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Biomarker responses in the clam *Ruditapes philippinarum* and contamination levels in sediments from seaward and landward sites in the Lagoon of Venice

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ABSTRACT

A multibiomarker approach was used to assess effects of different environmental conditions in the clam *Ruditapes philippinarum*. Bivalves were monthly collected from March 2009 to February 2010 in two sites of the Lagoon of Venice differently influenced by both anthropogenic impact and natural conditions: a seaward site (Punta Sabbioni, PS), close to the Lagoon inlet of Lido, characterised by high hydrodynamism and influenced by intense passage of ships, and a landward site (Canale Dese, CD) characterised by low hydrodynamism and influenced by both riverine inputs and agricultural waste waters. Various biomarkers were measured at cellular, tissue and organism level: total haemocyte count, pinocytotic and haemolymph lysozyme activities, gill acetylcholinesterase activity (AChE), superoxide dismutase and catalase activities in both gills and digestive gland, condition index and survival-in-air. Water temperature, pH and salinity values and total chlorophyll concentrations were measured. In addition, grain size, organic matter content, and concentrations of p,p′-DDT (dichlorodiphenyltrichloroethane) and its 5 homologues, 4 HCHs (hexachlorocyclohexanes), 13 PBDEs (polybrominated diphenyl ethers), 13 PCBs (polychlorinated biphenyls) and 18 PAHs (polycyclic aromatic hydrocarbons) were seasonally measured in surface sediments of the two sampling sites. Immunomarkers, as well as AChE activity, highlighted an overall better condition for clams from PS, whereas a different response was provided by the biomarkers measured at organism level, condition index increasing and survival-in-air decreasing in PS clams. No marked differences in sediment contamination levels (except for PAHs) were observed between CD and PS. Overall, results obtained demonstrated that biomarkers measured allowed to discriminate the two sampling sites. Considering that the contamination levels of sediments were similar between the two sampling sites, influence of abiotic factors cannot be excluded in influencing biological responses measured in clams.

1. Introduction

The Lagoon of Venice is a peculiar estuarine ecosystem of about 550 km^2 adversely affected by industrial contaminants from the industrial zone of Porto Marghera, urban untreated sewage from the cities of Venice and Chioggia and agricultural pollutants (Frignani et al., 2001a,b; Guerzoni et al., 2007; Parolini et al., 2010; Pojana et al., 2007). Parolini et al. (2010) have recently found the presence of a widespread contamination due to persistent organic pollutants in sediments from 8 sites of the Lagoon, even if sediment contamination was not always related to the localization of the expected emission sources. In that study, a non-negligible environmental hazard for benthic invertebrates has been suggested, as most of the sampling sites showed a possible moderate to high toxicity for the sediment-dwelling organisms (Parolini et al., 2010). However, other forms of disturbance for biocenosis, such as fishing and marked variations in seawater temperature and salinity, occur in the Lagoon. In this context, efforts have been addressed to developing feasible, sensitive, reproducible, and cost-effective assays for measuring biological effects of stressors in organisms.

Biomarkers, defined as quantitative measures of changes at differing biological levels indicative of exposure to stressors, have been proposed as sensitive early warning signals in environmental quality assessment (McCarthy and Shugart, 1990). Numerous biomarkers have been proposed at differing levels of the biological organisation for biomonitoring estuarine environments and to detect signs of impaired health in aquatic organisms. Among sentinel species, bivalve molluscs are recognised as appropriate

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indicator of environmental quality (Cajaraville et al., 2000). With regard to the Lagoon of Venice, the clam *Ruditapes philippinarum* has extensively been used in biomonitoring studies, being a filter-feeding mollusc living in sandy-mud bottoms of the Lagoon and thus representing a useful species to evaluate environmental quality (Boscolo et al., 2007; Da Ros and Nesto, 2005; Marin et al., 2001; Matozzo and Marin, 2007; Matozzo et al., 2005, 2010; Moschino et al., 2010). In a recent survey, biomarker responses of clams collected in 8 sites of the Lagoon provided controversial results, even if quite similar contamination levels were recorded among the sampling sites (Matozzo et al., 2010). In particular, the resistance to air exposure of clams from Canale Dese (close to the inner border of the Lagoon and influenced by agricultural waste waters) was shown to increase significantly with respect to that of animals from the other sampling sites, suggesting a good health status of clams. However, other biomarker responses, acetylcholinesterase (AChE) activity in particular, did not provide the same indication (Matozzo et al., 2010). Results of that study suggested that other environmental factors – not only pollutants – potentially influenced biomarker responses in *R. philippinarum*.

On the basis of those results (Matozzo et al., 2010), in the present study a multibiomarker approach was used to assess effects of different environmental conditions in the clam *R. philippinarum* collected monthly (March 2009–February 2010) in two sites of the Lagoon of Venice differently influenced by both anthropogenic impact and natural conditions: a landward site (Canale Dese, CD), and a seaward site (Punta Sabbioni, PS) close to the Lagoon inlet (see Section 2 for details). In addition, six different classes of persistent organic pollutants (POPs), such as p,p′-DDT (dichlorodiphenyltrichloroethane) and its 5 homologues, 4 HCHs (hexachlorocyclohexanes), 13 PBDEs (polybrominated diphenyl ethers), 13 PCBs (polychlorinated biphenyls) and 18 PAHs (polycyclic aromatic hydrocarbons) were seasonally measured in sediment from the two sampling sites.

2. Materials and methods

2.1. Sampling sites and collection of clam, seawater and sediment samples

At least 250 clams (mean shell length: 3.7 ± 0.4 cm) per site were monthly collected from March 2009 to February 2010 by a mechanical rake in two sites of the Lagoon of Venice (Fig. 1):

- *Canale Dese* (45°31′ latitude N; 12°23′ longitude E), close to the inner border of the Lagoon, far from urban and industrial sources of pollution, characterised by both low hydrodynamism and slow water renewal, and influenced by riverine inputs, agricultural waste waters and heavy boat traffic.
- *Punta Sabbioni* (45°26′ latitude N, 12°25′ longitude E), close to the Lagoon inlet of Lido, characterised by both high hydrodynamism and quick water renewal, and influenced by intense passage of ships.

During sampling, water temperature, pH and salinity values and total chlorophyll concentrations (Parsons et al., 1984) were measured. At each sampling site, three replicate sediment samples were seasonally (January, March, June and September) collected using a Van-Veen grab and a volume of 100–150 cm^3 was taken from the upper 10 cm of each sample, and frozen at −20 °C until analysis. Sediment grain size and organic matter content were measured according to Buchanan (1984) and Gaudette et al. (1974), respectively. In addition, sediment contamination levels were determined. Due to technical problems, clam sampling was not performed in August at PS, and in November at both the sampling sites.

2.2. Tissue collection

In order to measure biomarkers at both cellular and tissue level, 5 pools of 10 clams each were prepared. In total, 50 clams per site were monthly used. From each pool of 10 clams, haemolymph, gills, and digestive gland were collected as follows:

- *Total haemocyte count (THC), pinocytotic activity and haemolymph lysozyme activity determination*: haemolymph (at least 200 μl/clam) was collected from the anterior adductor muscle with a 1-ml plastic syringe and placed in Eppendorf tubes in ice. Each haemolymph pool was then divided into 3 aliquots to measure immunomarkers.
- *Measurement of AChE, superoxide dismutase (SOD) and catalase (CAT) activities*: after haemolymph sampling, shell was opened, gills and digestive gland were excised, pooled (5 pools) and then placed in tubes in ice. Aliquots from each pooled tissue were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

At organism level, two distinct groups of 30 clams each were monthly used to measure condition index (CI) and survival-in-air.

2.3. THC determination

A Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA) was used to determine THC. Pooled haemolymph (250 μl) was added to 19.75 ml of 0.45 μm-filtered seawater (FSW). THC results were expressed as number of haemocytes ($\times 10^6$)/ml haemolymph.

2.4. Pinocytotic activity: NR uptake assay

The cationic probe Neutral Red (NR) was used to evaluate the capability of haemocytes to carry out pinocytosis, as indicated in previous studies (Cajaraville et al., 1996; Matozzo et al., 2002). Pooled haemolymph was centrifuged at $780 \times g$ for 10 min. Haemocytes (at a final concentration of 10^6 cells/ml) were resuspended in an equal volume of 8 mg/l NR dye (Merck) solution in FSW, and incubated at room temperature for 30 min. They were then centrifuged at $780 \times g$ for 10 min, resuspended in distilled water, sonicated at 0 °C for 30 s with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. Supernatant, corresponding to haemocyte lysate (HL) was collected for the NR uptake assay. Absorbance at 550 nm was recorded on a Beckman 730 spectrophotometer. Results were expressed as optical density per ml haemolymph (OD/ml haemolymph).

