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Qualità del sedimento lagunare a supporto della sua gestione sostenibile

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Risultati dei biomarkers

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1 Summary

Marine environments are a sink for pollutants deriving from inland and coastal activities. Human activities have been changing the morphology and hydrodynamics of the lagoon of Venice, with consequences for the characteristics of the sediments, including the presence of pollutants. Sediments are a peculiar compartment, as they can accumulate relevant amounts of chemicals, which interact with inorganic and organic matter in the seafloor. The channels of the Lagoon often undergo dredging to allow vessels to reach the harbours, and more recently the construction of the MoSE with unknown effects on the biota.

This work aimed at assessing the toxicological effects of sediments dredged from channels of the lagoon of Venice. A sediment toxicity bioassay was performed using the Manila clam (*Ruditapes philippinarum*) as a bioindicator.

In this deliverable, the results of a battery of biomarkers indicative of cell damage, oxidative stress and damage, neurotoxicity and detoxification capability of clams exposed to sediments samples from 6 sites in the Venice lagoon are reported. Five sediments have been sampled along a transect between the city of Venice and the industrial area of Porto Marghera. The sixth sediment sample was collected in an area with supposed low contamination levels, and therefore it has been selected as a reference site.

Two different experiments were carried out using sediments sampled in different locations and times to assess potential variation in sediment toxicity and the different sensitivity of clams according to both the potential seasonality of the contaminant presence/bioavailability in the sediments and the gametogenic phase of clams. The first experiment was carried out in November 2020 when clams were in a resting phase, whereas the second one was performed in March 2021, during early gametogenesis of bivalves. Both experiments were conducted following the same experimental design. Biomarkers of immune responses, antioxidant defences, and detoxification of pollutants were analysed in haemolymph, gills and digestive gland of clams after 3 and 14 days of exposure.

Differences between times of exposure (3 and 14 days) and among sampling sites (1 to 6) were revealed, but a clear pattern of variation could not be identified. Indeed, no univocal responses of clams between the two experiments (November 2020 vs March 2021) were observed. The results obtained suggested homogeneous contamination levels in the sampling sites, including the reference one (#6). Overall, differences between the two experiments due to reproductive phase and/or contamination levels have been highlighted. To fully understand the results reported here, chemical analyses of the hazardous compounds present in sampled sediments must be considered.

2 Riassunto

Gli inquinanti derivanti dalle attività umane interne e costiere si possono accumulare negli ambienti marini. Le attività antropiche modificano la morfologia e l'idrodinamica della laguna di Venezia, con conseguenze sulle caratteristiche del fondale lagunare, inclusa la presenza di inquinanti. I sedimenti sono un comparto peculiare in quanto possono accumulare quantità rilevanti di sostanze chimiche, che interagiscono con la materia inorganica e organica del fondo marino. I canali della laguna sono spesso sottoposti a dragaggio per permettere alle navi di raggiungere i porti, e più recentemente è iniziata la costruzione del MoSE con effetti sconosciuti sul biota.

Questo lavoro ha avuto lo scopo di valutare gli effetti tossicologici dei sedimenti dragati dai canali della laguna di Venezia. È stato adottato un test di tossicità dei sedimenti utilizzando la vongola filippina (*Ruditapes philippinarum*) come bioindicatore.

In questa deliverable sono riportati i risultati di una batteria di biomarcatori indicativi di danno cellulare, stress e danno ossidativo, neurotossicità e capacità di detossificazione delle vongole esposte a sedimenti campionati in 6 siti della laguna di Venezia. Cinque sedimenti sono stati campionati lungo un transetto tra la città di Venezia e la zona industriale di Porto Marghera. Un sesto sedimento, considerato il sito di riferimento, è stato campionato in un'area con un livello di contaminazione presumibilmente basso.

Sono stati condotti due differenti esperimenti esponendo le vongole a sedimenti campionati in due diversi momenti dell'anno (inverno e primavera). Questo disegno sperimentale è servito a valutare le variazioni temporali della tossicità dei sedimenti (potenzialmente causate da una diversa presenza/biodisponibilità dei contaminanti nel sedimento nei due periodi considerati) e della sensibilità delle vongole (potenzialmente influenzata del ciclo gametogenetico). Il primo è stato effettuato a novembre 2020, durante la fase di riposo sessuale delle vongole; il secondo è stato effettuato a marzo 2021, utilizzando vongole nella fase iniziale di gametogenesi. Entrambi gli esperimenti sono stati condotti seguendo lo stesso disegno sperimentale.

Nell'emolinfa, nelle branchie e nella ghiandola digestiva delle vongole esposte per 3 e 14 giorni, sono stati misurati *biomarker* indicativi delle difese antiossidanti, delle risposte immunitarie e della capacità detossificante degli animali. La batteria di biomarcatori utilizzati ha fornito una valutazione comparativa della qualità dei sedimenti dragati.

Sono state osservate differenze tra i tempi di esposizione (3 e 14 giorni) e tra i siti di campionamento, ma non è stato possibile identificare una chiara tendenza di variazione. Le risposte biologiche analizzate hanno evidenziato sia un aumento che una diminuzione nel tempo per i diversi tessuti, per i diversi biomarcatori e i diversi siti, in maniera non univoca tra le due esposizioni (novembre 2020 vs marzo 2021). I risultati suggeriscono livelli di contaminazione omogenei nei siti indagati, compreso quello di riferimento (n. 6). Nel complesso, sono state evidenziate differenze tra le due esposizioni dovute alla fase riproduttiva e/o ai livelli di contaminazione. Per comprendere appieno i risultati qui riassunti, dovranno essere considerate anche le analisi chimiche dei composti eventualmente presenti nei sedimenti.

