1,360 kg of herbicides and, in 2002, about 510 kg (Munaron 2004). For most herbicides that were studied, summer (July, August, and September) corresponded to the period when quantities of herbicides provided by the Charente River

Table 1 Pollutant contents in oysters and physicochemical and biological parameters of the reference (Bouin, R) and contaminated sites (Mus du Loup, C1; Boyard, C2; Les Palles, C3) in summer (S), autumn (A), and winter (W) 2008

	National median	R			C1			C2			C3		
		S	A	W	S	A	W	S	A	W	S	A	W
Pollutant contents^a													
Metals (mg.kg⁻¹)													
Ag	7.69	19.50			12.30			16.20			29.90		
Cd	1.61	1.75			3.73			2.75			3.82		
Cr	0.84	1.07			1.23			1.04			1.29		
Cu	206	385			262			281			500		
Hg	0.20	0.19			0.32			0.32			0.36		
Ni	1.01	1.30			1.28			1.22			1.22		
Pb	1.30	1.30			1.60			1.45			1.55		
V	1.32	1.32			1.75			1.33			1.72		
Zn	2265	2360			2435			2680			4020		
Pesticides (µg.kg⁻¹)													
DDT, metabolites	5.22	5.76			8.31			4.28			5.03		
Lindane	0.74	0.39			0.90			0.79			0.46		
PAHs (µg.kg⁻¹)													
Fluoranthene	27.0	20.2			33.7			27.8			23.5		
Physicochemical parameters													
Temperature (°C) ^b	n.a.	18.39± 1.73	15.33± 2.89	8.83± 2.18	19.93± 2.09	15.59± 3.12	9.76± 2.64	19.26± 1.96	15.58± 3.48	9.36± 1.97	20.13± 1.98	16.35± 3.49	8.01± 1.56
Temperature (°C) ^c	n.a.	n.a.	n.a.	n.a.	19.40	18.90	8.20	20.08	19.20	7.85	20.50	18.65	7.10
Biological parameters													
Chlorophyll a (mg.m ⁻³) ^b	n.a.	1.62± 1.91	1.04± 0.91	1.11± 0.90	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.35± 3.28	2.39± 1.41	0.80± 0.37
Chlorophyll a (mg.m ⁻³) ^c	n.a.	n.a.	n.a.	n.a.	2.58	1.65	0.62	2.83	3.43	1.045	2.35	1.72	1.12
Condition index ^d	n.a.	31.85± 5.43	23.90± 1.42	17.15± 1.42	55.19± 1.63	30.74± 3.27	17.52± 0.94	40.17± 1.49	19.22± 1.93	16.73± 2.42	42.93± 3.02	n.a.	19.71± 1.94

n.a. not available

^a Mean values adapted from 2003 to 2007 data of the website “Site Ifremer consacré à l’environnement littoral”: <http://www.ifremer.fr/envlit/>

^b Mean values ± SD adapted from 2000 to 2004 data of the website “Site Ifremer consacré à l’environnement littoral”: <http://www.ifremer.fr/envlit/>

^c Mean values of 2008, collected by the French network Razlec (Ifremer; http://www.ifremer.fr/lerpc/reseaux/hydro/resul_hydro.htm). Values correspond to the mean of July, September, and December of 2008 of two measurements carried out per month at the bottom and at the top of the water surface from C1 and sites near C2 and C3

^d Condition index from juvenile oysters was calculated by the method of Lobel and Wright (1982), and expressed in mg dry flesh weight/g dry shell weight

were the lowest, i.e., between 12 and 30 kg of herbicides per month and the peak concentrations occurred especially at times when treatments were applied within the watershed, e.g., in May 2001, 400 kg of herbicides were carried down to the estuary in 1 month. Peak concentrations from 0.1 to 1 µg L⁻¹ have been reported for herbicides such as atrazine, isoproturon, diuron, and glyphosate in the Marennes-Oleron Bay (Burgeot et al. 2007). Moreover, industrial releases bring many heavy metals in these same estuarine areas (Miramand et al. 2003). Indeed, increasing levels of heavy metals [silver (Ag), cadmium (Cd),