2.5. Haemolymph lysozyme activity assay

Lysozyme activity was quantified in cell-free haemolymph (CFH) as an indicator of lysosomal membrane stability. Pooled haemolymph was centrifuged at $780 \times g$ for 10 min. The supernatant, corresponding to CFH, was collected, frozen and stored at −80 °C before analyses. Fifty microlitre of CFH was added to 950 μl of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 5 min at room temperature. Results were expressed as μg lysozyme/mg protein. Protein concentrations in CFH were quantified according to Bradford (1976).

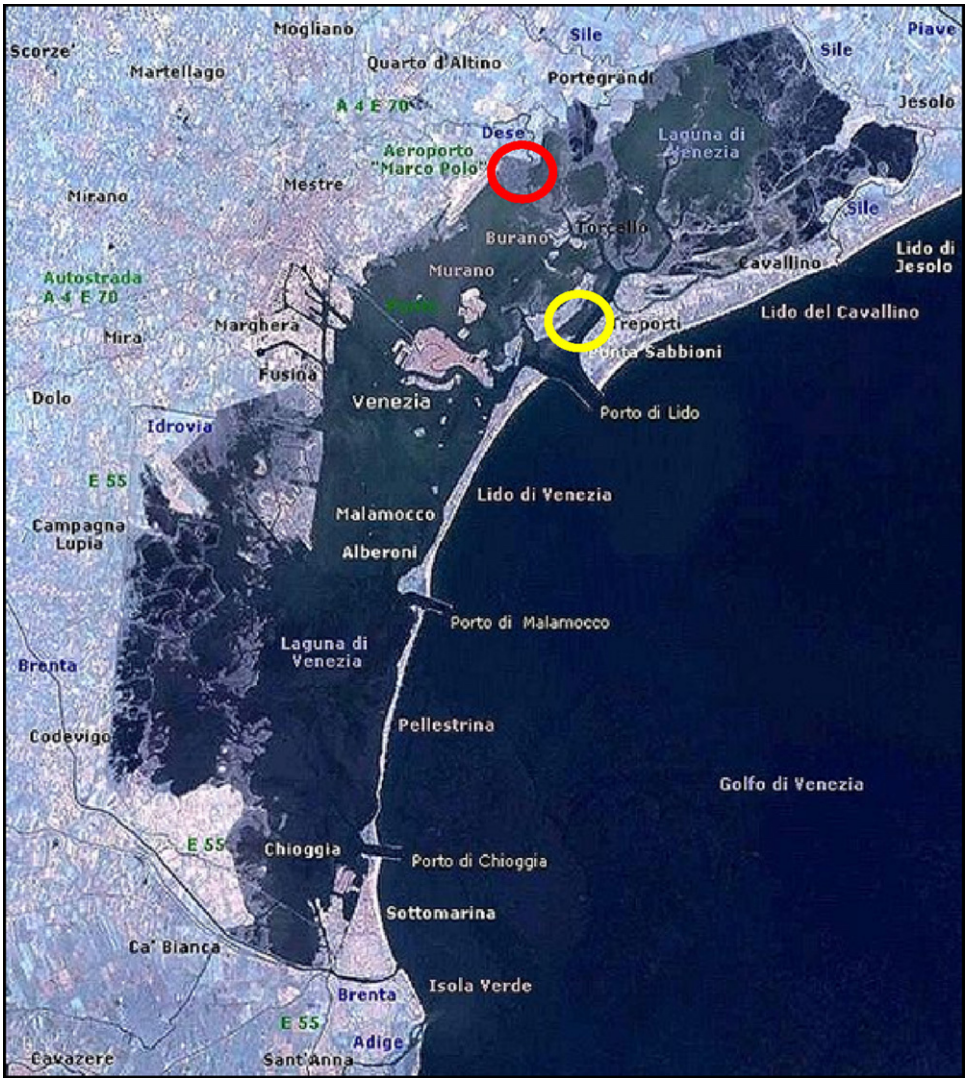

Fig. 1. Sampling sites in the Lagoon of Venice.

2.6. AChE activity

Gills were thawed on ice and homogenised (1:4, v:v) in 0.1 M Tris–HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT, Sigma) and 40 μg/mL aprotinin (Sigma), sonicated for 2 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12,000 × *g* for 45 min at 4 °C. Supernatant (SN) was collected for enzyme assay. AChE activity was determined according to the method of Ellman et al. (1961), adapted to the microplate reader (absorbance at 405 nm). Results were expressed as nmol/min/mg protein. Tissue protein concentrations were quantified according to Bradford (1976).

2.7. SOD activity assay

Total SOD activity was measured in gills and digestive glands in triplicate with the xanthine oxidase/cytochrome *c* method according to Crapo et al. (1978). Tissues were homogenised as described above, and the cytochrome *c* reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature. Enzyme activity was expressed as U/mg of proteins, one unit of SOD being defined as the amount of sample producing 50% inhibition in the assay conditions. The reaction mixture contained 46.5 μM KH_2PO_4/K_2HPO_4 (pH 8.6), 0.1 mM EDTA, 195 μM hypoxanthine, 16 μM cytochrome *c*, and 2.5 μU xanthine oxidase. Tissue protein concentrations were quantified according to Bradford (1976).

2.8. Catalase activity assay

Gill and digestive gland CAT activity was measured in triplicate following the method of Aebi (1984). Decreases in absorbance of a 50-mM H_2O_2 solution ($\varepsilon = -0.0436\,mM^{-1}\,cm^{-1}$) in 50 mM phosphate buffer (pH 7.8) and 10 μl of HL sample were continuously recorded at 240 nm at 10 s intervals for 1 min. Results were expressed in U/mg of proteins, one unit of CAT being defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2/min. Tissue protein concentrations were quantified according to Bradford (1976).

2.9. Survival in air

On their arrival at the laboratory, 30 clams from each sampling site were carefully washed in seawater, checked for vitality, and then kept in closed plastic boxes in humidity-saturated conditions, at a constant temperature of 17 °C. Mortality was recorded daily. Animals were considered dead when their shells gaped and failed to

shut again after external stimulus. LT_{50} (air exposure time resulting in 50% mortality) values were determined.

2.10. Condition index

The CI can give an indication of the general physiological status of the animals. It was calculated individually on 30 clams per site, according to the method of Walne (1976). Soft tissues were carefully separated from shells and washed in distilled water to remove dirt. Both soft tissues and shells were put in an oven at 60 °C for 48 h and then weighed. CI values are expressed as the ratio between the dry weight of soft tissues and the dry weight of shell × 100.

2.11. Chemical analyses

2.11.1. Reagents and standards

All solvents used were pesticide grade. Florisil (100–200 mesh) and anhydrous sodium sulfate were obtained from Fluka (Steinheim, Germany). Silica gel for column chromatography (70–230 mesh) was supplied by Sigma–Aldrich (Steinheim, Germany). Pesticide (Mix 164 and Mix 11), PCB (Mix 19, containing 14 CB congeners) and PAH (mix of 16 PAHs for US EPA method 610 plus 1-methylnaphthalene, 2-methylnaphthalene) standard solutions were purchased from Dr. Ehrenstorfer (Augsburg, Germany), while BDE-commonly occurring congeners mixture (PBDE-COC) and internal standards [$^{13}C_{12}$-labeled mixtures (PBDE-MXA and PDE-MXB composed by $^{13}C_{12}$-labeled BDE-47, 99, 153 and BDE-28, 154 and 183 respectively)], $^{13}C_{12}$-labeled p,p′-DDE and CB-209) were purchased from AccuStandard (New Haven, CT, USA).

2.11.2. Extraction and clean-up

Samples (about 2 g) were spiked with 10 μl (500 μg/l) of each recovery standard ($^{13}C_{12}$-labeled mixtures PBDE-MXA and PBDE-MXB; $^{13}C_{12}$-labeled p,p′-DDE and CB-209) before the 12 h extraction process, using 100 ml of acetone/*n*-hexane (1:1, v/v) mixture in a cold Soxhlet apparatus (FALC Instruments, Lurano, Italy). Clean-up was performed using a multilayer column (40 cm × 1.5 cm i.d.) made up of 10 g of silica gel (activated overnight at 130 °C, then partially deactivated with bi-distilled water, 5% W/W), 10 g of Florisil (activated for 16 h at 650 °C) and a third thin layer (about 0.5 cm) of activated (in HCl 18% for 30 min) powdered copper at the top, to remove sulfides. The column was washed with 100 ml of *n*-hexane/acetone/dichloromethane mixture (8:1:1, v/v). Two elutions were used to recover the analytes: the first by 50 ml of *n*-hexane, the second by 50 ml of *n*-hexane/dichloromethane mixture (1:1, v/v). Isooctane (1 ml) was added to the final sample, which was subsequently concentrated by rotary evaporator and subsequently under a gentle nitrogen flow to a final volume of 1 ml.