3 Introduction

3.1 Sediments in the lagoon of Venice

Marine sediments are a complex and heterogeneous matrix, composed of minerals, organic matter, interstitial water, and biological components and possess specific physico-chemical conditions. The superficial layer, comprehensive of the first few centimetres of sediment, is oxidized, it has a high positive redox potential, and it is home to a large portion of the marine macro- and meiobenthos (Hoffman et al. 2003). The lower layers are anoxic, with negative redox potential and are inhabited by anaerobic microorganisms and burrowing animals (Hoffman et al. 2003).

The sediment of the lagoon of Venice contains sand, silt and clay of alluvial and marine origin (Brambati et al. 2003). However, the overall morphology of the lagoon is changing. Overtime (1970-2000) the lagoon has changed from a more complex morphology with minor channels and elevations formed by saltmarshes and intertidal flats to a flatter and more open lagoon with less pronounced minor channels and elevations, weak siltation of main channels and decreased morphodiversity (Molinaroli et al. 2009). The reasons for the observed changes are multiple, but they are mostly due to anthropogenic alterations of the lagoon system (Molinaroli et al. 2009).

One of the sources of sediment loss is dredging. In the lagoon, a large amount of dredging is carried out, mostly for navigation purposes, moving about 1 million m^3/year of sediments on average. The depth of existing channels and harbours need to be maintained and thus are continuously dredged. Other reasons for dredging include the construction of the MOSE, dredging of new canals and enlargement of ports (Madricardo et al. 2018, Sarretta et al. 2010, Apitz et al. 2007, Teatini et al. 2017).

Dredging and dumping of the material have an environmental impact on the benthic communities due to changes in sea-floor integrity, increased turbidity and resuspension of sediment, which enhances the exchange of sediment-bound contaminants with water and resuspension of fine sediments, which are subsequently lost from the lagoon (Madricardo et al. 2018, Teatini et al. 2017).

The dredging of sediments in the lagoon of Venice is regulated by a protocol released by Italian Ministry of the Environment (Protocollo d'Intesa sui fanghi, 1993). The protocol expects to carry out the sediment characterization in terms of granulometry, chemical analyses to quantify toxic, hazardous and radioactive substances, as well as microbiological analyses. Thereafter, the sediments will be classified as toxic or non-toxic and, according to the classification, a different fate will follow. When the dredged material is highly toxic it has to be disposed of outside the lagoon in landfills. Non-toxic dredged sediment, instead, can be reused in the lagoon for morphological restoration of lost and degraded ecosystems like saltmarshes. However, due to high contamination of the lagoon, much of the dredged sediment is lost (Apitz et al. 2007).

3.2 Sediment toxicity testing

Sediment toxicity tests and analysis of biomarkers are an important ecotoxicological evaluation method to assess environmental pollution in sediments and its effects on the inhabiting biota.

Estimating the toxicity of sediments is important because they play a crucial role in the cycle of organic matter and elements in the ecosystem and provide habitat for many benthic species. They also act as a major pollutant reservoir because many compounds of anthropogenic origin, like metals and organic contaminants, tend to accumulate in the sediments, by absorbing or adsorbing to sediment particles or the contained organic matter. This can harm the organisms that inhabit the sediment (Hoffman et al. 2003, Burton et al. 1992). In addition, many contaminants accumulated in deeper layers of the sediment may be brought back to the surface layers and into the overlying water by anthropic or abiotic disturbance of the

sediment or due to bioturbation by burrowing macrofauna (Hoffman et al. 2003). A wide variety of organisms can be employed for the tests, including microorganisms, such as bacteria and microalgae, macroalgae, plants and animals. Different organisms have different mechanisms of exposure to the sediment and the contained compounds, depending on their burrowing behaviour and feeding strategy. Epibenthic organisms are primarily exposed to the overlying water, while infaunal organisms are exposed to pore water. Ingestion of sediment is another important exposure route and depends on the feeding strategy of the organism. Bivalves have a wide application in sediment toxicity tests, as they meet most criteria for a suitable test organism.

The Manila clam (*Ruditapes philippinarum*) is widely recognized as a suitable bioindicator species (Byrne et al. 2001, Won et al. 2016, Moschino et al. 2012). It lives in sandy-muddy bottoms from intertidal to subtidal zones. Due to their feeding behaviour, clams can be used as an indicator for water and sediment quality, and their tissues or body fluids are suitable to evaluate the accumulation of contaminants as well as their effects (Moschino et al. 2012, Nerlović et al. 2016, Won et al. 2016).

4 Materials and methods

4.1 Area of interest and samples

The sediment of six different sites in the lagoon of Venice was collected in November 2020 and March 2021 (fig.1). Five sampling stations (1-5) were in the Vittorio Emanuele III canal. Site 1 was the closest to the port of the historic city of Venice, whereas site 5 was the closest to the industrial area of Porto Marghera on the mainland (fig. 2). These sites are characterized by high anthropogenic influence, mainly due to urban input from the city centre and boat traffic (the first two sites), and industrial activities (the last two sites).

Site 6, chosen as the reference site, was far from the other sampling stations as it was located in the San Felice canal in the northern basin of the lagoon. This site is characterized by potentially less anthropogenic influence.

Four samples of sediment were collected at each site using a core drill-sampling tool operated by dredge. Each core tube had a diameter of 8,55 cm and length of 1 m.