chromium (Cr), copper (Cu), nickel (Ni), vanadium (V), and zinc (Zn)], have been reported in Marennes-Oleron Bay (Miramand et al. 2003). Similarly, these authors reported high PAHs contents in sediments and organisms from the Marennes-Oleron Bay. Some pollutant contents have been reported in sites chosen for the present study: high Cd, Hg, dichlorodiphenyltrichloroethane (DDT) and metabolites, lindane, and fluoranthene contents have been detected in C1, high Cd, Hg, and lindane contents have been detected in C2, and high Ag, Cd, Hg, and Zn contents have been detected in C3 (Table 1). These pollutants are, therefore,

likely to strongly influence the biomarkers levels in oyster tissues and fluids.

Among all biomarkers assessed in the reference site, only SOD levels in gills and digestive gland, GPx levels in gills, and PO levels in plasma displayed low variations between seasons. Interestingly, some of these biomarkers were modulated depending on the season and, this, for the three contaminated sites. Therefore, modulations on biomarker responses could be due to (1) abiotic or biotic factors in this area such as temperature, salinity, pH, or food supply (Khessiba et al. 2005; Kirchin et al. 1992; Lima et al. 2007; Prevodnik et al. 2007; Verlecar et al. 2007), (2) modulations of contaminant contents through seasons (Niyogi et al. 2001), and/or (3) an additive effect of temperature variations and contaminant contents in these areas. Indeed, Ringwood et al. (2002) reported a correlation between lysosomal destabilization in deployed oysters *Crassostrea virginica* and tissue metal concentrations. The authors suggested that adverse effects observed in winter could be due to seasonal differences in physicochemical factors, such as reduced levels of acid volatile sulfides, which could increase the bioavailability of metals during winter. In the present study, differences in condition index were observed depending on the season and the site. Even if differences observed in the reference site suggest that this parameter do not induce modulations of the biomarkers that were selected for the present study, differences of condition index were observed between the reference and the contaminated sites. Indeed, when comparing condition index between reference and contaminated sites, this parameter was higher in summer in the three contaminated sites, higher or lower in autumn depending on the site, and higher in winter in one contaminated site (Table 1). These differences may be due to differences in food supply or environmental stressors such as changes in temperature. However, temperature data do not reveal differences between sites and chlorophyll *a* content in the water column is not always correlated with condition index of oysters (Table 1). Hence, differences in condition index may be due to differences in food composition between sites. However, while results suggest a negative correlation between condition index and GPx levels in gills and PO levels in plasma, no correlation is observed in autumn and winter. Moreover, the presence of pollutants may also induce differences in condition index. Lannig et al. (2006) reported no significant change in condition, survival rate, and lipid peroxidation in oysters, *C. virginica*, exposed to elevated temperature. However, in oysters exposed to both temperature and Cd stresses, high mortality, low condition index, and elevated lipid peroxidation were observed, implying a synergism between elevated temperatures and Cd stress on their energy metabolism. In the Marennes-Oleron area, the period when herbicides are sprayed on the

crops, combined with rainfall events, coincides with the oyster-breeding season, and these events have been suggested to influence summer oyster mortalities (Munaron 2004; Munaron et al. 2006). Therefore, more special attention should be carried out between condition index, food composition, pollutant contents, and response of biomarkers.

In conclusion, response biomarkers were modulated depending on (1) the oyster body compartment, (2) the season, and (3) the site. Therefore, results of this work suggest that SOD in gills and digestive gland, GPx in gills, and PO levels in plasma could be biomarkers of interest to assess the impact of pollutants on health status in juveniles of *C. gigas*. Further similar studies should be done on these biomarkers in other contaminated sites (e.g., Valdez Domingos et al. 2007). Moreover, similar studies should be carried out with other biomarkers in this sentinel species in order to establish baseline levels of different biomarkers of interest for coastal pollution monitoring studies.

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