2.11.3. Instrumental analysis

An aliquot of 2 μl was injected twice into a GC chromatograph (TRACE GC, Thermo-Electron, Texas, USA) equipped with a programmed temperature vaporizer (PTV) injector and coupled with a PolarisQ Ion Trap mass spectrometer (Thermo Electron), using an AS autosampler (Thermo Electron). All the analytes were separated by an Rtx-5MS (Restek, Bellefonte, PA, USA) capillary column (30 m length, 0.25 mm i.d., 0.25 μm df). Silanized glass liners (1 ml of volume) were used. Each analytical determination was carried out following instrumental conditions specific for each chemical class: we analyzed pesticides, PCBs and PBDEs by using MS/MS mode, while PAHs were analyzed in SIM (Selective Ionization Monitoring) mode. All gas-chromatographic conditions for the determination of selected POPs were reported in detail by Binelli et al. (2007, 2008, 2009) and Sarkar et al. (2008).

2.11.4. Quality control and quality assurance

A procedural blank was run in parallel with every batch of 4 samples using anhydrous sodium sulfate (heated for 6 h at 550 °C). No organochlorine compounds (OCs) and PBDEs were detected in blanks. Since only few levels of the most volatile PAHs (naphthalene, 1-methylnaphthalene and 2-methylnaphthalene) were found, samples were corrected for these three compounds. Samples and blanks were spiked with 10 μl (500 μg/l) of internal recovery standards, namely two $^{13}C_{12}$-labeled mixtures (PBDE-MXA and PDE-MXB), $^{13}C_{12}$-labeled p,p′-DDE and CB-209, prior to the extraction procedures and recoveries over 85% were accepted. The method performance for OCs and PAHs was evaluated using standard reference material (SRM) from river sediments 1939a (NIST, Gaithersburg, MD, USA). The results for the reference material were within the range of certified values specified for the target compounds and the recoveries were on average greater than 75% for pesticides, 85% for PCBs and 74% for PAHs with respect to the reference values. PBDE method validation was carried out using river sediment (BROC-02) kindly provided by the Netherlands Institute for Fisheries Research (RIVO). Results were reported in Binelli et al. (2006), showing a relative standard deviation (RSD) less than 20% for all compounds. We performed quantitative analyses by using external multilevel calibration curves ($r^2 > 0.97$). The limits of detection (LODs) and limits of quantification (LOQs) were calculated by the signal-to noise ratio (3:1 and 10:1 respectively) for each chemical class and were reported in Binelli et al. (2007, 2008, 2009) and Sarkar et al. (2008).

2.12. Statistical analysis

Data were checked for normal distribution (Shapiro–Wilk's test) and homogeneity of variances (Bartlett's test). As ANOVA assumptions were not fulfilled, the non-parametric Mann–Whitney *U*-test was used for pair-wise comparisons between the two sites. All results were expressed as means ± standard error. Fisher's combined probability test (Fisher, 1932) was also performed, it being one of the most commonly used methods to test the overall significance of a set of independent *p*-values. The test combines *p*-values from independent tests into one statistic test having a chi-square (χ^2) distribution, using the following formula:

$$-2\sum_{i=1}^{m} \ln(p_i)$$

where *m* is the number of *p*-values from independent tests, p_i are the *p*-values.

In addition, the Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001) with 999 permutations was performed in order to examine statistically significant differences between the two sampling sites on the basis of the biomarker responses measured monthly at cellular and tissue level. Moreover, the PERMANOVA analysis was performed to verify influence of the sampling sites, sampling month and site/month interaction on the biomarker responses.

Principal Component Analysis (PCA) of the whole data set, including environmental parameters (temperature, salinity, pH, total chlorophyll, sediment grain size and organic matter), biomarkers and contaminant levels, was also performed.

In the survival-in-air test, LT_{50} values were determined according to Kaplan and Meier (1958) and the significance of differences between groups was tested using the Gehan and Wilcoxon test (Gehan, 1965). The software packages STATISTICA 9 (StatSoft, Tulsa, OK, USA) and PRIMER 6 PERMANOVA Plus (PRIMER-E Ltd., Plymouth, UK) were used for statistical analyses.

3. Results

3.1. Environmental parameters

Water temperature, salinity, pH and total chlorophyll values are reported in Table 1. Temperature and pH values did not differ markedly between the two sampling sites during the study, whereas salinity and total chlorophyll were markedly different. The sediment grain size also differed between the two sampling sites, silt (59%) and sand (60%) being prevalent in sediments from CD and PS, respectively. In addition, sediment organic matter was 2.63% at CD and 0.42% at PS.

3.2. Biomarker responses

Fisher's combined probability test (p-values are shown in each figure) revealed statistically significant differences in biomarker responses (except for digestive gland catalase activity) between clams from CD and PS.

Significantly lower THC values were observed in clams collected at Canale Dese than in those from PS in April ($p < 0.01$), May ($p < 0.01$), June ($p < 0.05$), October ($p < 0.01$), January ($p < 0.01$) and February ($p < 0.05$) (Fig. 2). Conversely, in July THC was significantly higher ($p < 0.05$) in bivalves from CD with respect to those from PS.

Clams sampled at CD had significantly reduced pinocytotic activity, with respect to those of animals from PS in April ($p < 0.01$), May ($p < 0.01$), June ($p < 0.05$), July ($p < 0.01$) and October ($p < 0.05$) (Fig. 3). Conversely, a significantly lower pinocytotic activity was recorded in March ($p < 0.01$) and December ($p < 0.05$) in clams from PS.

Haemolymph lysozyme activity showed a similar pattern of variation between clams from CD and those from PS (Fig. 4). Significantly ($p < 0.05$) higher lysozyme activity was recorded in April, May, July and September in clams collected at CD, than in those from PS.

Significantly lower AChE activity was recorded in gills of clams collected in June ($p < 0.05$), September ($p < 0.01$) and December ($p < 0.01$) at CD, than in those of animals from PS (Fig. 5).

Gill SOD activity was significantly higher in clams collected at PS in March ($p < 0.05$), May ($p < 0.01$), June ($p < 0.01$) and September ($p < 0.01$), with respect to those from CD (Fig. 6A). Likewise, digestive gland SOD activity was statically higher in clams collected at PS in March ($p < 0.01$), April ($p < 0.05$), June ($p < 0.01$) and December ($p < 0.05$), when compared with that of animals from CD. Conversely, in January and February, digestive gland SOD activity was shown to increase significantly ($p < 0.05$ and $p < 0.01$, respectively) in clams from CD, with respect to those from PS (Fig. 6B). Gill CAT activity was significantly ($p < 0.01$) higher in clams collected at PS in October and February, with respect to those from CD, whereas in June and January the enzyme activity was significantly ($p < 0.01$) higher in clams from CD, than in those from PS (Fig. 7A). No significant differences were found in digestive gland CAT activity (Fig. 7B).

The pairwise PERMANOVA analysis revealed statistically significant differences between CD and PS in each sampling month (Table 2). In addition, PERMANOVA revealed a significant influence of the sampling site, of the sampling month and of the site/month interaction on biomarker responses (Table 2).

The resistance to air exposure of clams from CD generally increased significantly ($p < 0.001$) with respect to that of animals from PS (Fig. 8). The highest LT_{50} value (9 days) was recorded in clams collected at CD in March 2009, December and January 2010, whereas the lowest value (4 days) was found in clams collected at PS in June and July 2009.

CI values are shown in Fig. 9. Clams from PS generally had significantly higher CI than those collected at CD, except in March and July. The highest and the lowest CI values were recorded in PS clams in May and March, respectively. A seasonal trend of CI was

Table 1
Values of temperature, salinity and pH, and total chlorophyll concentrations in seawater samples from the two sampling sites; n.a., not available.

	March 2009	April 2009	May 2009	June 2009	July 2009	August 2009	September 2009	October 2009	November 2009	December 2009	January 2010	February 2010
Temperature (°C)												
Canale Dese	9.8	18	14.7	24.5	26.9	23.9	21.1	14.5	n.a.	9.2	6.9	5.9
Punta Sabbioni	9.2	15.3	20	21.5	25	27.2	22.2	16	n.a.	9.3	7.5	5
Salinity (psu)												
Canale Dese	15.29	10.81	9.17	23.82	13.3	19.72	19.72	24.14	n.a.	10.61	17.06	10.8
Punta Sabbioni	32.12	30.36	33.44	32.23	30.46	33,8	33.8	34.42	n.a.	20.53	28.22	30.91
pH												
Canale Dese	8.03	8.07	8.15	8.25	8.45	8.41	8.32	8.45	n.a.	7.95	8.19	8.43
Punta Sabbioni	8.16	8.11	8.22	8.11	8.29	8.35	8.18	8.18	n.a.	8.1	8.26	8.39
Total chlorophyll (mg/m^3)												
Canale Dese	8.56	6.14	30.27	13.35	14.69	53.64	12.44	6.38	n.a.	3.59	23.93	22.33
Punta Sabbioni	2.24	3.97	4.81	2.06	5.47	2.97	3.59	2.04	n.a.	2.53	13.10	1.60

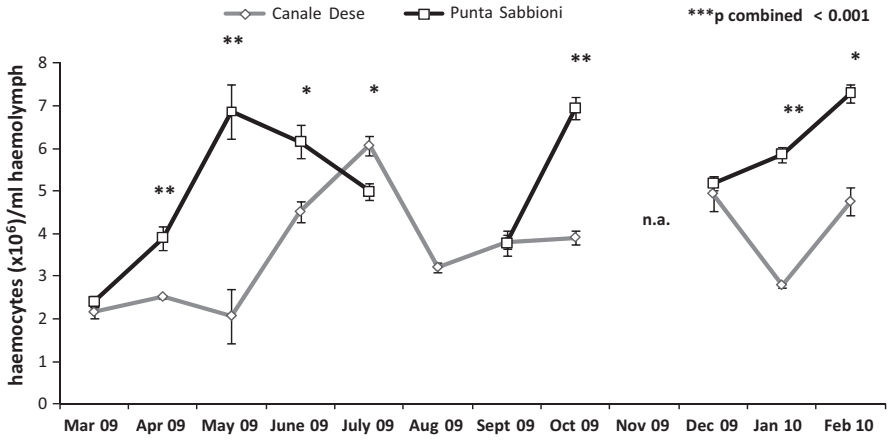

Fig. 2. THC values, expressed as number of haemocytes ($\times 10^6$)/ml of haemolymph, in *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^*p < 0.05$; $^{**}p < 0.01$; n.a., not available. $N = 5$.