Figure 1. Sampling stations 1-6 in the lagoon of Venice.

After arriving in the laboratory, the core samples were extruded from the tubes; replicate cores from the same site were mixed and then stored at -80 °C for a few weeks before experiments.

Grain size and the organic matter content were evaluated on samples collected from each of the six sites in the first sampling campaign (November 2020).

4.2 Sediment characterization

Determination of organic matter in the sediment was performed in triplicate by titration, following the method of Gaudette et al. (1974). After thawing, sediment samples were dried in an oven, crushed by mortar and sieved on 500 µm mesh. Approximately 0.5 g of sediment was weighed and placed in a flask. To the sample were added in the following order: 10 ml of potassium dichromate 1N, 20 ml of sulfuric acid 96%, the volume was increased to 200 ml with distilled water, 10 ml of phosphoric acid 85%, 5 ml of sodium fluoride and 15 drops of diphenylamine (2.5% in sulfuric acid 96%) which acts as an indicator. Titration was performed with 0.5 N ammonium ferrosulfate noting the volume of the titrant when the sample turned from black to emerald green. The result was expressed as % organic matter.

Grain size analysis was performed in triplicate according to the method proposed by Buchanan (1984). Once thawed, approximately 15 grams of sediment was air dried and transferred to a beaker with 100 ml of 6% hydrogen peroxide. The sediment was then rinsed with distilled water over a paper filter (Whatman® #50) in a Buchner funnel and after recovering the sediment, with 200-300 mL of distilled water, 10 mL of sodium hexametaphosphate solution (6.2 g/l) was added and allowed to react for 12 hours at room temperature. The sediment was transferred to a 63 µm sieve by sieving directly into the water, and then was transferred to an oven at 100 °C for 12 hours. Sediment above 63 µm was then passed through sieves of 2000 µm, 500 µm, and 125 µm mesh size from Endecotts S.p.A. (London), with subsequent weighing of the individual fractions. Fractionation of granules smaller than 63 µm was evaluated using different sedimentation times, based on the concept that larger particles sediment faster. In detail, the sediment was dispersed and shaken repeatedly in a cylinder containing exactly 1 L of distilled water (Fig. 12). A volumetric pipette was then used to collect 20 mL of suspended sediment at a depth of 20 cm from the surface at predetermined times representing the different fractions of the sediment still in suspension. The sediment fractions were then expressed as percentages divided in: shell (>2000 µm), coarse sand (2000-500 µm), medium sand (500-125 µm), fine sand (125-62 µm), silt (62-3.9 µm), and clay (<3.9 µm).

4.3 Clams

The Manila clams (*Ruditapes philippinarum*) were obtained from an aquaculture facility located in the southern basin of the lagoon of Venice. Before the exposure, they were measured (mean size ± standard deviation: 1st experiment 39.0 ± 4.4 mm, 2nd experiment: 36.21 ± 3.90 mm) and acclimatized to the laboratory conditions for 10 days. Clams were maintained in large aquaria (70 l), with a 10-15 cm uncontaminated sandy bottom and aerated natural seawater (salinity of 35 ± 0.5 and temperature of 10 ± 0.5 °C (1st experiment) or 12 ± 0.5 °C (2nd experiment)), and fed with a microalgae mixture (*Isochrysis galbana*, *Phaeodactylum tricornutum, Tetraselmis chui)*, at a final concentration of 130000 cells/l.

4.4 Exposure of clams to the sediments

Clams were exposed to sediment samples in two different experiments performed in November/December 2020 and March/April 2021, two weeks after sediment collection in the lagoon of Venice, and during two different phases of the gametogenic cycle of clams, namely the resting phase (November/December) and early gametogenesis (March/April).

In order to carry out the toxicity test, the sediments were placed in 28x50x30 cm (BxHxL) glass tanks. In both experiments, for each sampling site, 2 replicate tanks were prepared.

The sediment samples were thawed and mixed thoroughly before they were added into the tanks that were then filled up with seawater. Sediment to water ratio was about 1:6 (5 l of sediment and 30 l of water per tank). Natural seawater collected from the lagoon of Venice near Chioggia was used during the whole experiments. Before being released into the tanks, it was stored in a tank to stabilize the temperature

required for the experiment (10°C or 12°C, respectively for the 1st or 2nd experiment). The experimental tanks were maintained at a constant temperature inside a thermostatic room.

Each tank was provided with an aerator to properly oxygenate the seawater and covered with a Plexiglas slab to reduce evaporation.

One day after the sediment and water were added to the tanks, the clams were placed on the sediment, ensuring animals of similar size in each tank. In both experiments, for each site, 57 and 58 animals were added to the first and second tank, respectively. Clams were exposed to sediments for 3 (short-term exposure) and 14 days (long-term exposure).

Every 48 hours the seawater in the experimental tanks was changed by renewing 15 l of water and two water samples were taken out of each tank for the nutrient and sulphide analyses.

The animals were fed every 48 hours, after the water change, by adding 200 ml of a microalgae mixture (*Isochrysis galbana*, *Phaeodactylum tricornutum, Tetraselmis chui*) into each tank, at a final concentration of 130000 cells/l.

Seawater physico-chemical parameters were measured daily. Seawater temperature was determined with a thermometer with a precision of 0.1°C. Density was determined using densimeters and Knudsen tables were used for the calculation of salinity. pH was measured with a benchtop pH-meter (CRISON, mod. GLP 22). Dissolved oxygen was measured through the Fiber-Optic Oxygen Meter Piccolo2 (Pyro Science GmbH, Aachen, Germany) in glass vials provided with an oxygen sensor spot (OXSP5, Pyro Science GmbH).