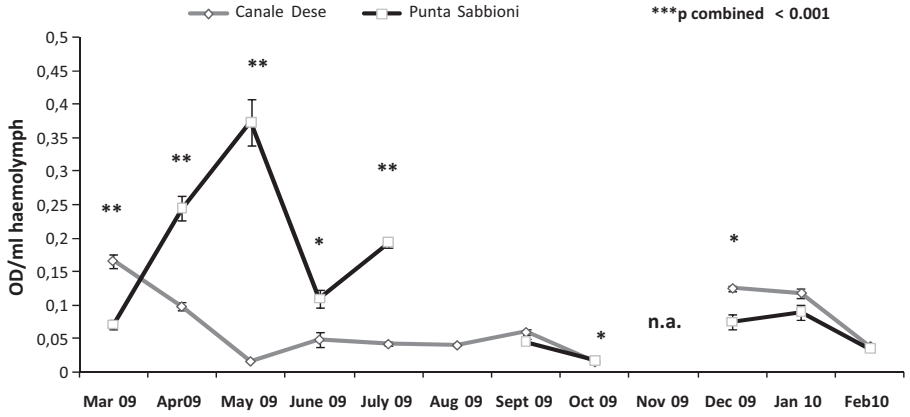

Fig. 3. Pinocytotic activity, expressed as OD/ml of haemolymph, in *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^*p < 0.05$; $^{**}p < 0.01$; n.a., not available. $N = 5$.

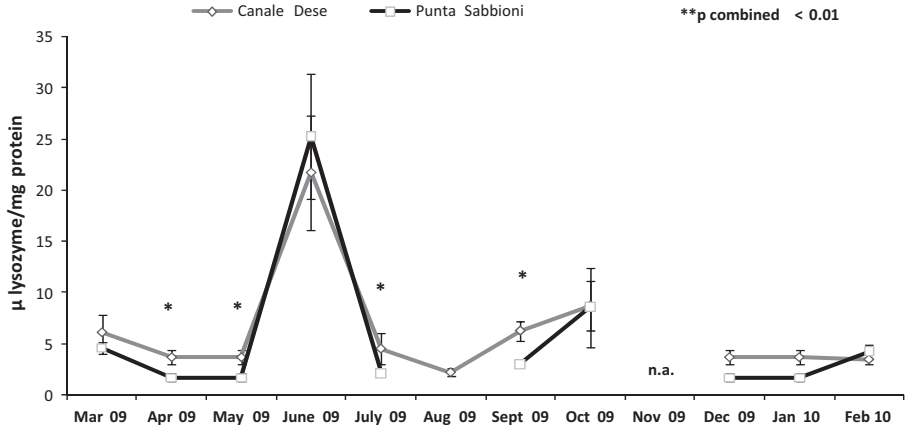

Fig. 4. Haemolymph lysozyme activity, expressed as µg lysozyme/mg protein, in *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^*p < 0.05$; n.a., not available. $N = 5$.

also found: it was higher in spring/summer (April/July) and lower in autumn/winter (October/January), although with a slight temporal shift between the two sampling sites.

3.3. Chemical analyses

The entire data set obtained for the six different classes of POPs monitored at CD and PS is shown in Table 3. The contamination pattern was very similar, showing the lack of influence due to the flux of pollution from the numerous sources present in the Northern part of the Lagoon. As expected, PAHs were the main class of pollutants found both at CD and PS, and their levels ranged between 31 ng/g dry weight and 299 ng/g dry weight. The main PAHs found in the sediments from the two sites were phenanthrene, pyrene, fluoranthene and naphthalene. To evaluate PAH sources, the eighteen PAHs analyzed were divided into two groups, representing

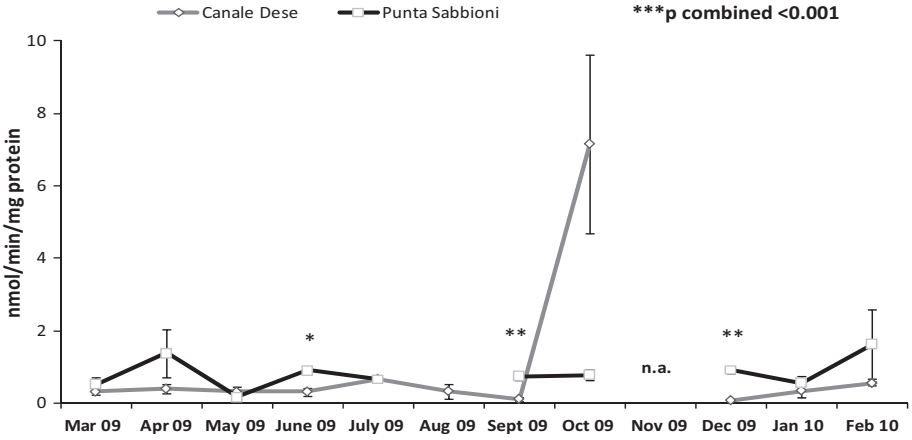

Fig. 5. AChE activity, expressed as nmol/min/mg protein, in gills from *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^{*}p < 0.05$; $^{**}p < 0.01$; n.a., not available. $N = 5$.

PAHs containing two to four rings (low molecular weight, LMW), and five to six rings (high molecular weight, HMW), respectively. The LMW/HMW ratio was then calculated to allow the identification of the origin of PAH pollution (Xu et al., 2007). Interestingly, the two sites are dominated by PAHs with different ring-condensed structure: CD showed a LMW/HMW ratio much lower than 1 for almost all the seasons (excepted for Spring = 2.42), pointing out a pyrogenic origin of PAH pollution. Conversely, at PS, PAHs exhibited a clear petrogenic origin with the exception of Winter 2010 (LMW/HMW ratio = 0.58). Thus, the main sources of PAHs in this site is mainly due to the direct discharge of hydrocarbons, instead of the incomplete combustion due to traffic, shipping and power plants, as on the contrary happens at Canal Dese, located near to the coast.

The other chemical classes showed very low concentrations at the two sites, with values lower than 5 ng/g dry weight, indicating a negligible intake of these pollutants from the watershed. It is of particular interest the p,p′-DDT value measured in Spring 2009 at the two sampling stations, higher than its relative metabolites (p,p′-DDD and p,p′-DDE). This suggests a recent intake of the parent compound from the watershed, although since 1978 the p,p′-DDT use for agricultural purposes has been banned in Italy.

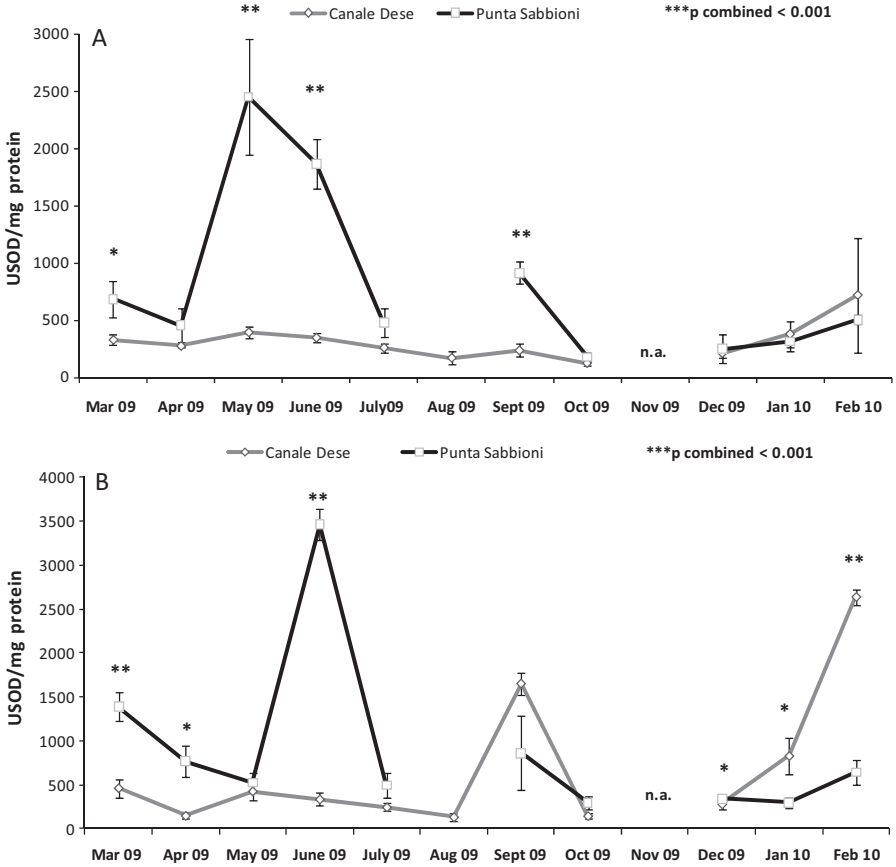

Fig. 6. SOD activity, expressed as U/mg of proteins, in gills (A) and digestive gland (B) of *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^{*}p < 0.05$; $^{**}p < 0.01$; n.a., not available. $N = 5$.

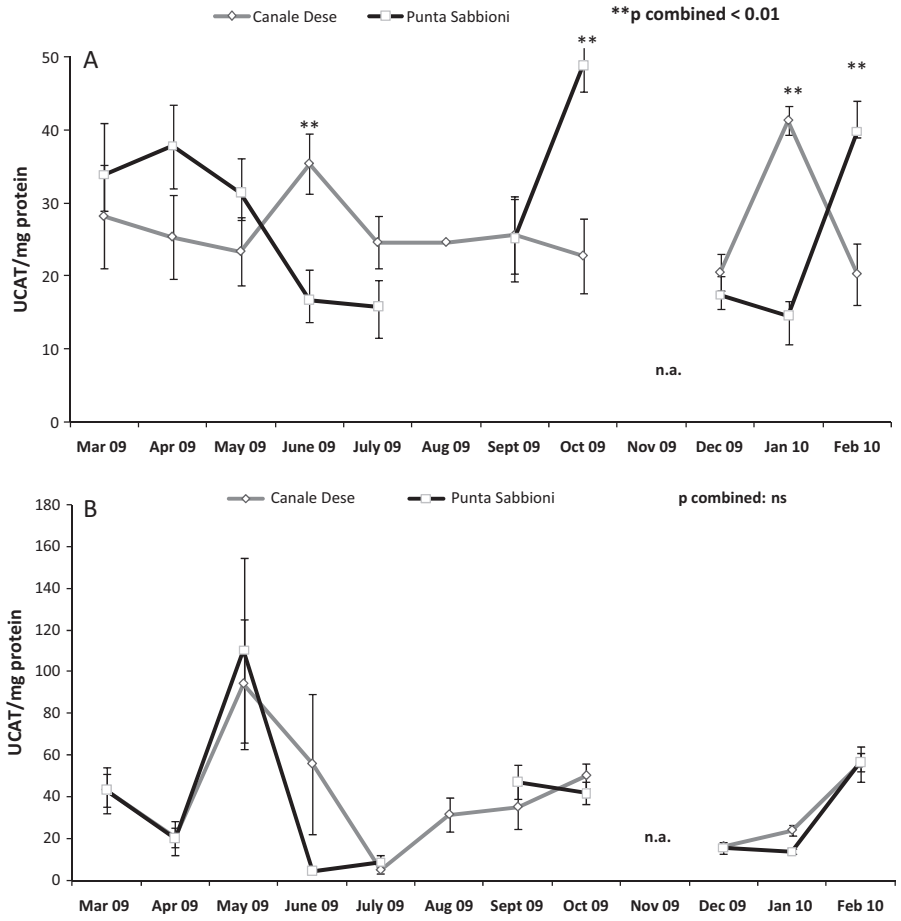

Fig. 7. CAT activity, expressed as U/mg of proteins, in gills (A) and digestive gland (B) of *R. philippinarum*. Values are means ± SE. Asterisks: significant difference. $^{**}p < 0.01$; n.a., not available. $N = 5$.

Table 2
PERMANOVA analysis. Pairwise comparisons (above) and overall results (below); n.a., not available.

Sampling month	Canale Dese vs. Punta Sabbioni
March 2009	$p = 0.008$
April 2009	$p = 0.010$
May 2009	$p = 0.009$
June 2009	$p = 0.005$
July 2009	$p = 0.014$
August 2009	n.a.
September 2009	$p = 0.012$
October 2009	$p = 0.006$
November 2009	n. a.
December 2009	$p = 0.007$
January 2010	$p = 0.008$
February 2010	$p = 0.008$

Source	df	SS	MS	Pseudo-*F*	*P* (perm)	perms
Site	1	36.917	36.917	9.6988	0.001	996
Month	10	263.64	26.364	6.9262	0.001	998
Site × month	9	207.4	23.044	6.0541	0.001	997
Res	84	319.74	3.8064			
Total	104	832				

Notwithstanding the low values of PBDEs, special attention should be given to these chemicals, as since several BDE congeners were found in the Lagoon sediments, mainly at CD. However, it has to be taken into account that the most toxic and dangerous congener (BDE-209) was not measured because of the very extreme GC condition needed for its identification (Binelli et al., 2006). Thus, the levels of total PBDEs measured are probably underestimated and will need further attention in the next years in this very fragile aquatic ecosystem.

Overall, no clear seasonal differences in contamination patterns between the two sites were observed, excepted in Summer 2009, when an increase in PAH levels was found at CD, whereas a decrease in PAHs, HCB and HCHs was detected at PS.

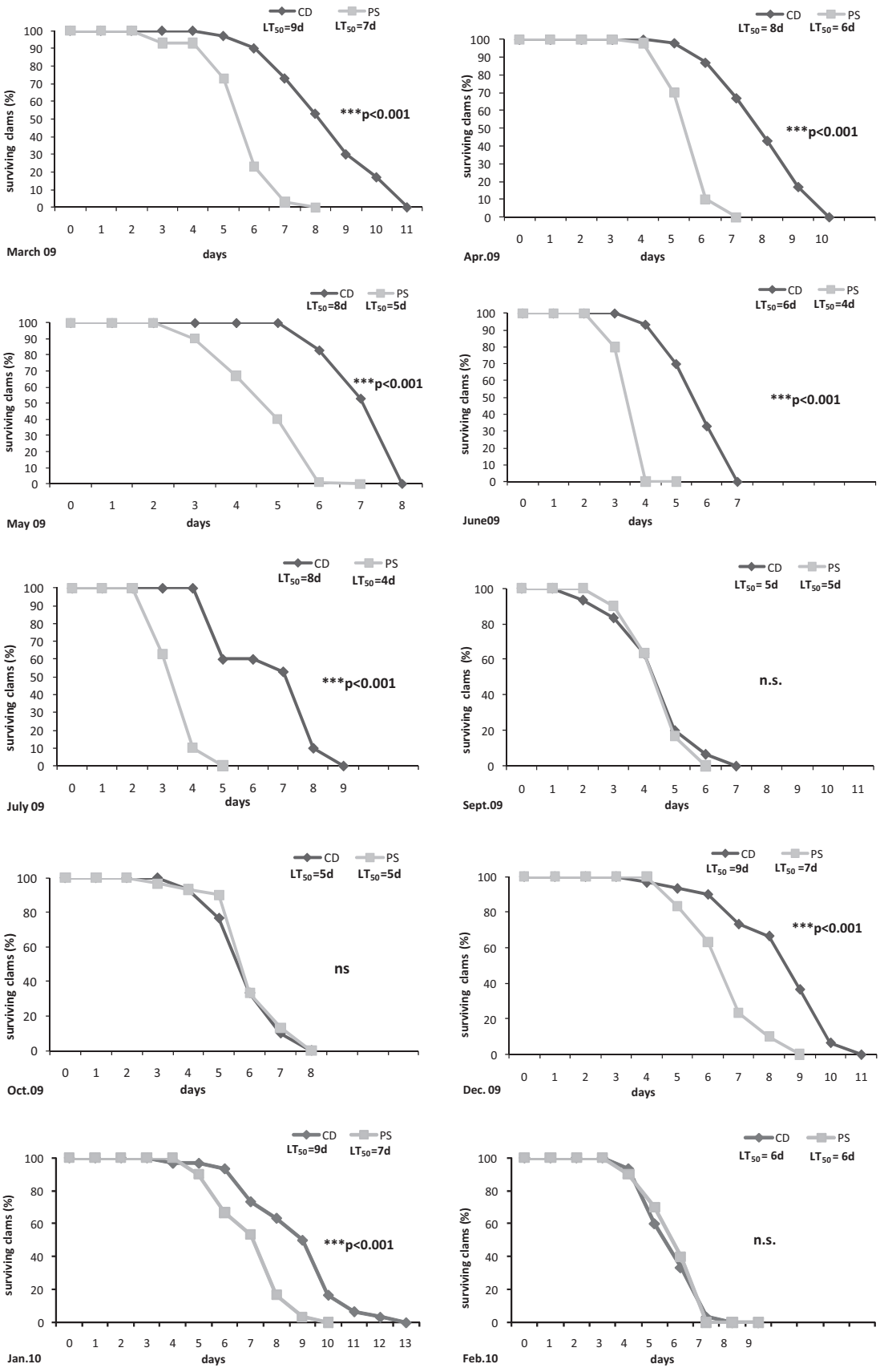

Fig. 8. Survival in air response of *R. philippinarum*, expressed as percentage of clams surviving at each day. LT_{50} values (in days) are shown. $^{***}p < 0.001$; n.s., not significant. $N = 30$.