4.5 Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. Twenty clams per site and per sampling time (3 and 14 days of exposure), 10 from each of the two replicate tanks per sediment, were used. Clams were then pooled into 5 groups of 4 animals each. A volume of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, haemocyte proliferation (XTT) and lactate dehydrogenase (LDH) activity. The remaining part of pooled haemolymph was then centrifuged at 780 × *g* for 10 min, the supernatant was discharged, while the pellets (haemocytes) were resuspended in distilled water to obtain haemocyte lysate samples. The lysate was then collected, frozen in liquid nitrogen and stored at − 80 °C until analyses (lysozyme, acid phosphatase and alkaline phosphatase activities and nitric oxide content assays).

Gills and digestive gland from clams were then excised, pooled to obtain ten different pools of five animals each, divided in aliquots, frozen in liquid nitrogen, and stored at − 80 °C until analyses.

4.6 Haemocyte/haemolymph parameters

THC, as well as haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). Briefly, 20 µL of haemolymph were added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^7)/mL of haemolymph, while haemocyte diameter and volume were expressed in µm and picolitres (pL), respectively.

Haemocyte proliferation was evaluated using the *Cell proliferation* Kit II, Roche (a commercial kit), as described in our previous study (Matozzo et al., 2012). In brief, a volume of 200 µL of the mixture provided by the kit was added to 400 µL of pooled haemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was then recorded using a Beckman 730 spectrophotometer. The results were normalised to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

The commercial *Cytotoxicity Detection* Kit, (Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph. Pooled haemolymph (500 µL) from each experimental condition was centrifuged at 780 x g for 10 min, and the supernatant was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

The lysozyme activity was measured in haemocyte lysate (HL) from pooled haemolymph (500 µL). HL was obtained as described above. Briefly, 50 µL of HL 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 3 min at room temperature. The results were expressed as µg lysozyme/mg of protein. Protein concentrations in the HL were quantified according to Bradford (1976).

Nitric oxide content was measured in haemocytes according to the in-house Griess method proposed by Yucel et al. (2012), after some modifications. Measurements were performed in microwell plates using a microplate reader. Pellets (=haemocytes) of haemolymph samples were centrifuged and supernatant was eliminated before they were frozen. Two replicates were measured for each sample. The pellets were resuspended in 400 ml phosphate-buffered saline (PBS, 0.01 M) and sonicated for 30 seconds at 90% wave amplitude. They were then centrifuged at 12000 x g and 4°C for 30 minutes. In each well of the plate, the sample supernatant (or PBS for the blank) was placed, then, at the ratio 1:1:1, sulphanilamide (1% in 5% hydrochloride acid) and finally N-(1-Naphthyl) ethylene diamine (NEDD, 0,1%) were added. After mixing and incubating for 30 min at 37°C, the plate was read at 540 nm. The NO content was quantified using a standard curve constructed by measuring known concentrations (0, 2.5, 5, 7.5, 10, 12.5, 25, 50 and 100 µM) of sodium nitrite ($NaNO_2$, 10 mM in PBS buffer). The results were expressed as nM/mg protein.

The acid phosphatase assay has been performed according to the method of Mazorra et al. (2002).

Briefly, the enzyme activity was measured in cell-free haemolymph samples. Two replicates per sample were prepared. Measurements were carried out in well plates using a microplate reader (Ivymen Optic System, mod. 2100-C). The supernatants and citrate buffer (pH 4.8) for the blanks were mixed 1:1 in the wells of the plate with the substrate solution, consisting of 5 mg 4-Nitrophenile phosphate in 2.5 mL citrate buffer, that was incubated at 37°C for 1 hour. In two extra wells of the plate, a standard solution (p-nitrilphenole 10 mM diluted 1:199 in NAOH 0.5 N) was inserted. The plate was then incubated for 10 minutes at 37°C. After that, the stop solution (NAOH 0,5 N) was added 1:2 to all the wells except the standards. Lastly, the plate was read at 405 nm. The results were expressed as units (U) of acid phosphatase/mg protein. One unit of acid phosphatase was defined as the amount of enzyme that hydrolyses 1 µmol of 4-Nitrophenile phosphate/min at 4.8 pH and 37 °C.

The alkaline phosphatase activity was measured in well plates with the well plate reader on cell-free haemolymph samples. Two replicates of each sample were performed. For the analysis, the method of Ross et al. (2000) was used. Briefly, the supernatant of each sample was mixed 1:1 with the substrate solution, consisting of 5 mg p-nitrophenyl phosphate in 3.37 ml ammonium bicarbonate buffer (100 mM with 1 mM magnesium chloride, pH 7.8, incubated for 30 min at 30°C). A blank was prepared using ammonium bicarbonate buffer instead of the sample. After incubating the plate for 1 hour at 30°C, the absorbance was measured at 405 nm using a microplate reader. The enzyme activity was expressed in U/mg protein, with 1 unit of alkaline phosphatase defined as the amount of enzyme that hydrolyses 1 µmol of 4-Nitrophenile phosphate/min at 7.8 pH and 30 °C.

4.7 Gill and digestive gland enzyme activity assays

Before analyses, gill and digestive gland samples were homogenized in homogenization buffer (TRIS-HCl 10 mM, pH 7.6, KCl 0.15 M, Sucrose 0.5 M, with protease inhibitors) using a homogenizer (TissueLyser LT, Qiagen) for 5 minutes at 50 Hz oscillation frequency. After 30 minutes of centrifugation at 12000 x g and

4°C, the supernatant (SN) was used for analyses. The protein concentration in SN samples was quantified according to Bradford (1976).

Total superoxide dismutase (SOD) activity was measured in both gills and digestive gland in triplicate using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg protein, one unit of SOD has been defined as the amount of sample causing 50% inhibition under the assay conditions.

Catalase (CAT) activity was measured in gills and digestive gland SN in triplicate following the method proposed by Aebi (1984). The enzyme activity in a volume of 30 µL of tissue SN were measured at 240 nm and expressed as U/mg protein; one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min.

Glutathione reductase (GR) activity was evaluated according to Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg protein.

The method of Ellman et al. (1961) was used to measure acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in gill SN (25 µL), following the colorimetric reaction between acetylthiocholine or butyrylthiocholine, respectively, and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein.

Glutathione S-transferase (GST) activity was measured in digestive gland SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg protein.

The glyoxalase type 1 activity was measured spectrophotometrically on digestive gland samples according to the method proposed by Principato et al. (1983). Briefly, phosphate buffer (100 mM KH2PO4, pH 6.8), GSH (100 mM) and metylglyoxal (2%) were mixed 84:10:1:5 with the sample supernatant. The increase in absorbance, due to S-D-lactoylglutathione formation catalysed by the enzyme, was measured at 240 nm for 30 seconds in at least two replicates per sample. The blank was prepared using phosphate buffer instead of sample. The results were expressed in µm/min/mg protein.

Oxidative damage in gills and digestive gland was measured through the protein carbonyl content (PCC) assay. Briefly, PCC was measured in duplicate using the method of Mecocci et al. (1999) following the reaction with 2,4-dinitrophenylhydrazide (DNPH). Results were expressed as nmol carbonyl group/mg protein.

4.8 Seawater analyses

To measure the ammonia (NH_3), nitrite (NO_2^-), and orthophosphate (PO_4^-) content, seawater samples from each tank were collected in plastic bottles every 48 hours before the water renewal and stored at -20°C until the analyses. To measure the amount of ammonia present in the water samples, the indophenol blue method was employed (Solorzano, 1969). Each sample was mixed 50:2:2:5 with phenol solution (20 g phenol in 200 ml 95% v/v ethyl alcohol), then nitroprusside (1 g sodium nitroprusside in 200 ml distilled water) and finally oxidizing solution. The oxidizing solution consisted of 4:1 alkaline reagent (100 g sodium citrate tribasic dihydrate and 5 g sodium hydroxide in 500 ml distilled water) and sodium hypochlorite solution (1.5 N). After the samples were incubated for 1 hour at room temperature, the absorbance was determined with the spectrophotometer at 640 nm against the blank prepared with distilled water. To quantify the amount of ammonia in the sample, a standard curve generated by measuring known concentrations of ammonium chloride (0.15, 0.6, 1.5, 3, 9, 13.5, 18, 27, 36 µM) was used. The results were expressed in µmol/l. The analysis of nitrites is based on the formation of a pink azo dye derived from a series of reactions, known as Griess reaction. The method is reported in Pearsons et al. (1984). For the analysis, the water sample was mixed 50:1:1 with sulphanilamide (5 g sulphanilamide in 50 ml hydrochloric acid and 500 ml distilled water) and N-(1-naphtyl)ethylenediamine dihydrochloride (NNEDDC, 500 mg in

500 ml distilled water). First the sulphanilamide was added to the sample, incubated for 5 minutes and then mixed with NNEDDC, allowing the reaction to take place for 10 more minutes. The samples were measured against the blank prepared with distilled water with the spectrophotometer at 543 nm. To quantify the amount of nitrates in the sample, a standard curve generated by measuring known concentrations of sodium nitrate (0.2, 0.5, 1, 1,5, 2, 3 µM) was used. The results were expressed in µmol/l. The amount of orthophosphates in the water samples was detected using the molybdenum blue method, as described in Pearsons et al. (1984). In this analysis the orthophosphates are measured spectrophotometrically using a mixed reagent, that consisted 1:2.5:1:0.5 of ammonium molybdate, sulfuric acid, ascorbic acid (2.7 g in 50 ml water) and antimony potassium tartrate solution (0.34 g in 250 ml distilled water). The mixed reagent was added 1:10 to the water sample or to seawater from the cistern for the blank. After 5 minutes of incubation, the absorbance of the samples was read against the blank at 882 nm. To quantify the amount of orthophosphates in the sample, a standard curve constructed by measuring known concentrations of monopotassium phosphate in sea water (0.2, 0.5, 1, 1,5, 2, 3 µM) was used. The results were expressed in µmol/l.

To measure the amount of sulphides liberated from the sediment into the tank water, the method described by Pearsons et al. (1984) was followed. Every 48 hours, water samples were collected in glass bottles 10-15 minutes after each water change. In the bottle, at the ratio of 1:1:500, zinc acetate (2N), NaOH (6 N) and then seawater was added without creating bubbles. The bottles were mixed well until a precipitate was visible and then stored at 4°C in the dark until the analysis was conducted. For the analysis, the samples were mixed thoroughly, and an aliquot was added, in the 12.5:1 ratio, to a mixed reagent. This reagent consisted of 2 g of N, N-dimetyl-p-phenylenediamine in 500 ml HCl (6N), mixed 1:1 with 3 g iron chloride in 500 ml HCl (6N). Two replicates per sample were made and blanks were prepared in the same way using filtered sea water instead of sample as a control for seawater contamination. The samples were mixed twice by inversion, incubated for 20 minutes in the dark and mixed twice again. After this, the absorbance was measured at 670 nm with the spectrophotometer against a blank of only distilled water. The concentration in µmol/ml was quantified using a standard curve constructed by measuring known concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30 and 50 µM) of Na2S*9 H_2O in oxygen-free distilled water, prepared and read in the same way as the samples.