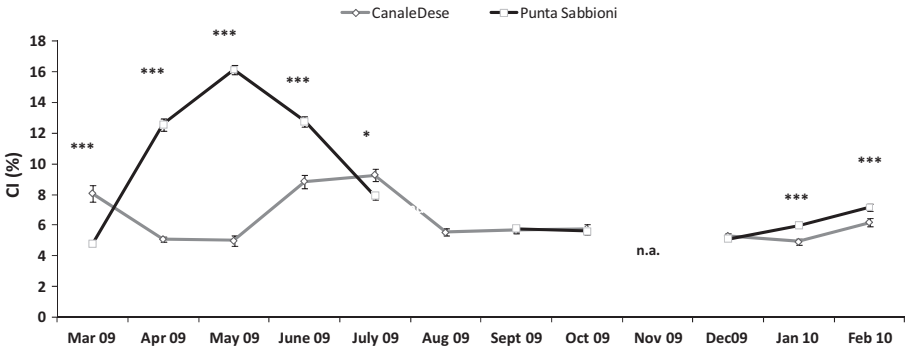

Fig. 9. Condition index of *R. philippinarum* collected at Canale Dese and Punta Sabbioni. Values are means ± SE. Asterisks: significant difference. $^{*}p < 0.05$; $^{***}p < 0.001$; n.a., not available. $N = 30$.

Results of PCA analysis are shown in Fig. 10. The first two PCA axes account for 63.2% of variability (PC1 for 43.1%, and PC2 for 20.1%). PC1 splits samples from the two sites, CD on the right, PS on the left. PC2 tends to separate on a seasonal basis: lower spring–summer samples, upper the other ones, with the exception of CD1 laying close to zero (Fig. 10A). Silt, total chlorophyll, organic matter and clay vectors are linked with CD samples, CD1 in particular; salinity and sand are diametrically opposite to these environmental variables (Fig. 10A). All vectors of contaminant levels in sediment are linked with CD samples and associated with environmental parameters. PAHs, HCB and HCHs join in CD1 and CD6, PBDEs in CD6 and DDTs in CD9 (Fig. 10B). Among biomarkers, LT_{50}, haemolymph lysozyme and digestive gland SOD activities link with CD samples, while gill SOD activity, pinocytosis, CI and THC with PS ones (Fig. 10C). LT_{50} and CI are opposite along PC1, showing an inverse relationship between them. The same pattern is showed by AChE and CAT. AChE is associated with DDTs in sediments and with PS1 and CD9.

4. Discussion

The aim of the present study was to evaluate effects of differing environmental conditions in the clam *R. philippinarum* from both a seaward site and a landward site in the Lagoon of Venice, using a suite of biomarkers. Levels of some priority organic pollutants were also measured in sediments from the two sampling sites.

Numerous studies have demonstrated that bivalve haemocytes play a key role in internal defence against pathogens and foreign materials. Consequently, alterations in haemocyte functionality may increase the susceptibility of animals to diseases, potentially reducing animal survival. In the present study, THC values were generally lower in clams from CD than in those from PS. As well known, molluscs have an open circulatory system and haemocytes are distributed in both the vascular system and tissues. Increases in THC are generally considered as a consequence of proliferation or movement of cells from tissues into haemolymph, whereas decreases may be due to cell lysis or increased movement of cells from haemolymph to tissues (Pipe and Coles, 1995). In this study, low THC values recorded in clams from CD were probably due to exposure of animals to contaminants able to reduce markedly the number of circulating haemocytes. In this regard, it is important to highlight that higher levels of PCBs and PAHs were found in sediments of CD than in those of PS. Likewise, reduced THC was observed in clams from sites (mainly Marghera, Fusina and Palude del Monte) influenced by high levels of PCBs and PAHs (Matozzo et al., 2010). Conversely, increases in THC were recorded in mussels (*Mytilus galloprovincialis*) from the Lagoon of Venice indicating that high levels of contaminants (heavy metals) were correlated with enhanced THC values (Pipe et al., 1995). In the clam *Mya arenaria*, THC was markedly reduced in clams from sites influenced by both ferry traffic and discharges of aluminium smelters and pulp and paper mills, as well as of four townships discharging their wastewaters into the Saguenay Fjord (Québec, Canada) (Gagné et al., 2008). In any case, influences of abiotic factors on THC of *R. philippinarum* cannot be excluded. Among environmental parameters measured in the present study, salinity showed marked differences between the two sampling sites, being lower at CD than PS. As a consequence, it can be hypothesised that low salinity influenced negatively THC values in clams from CD. With regard to this, Reid et al. (2003) demonstrated that THC increased with increasing salinity values, from 20 psu (control) to 30 and 40 psu in *R. philippinarum*. The THC increased directly with salinity levels also in the Taiwan abalone *Haliotis diversicolor supertexta* (Cheng et al., 2004). Overall, the high THC values observed in the present study in clams from PS indicated an activated immunological response, whereas the low THC values recorded in clams from CD suggested an immunesuppression in animals.

In the immune responses of molluscs, phagocytosis is the main internal defence mechanism against pathogens and foreign materials. In the present study, pinocytosis was evaluated by the NR uptake assay as a surrogate analysis of haemocyte capability to engulf foreign materials, as previously suggested (Cajaraville et al., 1996; Matozzo et al., 2002). Results demonstrated that clams from CD had a lower pinocytotic activity than those from PS, suggesting a reduced capability of haemocytes to engulf foreign particles. As for THC, it can be hypothesised a negative effect of contaminants, mainly PAHs, on pinocytotic activity of haemocytes. Several laboratory studies have demonstrated that phagocytosis may be affected by exposure to xenobiotics. For example, exposure for 2 and 4 weeks to a mixture of PAHs (anthracene, fluoranthene, and phenanthrene) was shown to inhibit significantly the phagocytic activity in *Mytilus edulis* (Grundy et al., 1996). In field studies, Sami et al. (1992) and Pipe et al. (1995) observed reduced phagocytic activity in molluscs from contaminated sites compared with that of bivalves from reference sites. Significant reductions in both activity and efficiency of phagocytosis were also observed in *M. arenaria* exposed to four different sources of PAHs (aluminium smelter soot, sediment from an industrial discharge pound, charcoal fine particles, and dietary) (Frouin et al., 2007). The percentage of haemocytes that had ingested fluorescent particles (indicative of phagocytic activity) reduced significantly in clams (*M. arenaria*) from polluted sites when compared to the reference site in the Saguenay Fjord (Gagné et al., 2008). In any case, influence of salinity (markedly different between the two sampling sites) on pinocytosis cannot be excluded in the present study. Extreme (high and low) salinities have been shown to induce significant variations in phagocytosis in several bivalve species. For example, in *Chamelea gallina*, both the highest and the lowest salinity caused a significant decrease in phagocytic activity of haemocytes (Matozzo et al., 2007). In *R. philippinarum*, low water salinity induced a decrease in the phago-

Table 3
Concentrations of POPs in surface sediments collected seasonally at the two sampling sites; <d.l. = below detection limit.