4.9 Statistical analysis

Statistical differences in the biomarker responses and the water quality depending on sediment sampling time, site and day of exposure have been investigated by a three-way analysis of variance (ANOVA). Before the analysis, the data were tested for normality and homoscedasticity with the Shapiro-Wilk test and the Breusch-Pagan test, respectively. Not normal data were log-transformed. Multiple pair-wise comparisons of the biomarker responses among times and sites were performed using the Tukey HSD test.

The differences between sediment sampling time, sites and day of exposure were visualized with a principal component analysis (PCA). To test whether differences among clusters were significant, a non-parametric permutational multivariate analysis of variance (PERMANOVA, with 9999 permutations) was performed, applying the Euclidean distance matrix on the raw data.

The analyses and graphics were done using RStudio software (version 4.1.2, R Core Team, 2021).

5 Results

5.1 Sediment characterization

From the analysis carried out in the sediment samples collected in November 2020, a high percentage of organic matter was observed in the first five sediments, in particular the fourth and fifth ones have the highest values of about 3%; on the contrary, there is a low amount, less than 0.5%, in the sixth sediment (Table 1).

A slight increase in the clay percentage was observed from the first to the fifth sediment, while a low percentage is present in the sixth (Table 2). Silt is the predominant component in the first five sediments, while it is a smaller fraction in the sixth, where most is medium sand. In addition, the latter sampling site shows a considerable amount of shell fragments compared to the others and coarse sand. Sediments 2, 3, and 6 show more fine sand than the others.

Table 1. Organic matter content in sediments from the six sampling sites collected in November 2020.

Site	Organic matter content (in %)
1	1,22 (± 0,18)
2	2,17 (± 0,05)
3	2,52 (± 0,06)
4	3,07 (± 0,10)
5	3,21 (± 0,10)
6	0,24 (± 0,04)

Table 2. Grain-size distribution in sediments from the six sampling sites collected in November 2020.

Grain-size distribution (%)	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Coarse sand (>2000-500 µm)	0,55	0,42	0,94	0,11	0,39	13,73
Medium sand (500-125 µm)	0,93	12,76	2,79	0,55	1,22	71,79
Fine sand (125-62 µm)	1,91	13,61	7,82	1,23	0,91	7,40
Silt (62-3,9 µm)	95,46	70,82	85,80	94,12	93,98	5,85
Clay (< 3,9 µm)	1,16	2,39	2,65	3,99	3,50	1,23

5.2 Haemocyte biomarkers

All haemocyte biomarkers were significantly different between experiments (Figure 2). In particular, THC was slightly higher in the first experiment and in particular in clams from site 1 and 6, while cell diameter and volume were lower. Cytotoxicity (LDH activity) and haemocyte proliferation (XTT assay) were lower in the first experiment compared to the second, indicating that the immunological defences were not activated. However, higher oxidative damage (nitric oxide content) was detected in the haemocytes of the first experiment.

THC, LDH, XTT, Acid and alkaline phosphatase and nitric oxide content were significantly different among sites, but a clear pattern cannot be identified in the graphs. Indeed, the interactions between factors Experiment:Site (interaction), Experiment:Day (interaction) and Site:Day (interaction) are often statistically significant, complicating the general picture.

Factors	THC	Diameter	Volume	LDH	Cell proliferation	Lysozyme	Acid phosphatase	Alkaline phosphatase	Nitric oxide
Experiment	$F_{(1,96)}$=59.95 p<0.001	$F_{(1,96)}$=32.18 p<0.001	$F_{(1,96)}$=32.04 p<0.001	$F_{(1,96)}$=94.25 p<0.001	$F_{(1,96)}$=126.46 p<0.001	$F_{(1,96)}$=28.39 p<0.001	$F_{(1,96)}$=246.33 p<0.001	$F_{(1,96)}$=6.16 p=0.015	$F_{(1,96)}$=146.97 p<0.001
Site	$F_{(5,96)}$=5.66 p<0.001	$F_{(5,96)}$=0.99 p=0.427	$F_{(5,96)}$=1.02 p=0.410	$F_{(5,96)}$=2.32 p=0.049	$F_{(5,96)}$=3.476 p=0.006	$F_{(5,96)}$=0.44 p=0.819	$F_{(5,96)}$=3.63 p=0.005	$F_{(5,96)}$=3.53 p=0.006	$F_{(5,96)}$=14.34 p<0.001
Day	$F_{(1,96)}$=11.20 p=0.001	$F_{(1,96)}$=3.12 p=0.080	$F_{(1,96)}$=3.07 p=0.083	$F_{(1,96)}$=4.21 p=0.043	$F_{(1,96)}$=0.096 p=0.757	$F_{(1,96)}$=2.57 p=0.113	$F_{(1,96)}$=1.69 p=0.196	$F_{(1,96)}$=8.42 p=0.005	$F_{(1,96)}$=0.016 p=0.898
Experiment:Site	$F_{(5,96)}$=3.28 p=0.009	$F_{(5,96)}$=1.97 p=0.090	$F_{(5,96)}$=1.91 p=0.100	$F_{(5,96)}$=0.64 p=0.667	$F_{(5,96)}$=1.59 p=0.170	$F_{(5,96)}$=0.81 p=0.549	$F_{(5,96)}$=1.12 p=0.358	$F_{(5,96)}$=3.23 p=0.010	$F_{(5,96)}$=5.52 p<0.001
Experiment:Day	$F_{(1,96)}$=13.29 p<0.001	$F_{(1,96)}$=5.92 p=0.017	$F_{(1,96)}$=6.04 p=0.016	$F_{(1,96)}$=12.08 p<0.001	$F_{(1,96)}$=4.01 p=0.048	$F_{(1,96)}$=0.63 p=0.429	$F_{(1,96)}$=0.11 p=0.741	$F_{(1,96)}$=12.028 p<0.001	$F_{(1,96)}$=27.92 p<0.001
Site:Day	$F_{(5,96)}$=6.09 p<0.001	$F_{(5,96)}$=1.09 p=0.370	$F_{(5,96)}$=1.08 p=0.376	$F_{(5,96)}$=1.14 p=0.483	$F_{(5,96)}$=3.89 p=0.003	$F_{(5,96)}$=0.58 p=0.711	$F_{(5,96)}$=2.45 p=0.039	$F_{(5,96)}$=2.89 p=0.018	$F_{(5,96)}$=1.46 p=0.212
Experiment:Site:Day	$F_{(5,96)}$=1.67 p=0.150	$F_{(5,96)}$=1.02 p=0.411	$F_{(5,96)}$=0.99 p=0.431	$F_{(5,96)}$=1.14 p=0.342	$F_{(5,96)}$=1.51 p=0.193	$F_{(5,96)}$=0.37 p=0.866	$F_{(5,96)}$=3.40 p=0.007	$F_{(5,96)}$=4.28 p=0.001	$F_{(5,96)}$=4.92 p<0.001

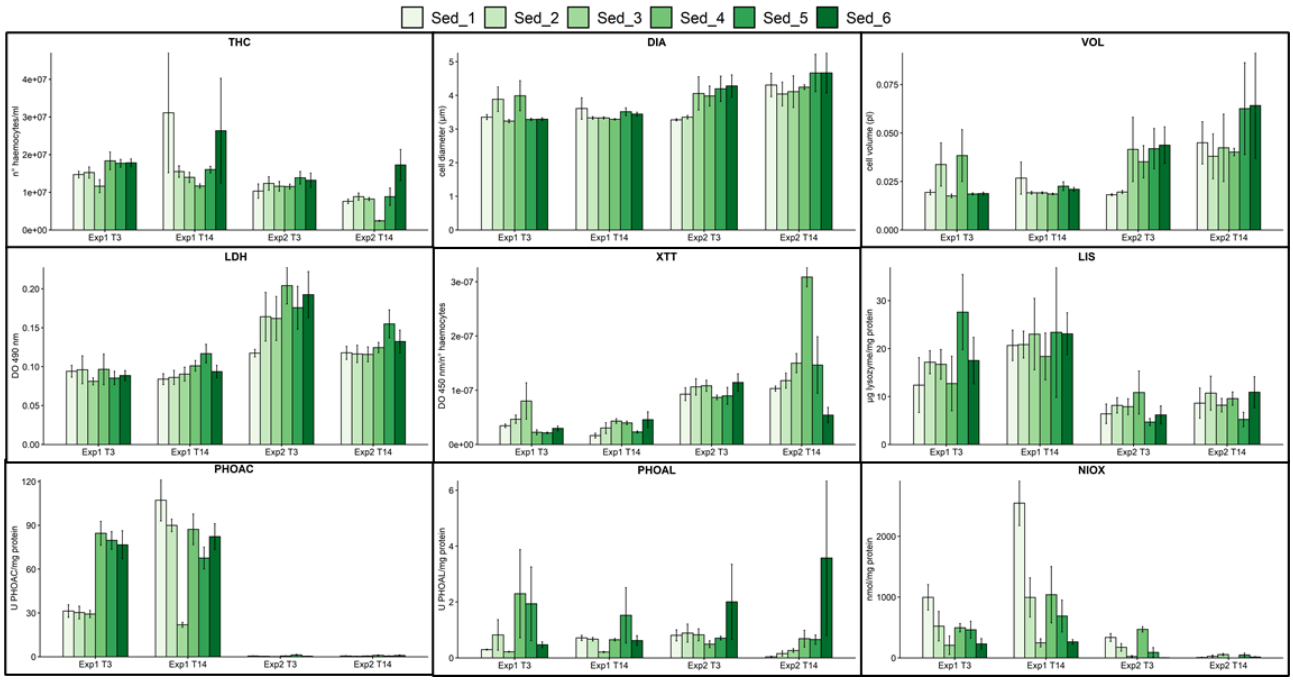

Figure 2. Results of cell biomarkers measured in haemocytes/haemolymph of clams (mean ± standard error) exposed for 3 and 14 days to sediments from the different sites (1, 2, 3, 4, 5, 6) during Novembre 2020 (Exp1) and March 2021 (Exp2). Total haemocyte count (THC); haemocyte diameter (DIA); haemocyte volume (VOL); *Cytotoxicity* (LDH); cell proliferation (XTT); lysozyme activity (LIS); acid phosphatase activity (PHOAC); alkaline phosphatase activity (PHOAL); nitric oxide content (NIOX). Results of the three-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

5.3 Gills biomarkers

Antioxidant defences were induced in the second exposure experiment compared to the first one. Indeed, CAT activity was significantly higher in the trials carried out in March 2021. This suggested a higher protection of the animals against oxidative damages to the proteins. Indeed, PCC values were significantly lower in the second exposure experiment compared to the first one (Figure 3).