Compound	Canale Dese (ng/g dry weight)				Punta Sabbioni (ng/g dry weight)			
	Spring 2009	Summer 2009	Autumn 2009	Winter 2010	Spring 2009	Summer 2009	Autumn 2009	Winter 2010
o,p′-DDE	0.032	0.010	0.006	0.013	0.013	0.006	0.009	0.022
p,p′-DDE	0.063	0.140	0.146	0.200	0.049	0.092	0.108	0.215
o,p′-DDD	0.021	0.018	0.022	0.031	0.034	0.016	0.027	0.081
p,p′-DDD	0.051	0.076	0.164	0.125	0.042	0.017	0.060	0.224
o,p′-DDT	0.017	0.012	0.019	0.059	0.049	0.061	0.032	0.138
p,p′-DDT	0.089	0.045	0.060	0.046	0.064	0.033	0.017	0.061
∑DDTs	0.272	0.301	0.417	0.476	0.253	0.226	0.253	0.741
α-HCH	<d.l.	<d.l.	<d.l.	<d.l.	0.010	<d.l.	<d.l.	<d.l.
β-HCH	0.037	0.008	0.036	0.038	0.036	0.020	0.027	0.035
γ-HCH	0.096	0.050	0.020	0.118	0.091	0.045	0.056	0.081
δ-HCH	<d.l.	0.010	0.005	0.016	<d.l.	0.009	0.009	0.022
∑HCHs	0.133	0.067	0.061	0.177	0.137	0.075	0.091	0.138
HCB	0.218	0.147	0.159	0.097	0.103	0.045	0.141	0.111
CB-18	0.119	0.103	0.138	0.038	0.131	0.055	0.161	0.080
CB-28 + 31	0.710	0.393	0.590	0.261	0.542	0.243	0.581	0.254
CB-52	0.303	0.187	0.271	0.331	0.116	0.055	0.250	0.224
CB-44	0.132	0.104	0.122	0.064	0.084	0.013	0.072	0.044
CB-101	0.215	0.161	0.402	0.357	0.111	0.035	0.123	0.293
CB-149	0.184	0.213	0.166	0.311	0.094	0.029	0.118	0.211
CB-118	0.208	0.209	0.303	0.395	0.045	0.016	0.106	0.283
CB-153	0.298	0.329	0.439	0.578	0.087	0.039	0.031	0.163
CB-138	0.099	0.280	0.390	0.496	0.067	0.026	0.036	0.118
CB-180	0.022	0.190	0.166	0.206	0.028	0.018	0.051	0.120
CB-170	0.065	0.126	0.028	0.067	0.019	0.019	0.014	0.070
CB-194	0.034	0.041	0.065	0.039	<d.l.	<d.l.	<d.l.	0.015
∑PCBs	2.391	2.335	3.080	3.144	1.326	0.550	1.544	1.876
Naphtalene	15.250	8.556	12.690	5.642	10.983	4.306	7.838	5.706
2-Methylnaphtalene	5.159	3.028	4.685	2.358	3.573	1.500	3.465	1.943
1-Methylnaphtalene	9.119	5.338	7.748	3.263	6.616	2.784	6.393	3.143
Acenaphtylene	0.736	0.390	0.332	0.494	0.592	0.439	0.992	0.525
Acenaphtene	3.825	2.090	3.074	1.160	2.497	0.827	1.995	0.445
Fluorene	15.280	7.435	9.972	4.198	6.947	2.844	6.952	2.712
Phenanthrene	42.003	35.993	35.224	15.309	29.004	11.611	30.700	12.594
Anthracene	5.860	4.310	4.137	2.197	3.021	1.283	3.654	1.727
Fluoranthene	10.076	44.807	22.889	20.103	6.185	2.763	9.940	8.293
Pyrene	6.869	28.650	13.680	13.039	3.655	1.686	7.175	6.428
Benz(α)anthracene	1.752	24.054	9.041	9.634	0.218	0.071	3.544	3.625
Chrysene	3.715	35.101	17.487	16.101	1.073	0.350	5.196	6.104
Benzo(β)fluoranthene	2.417	20.725	13.466	12.944	0.190	0.136	4.231	5.719
Benzo(k)fluoranthene	2.881	25.278	13.627	12.364	0.305	0.081	5.381	5.538
Benzo(α)pyrene	1.245	21.857	10.115	8.873	0.207	0.035	4.251	3.450
Indeno(1,2,3-cd)pyrene	1.053	10.431	5.793	6.607	<d.l.	<d.l.	1.512	2.264
Dibenz(a,h)anthracene	0.451	7.863	3.789	3.377	<d.l.	<d.l.	<d.l.	<d.l.
Benzo(g,h,i)perylene	1.485	13.583	8.317	7.612	0.223	0.192	1.789	3.187
∑PAHs	129.177	299.489	196.066	145.273	75.288	30.908	105.008	73.403
BDE-17	0.066	0.030	0.010	0.006	0.009	0.019	0.005	0.008
BDE-28	0.033	0.006	0.030	0.015	0.085	<d.l.	0.045	<d.l.
BDE-71	0.041	0.053	0.063	0.062	0.047	0.022	0.065	0.061
BDE-47	0.544	0.479	0.443	0.409	1.030	0.163	0.387	0.479
BDE-66	0.052	0.044	0.046	0.068	0.152	0.020	<d.l.	0.058
BDE-100	0.360	0.486	<d.l.	0.521	0.373	<d.l.	0.585	<d.l.
BDE-99	2.233	0.403	0.762	0.511	1.149	0.583	0.647	<d.l.
BDE-85	0.933	1.887	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
BDE-154	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
BDE-153	0.029	0.061	0.051	0.019	0.034	0.036	0.027	<d.l.
BDE-138	0.153	<d.l.	0.084	0.067	<d.l.	0.076	0.032	0.031
BDE-183	0.494	0.049	0.025	0.085	<d.l.	<d.l.	<d.l.	<d.l.
BDE-190	0.199	<d.l.	0.165	0.080	<d.l.	<d.l.	<d.l.	<d.l.
∑PBDEs	5.136	3.498	1.680	1.843	2.879	0.919	1.793	0.637

cytic capability of haemocytes (Reid et al., 2003). On the basis of results obtained in the present study, we can infer that low salinities induced significant reduction in pinocytotic capability of haemocytes. Moreover, the present study demonstrated that haemocyte pinocytotic activity was independent of THC values, being the increase in haemocyte number not always related to increases in the pinocytotic activity. The same results were obtained by Gagné et al. (2008).

In immune defence of bivalves, lysosomes play an important role, as they contain acid hydrolases able to degrade most cellular macromolecules to low-molecular weight products. Lysosomes also represent a target site for differing contaminants (Cajaraville et al., 2000). As a consequence, damage to lysosomes can negatively affect cell health. This is generally due to rapid weakening of the lysosomal membranes, and the consequent release of hydrolytic enzymes into the cytoplasm (Moore, 1985). In the present study,

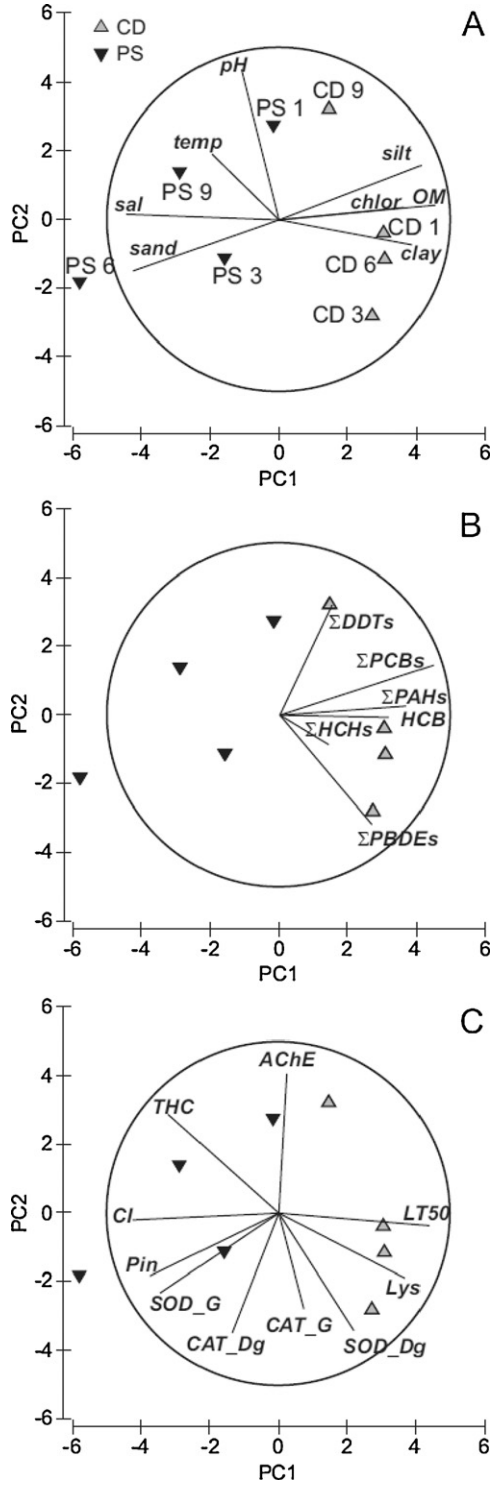

Fig. 10. PCA ordinations of whole data set, including environmental parameters, biomarkers and contaminant levels, with superimposed vectors of environmental (A), contaminant (B) and biomarker (C) variables. *Abbreviations*: CD, Canale Dese; PS, Punta Sabbioni; temp, temperature; sal, salinity; chlor, total chlorophyll; OM, organic matter; THC, total haemocyte count; AChE, acetylcholinesterase activity; CI, condition index; LT_{50}, air exposure time resulting in 50% mortality; Pin, pinocytosis; Lys, lysozyme activity; SOD, superoxide dismutase activity; CAT, catalase activity; G, gills; DG, digestive gland. The numbers (1, 3, 6 and 9) are the sediment sampling months.

release of lysozyme into the haemolymph was used as a measurement of lysosomal membrane fragility. Although lysozyme activity showed a similar variation pattern in clams from CD and in those from PS, higher lysozyme activity was generally observed in haemolymph from bivalves collected at CD, suggesting a lysosomal membrane destabilisation. This was probably due to the high levels of PAHs in sediment from CD. Likewise, a significant reduced lysosomal stability was recorded in haemocytes of cockles (*Cerastoderma edule*) from an area contaminated by heavy metals and PAHs (Galloway et al., 2004). Higher lysosomal membrane destabilisation was also recorded in haemocytes of *M. galloprovincialis* from the most polluted sites in the Lagoon of Venice, when compared with those from mussels collected at the reference site inside a farming area (Da Ros et al., 2002). In any case, a negative effect of low salinity can be hypothesised to explain lysosomal membrane destabilisation in clams from CD. In this context, a significantly higher lysozyme activity was previously observed in haemolymph of oysters (*Ostrea edulis*) acclimated at 25 psu, when compared to that of control bivalves (32 psu) (Hauton et al., 2000).

Several studies demonstrated usefulness of measurement of AChE activity in evaluating the effects of exposure to neurotoxic compounds in aquatic organisms. In the present study, lower AChE activity was generally recorded in gills of clams collected at CD, suggesting a possible exposure of bivalves to neurotoxic compounds, probably present in drainage waters from agricultural land. A reduced AChE activity has recently been recorded in clams (*R. philippinarum*) from Marghera, San Servolo and CD (Matozzo et al., 2010), confirming results of previous studies (Matozzo et al., 2005). Interestingly, in all these studies (the present and those of Matozzo et al., 2005, 2010) AChE activity values of clams collected at CD show the same order of magnitude (except for October 2009, in this study), resulting markedly lower than those recorded in clams from a small Lagoon inside a natural area along the coast of the Northern Adriatic, not far from the Lagoon of Venice, but without any connection with it (Matozzo et al., 2005). In previous studies, it has been demonstrated that AChE activity was reduced in animals from sites contaminated by organophosphorus pesticides (OPs). For example, clams (*Ruditapes decussatus*) harvested from a site near an agricultural area in the Lagoon of Bizerta (Tunisia) showed decreased AChE activity, mainly after periods of rain (Dellali et al., 2001). In *M. galloprovincialis*, alterations in AChE activity were also related to agricultural practices in areas where pesticides and biocides were frequently used (Escartin and Porte, 1997). Although a negative effect of OPs, carbamates and organochlorine pesticides on AChE activity may be hypothesised in the present study for clams from CD, chemical analyses cannot confirm this hypothesis, because we did not evaluate any OP compound. In any case, effects of other pollutants capable to inhibit AChE activity in *R. philippinarum* should be considered. In this regard, Solé et al. (2009) found a significant inhibition of AChE activity in the polychaete *Nereis diversicolor* and the clam *Scrobicularia plana* from the most polluted sites. However, in that study, the authors stated that AChE inhibition cannot be solely attributed to inputs of pesticides, and the presence of chronic exposure to metals, surfactants and other unknown chemicals from several origins (domestic, uncontrolled spillages) were possible causative agents. In addition, previous studies have demonstrated that AChE activity is modulated by other neurotoxic chemicals, including metals (Dellali et al., 2001; Durou et al., 2007; Moreira et al., 2006).

Mollusc defence systems against reactive oxygen species (ROS) include anti-oxidant agents, such as glutathione, glutathione peroxidase, superoxide dismutase (SOD) and catalase (CAT). Variations in their levels/activities have been proposed as useful biomarkers to assess susceptibility of organisms to oxidative stress (Soldatov et al., 2007). Indeed, it is well-known that various contaminants can induce oxidative stress in animals by enhancing ROS levels,

such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) and hydroxyl radical ($OH^•$). ROS can promote a sequence of reactions which produce compounds, such as free lipid radicals and hydroperoxides, extremely toxic to cells (Slater, 1984). In the present study, higher SOD and CAT activities (except digestive gland CAT activity) were generally found in gills and digestive gland of clams from PS, suggesting that animals had a higher efficiency to cope with oxidative stress than bivalves from CD. In this context, it is suggested that increases in the activity of antioxidant enzymes may reflect an adaptation of animals to the chronic exposure to high/moderate levels of contamination, since this would confer increased protection from oxidative stress (Cheung et al., 2001; Romero-Ruiz et al., 2003). Reduced antioxidant enzyme activities recorded in clams from CD were probably due to exposure of clams to both natural (i.e., salinity) and anthropogenic (i.e., PAHs) factors capable to affect antioxidant enzymes. It is well known that oxidative damage is an important mechanism of toxicity induced by PAHs (Altenburger et al., 2003), while it has been demonstrated that osmotic stress due to hyposalinity can strongly affect antioxidant enzyme activity in bivalves (Lau et al., 2004). In a recent study, gill CAT activity was shown to decrease in mussels caged at sites influenced by anthropogenic activities compared to reference sites on the Greek coastline (Tsangaris et al., 2010). Summarising, results obtained in the present study suggested a reduced antioxidant status of clams from CD.

The survival-in-air test is considered as one of the simplest, most feasible, sensitive, reproducible and cost-effective methods for evaluating the negative effects of environmental stress in euryoxic bivalves (de Zwaan and Eertman, 1996; Eertman and de Zwaan, 1994; Eertman et al., 1993). In the present study, higher tolerance to aerial exposure was generally recorded in clams collected at CD, with respect to those from PS. Likewise, in our recent study, we observed a reduced tolerance to aerial exposure and LT_{50} values in clams collected at Marghera, Cà Roman, San Servolo and Fusina, whereas animals from Palude del Monte and CD showed the highest tolerance (Matozzo et al., 2010). During air exposure, bivalves are under anaerobic metabolism, and glycogen represents the main source of energy (de Zwaan and Wijsman, 1976). Bivalves exposed to stressors generally show increased metabolism and require extra energy (de Zwaan and de Kock, 1988). Results of the present study are surprising, as clams from CD displayed generally the highest LT_{50} values, while had significant reductions in both immunomarkers and AChE activity. It can be hypothesised that high tolerance of CD clams to aerial exposure may represent an adaptive response of animals to peculiar environmental conditions, mainly related to salinity and total chlorophyll concentrations in seawater, and grain size and organic matter in sediments. The peculiar environmental conditions recorded at CD could disguise potential negative effects of contaminants, as suggested by Widdows et al. (1997) in relation to the high trophism of contaminated sites in the Lagoon of Venice. In addition, it has to be noted that in previous studies survival in air was not negatively affected in mussels, both transplanted and indigenous, from polluted sites in the Lagoon (Nasci et al., 2002; Nesto et al., 2004).

In environmental studies, the measurement of CI is used to evaluate general condition of bivalves (Lucas and Beninger, 1985). CI can show seasonal variations, being influenced by seasonal biotic and abiotic factors, including sexual maturity and food availability. In the present study, higher CI values were generally recorded in clams collected at PS, than in those from CD. In addition, CI showed a seasonal variation pattern in both sites, increasing in spring/summer and decreasing progressively from autumn to winter. Similar results were previously recorded in the same clam species (Marin et al., 2003). Gametogenesis of *R. philippinarum* begins in January and continues until May, ripe clams appear in April, and most spawning occurs in May, continuing during summer until September (Meneghetti et al., 2004). As a consequence, spawning may result in both weight loss and low CI. This may explain why, in the present study, lower CI values were recorded in autumn/winter, in the post-spawning phase of clams. The CI may also vary with food availability and diet quality (Mougraud et al., 2002). Interestingly, sediment organic matter and water total chlorophyll were more abundant at CD, where CI was lower. Although surprising, these results can be explained by the marked difference in shell weight of clams from the two sampling sites. Indeed, mean dry weight of soft tissues was similar between clams from CD and PS (0.69 g in clams from CD, 0.68 g in those from PS), whereas mean dry weight of shell differed markedly (11.86 g at CD, 7.37 g at PS), despite similar shell size. The reasons of this difference need to be more fully investigated. In any case, different methods of clam CI calculation, such as the ratio between the soft tissue dry weight and the length of shell, should be considered in future studies.

5. Conclusions

Results of the present study demonstrated that the suite of biomarkers measured in *R. philippinarum* at differing levels of biological organisation allowed us to discriminate clearly the two sampling sites. Although the contamination levels of sediments from CD and PS were rather similar (on the basis of the pollutants measured in this study at least), significant differences were observed in clam biological responses. PERMANOVA analysis revealed a strong influence of the factors "sampling month", "sampling site" and "sampling site/month interaction" on biomarker responses. Results obtained suggested that the peculiar environmental features of the two sampling sites (mainly related to salinity, total chlorophyll, and sediment grain size and organic matter) influenced clam biomarker responses. In our opinion, this aspect needs to be taken into account when biomonitoring studies are performed in sites of estuarine ecosystems having similar contamination levels, since abiotic factors, rather than contaminants, may influence animal biological responses.

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