In the first exposure experiment, AChE in clams exposed to sediments 1-5 showed values like those from the reference site #6 after 3 days of exposure, while a decrease of activity was recorded after 14 days of exposure. On the other hand, BChE showed an opposite pattern, with a decrease of activity at T3 but not at T14. With regards to the second exposure experiment, an induction of both AChE and BChE activities was recorded at T3 for clams exposed to all sediments, when compared to those exposed to sediment 6. At T14, values were more like the reference site.

The presence of contaminants able to affect enzyme activity more bioavailable for animals during the winter than the spring cannot be excluded.

Factors	SOD	CAT	GR	AChE	BChE	PCC
Experiment	$F_{(1,96)}$=0.006 p=0.936	$F_{(1,96)}$=1158.728 p<0.001	$F_{(1,96)}$=61.855 p<0.001	$F_{(1,96)}$=7.355 p=0.008	$F_{(1,96)}$=49.735 p<0.001	$F_{(1,96)}$=115.857 p<0.001
Site	$F_{(5,96)}$=5.671 p<0.001	$F_{(5,96)}$=1.777 p=0.125	$F_{(5,96)}$=3.377 p=0.007	$F_{(5,96)}$=1.630 p=0.159	$F_{(5,96)}$=1.367 p=0.244	$F_{(5,96)}$=3.340 p=0.008
Day	$F_{(1,96)}$=0.752 p=0.388	$F_{(1,96)}$=10.998 p=0.001	$F_{(1,96)}$=4.541 p=0.036	$F_{(1,96)}$=4.093 p=0.046	$F_{(1,96)}$=0.881 p=0.350	$F_{(1,96)}$=0.851 p=0.359
Experiment:Site	$F_{(5,96)}$=6.012 p<0.001	$F_{(5,96)}$=1.515 p=0.192	$F_{(5,96)}$=11.365 p<0.001	$F_{(5,96)}$=3.430 p=0.007	$F_{(5,96)}$=1.251 p=0.291	$F_{(5,96)}$=1.839 p=0.112
Experiment:Day	$F_{(1,96)}$=89.212 p<0.001	$F_{(1,96)}$=25.348 p<0.001	$F_{(1,96)}$=32.568 p<0.001	$F_{(1,96)}$=0.010 p=0.919	$F_{(1,96)}$=8.915 p=0.004	$F_{(1,96)}$=2.287 p=0.134
Site:Day	$F_{(5,96)}$=3.967 p=0.003	$F_{(5,96)}$=15.613 p<0.001	$F_{(5,96)}$=11.653 p<0.001	$F_{(5,96)}$=0.480 p=0.790	$F_{(5,96)}$=2.379 p=0.044	$F_{(5,96)}$=0.833 p=0.530
Experiment:Site:Day	$F_{(5,96)}$=2.198 p=0.061	$F_{(5,96)}$=18.215 p<0.001	$F_{(5,96)}$=1.738 p=0.133	$F_{(5,96)}$=1.692 p=0.144	$F_{(5,96)}$=3.564 p=0.005	$F_{(5,96)}$=1.212 p=0.310

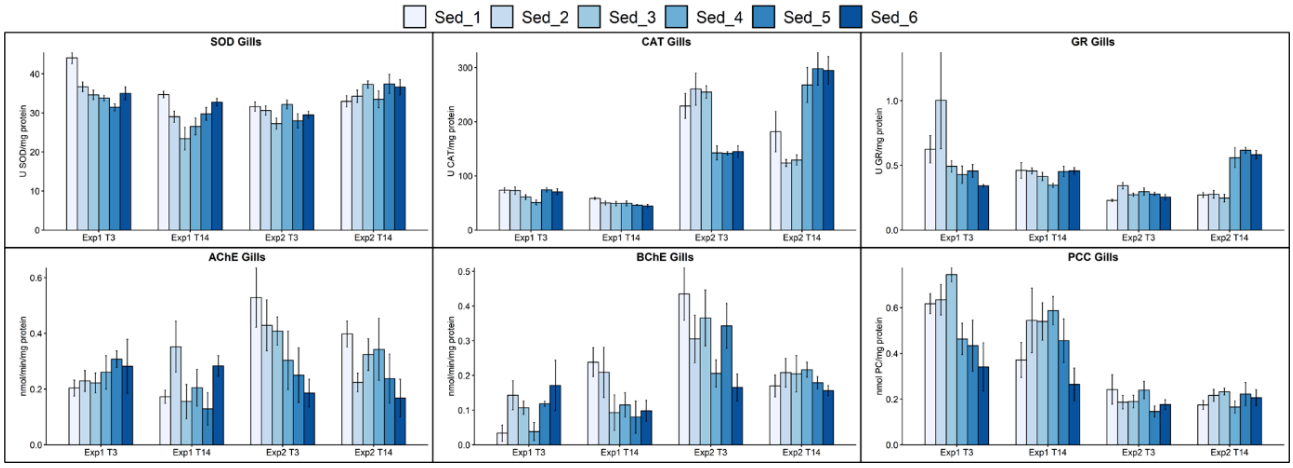

Figure 3. Results of gill biomarkers measured in clams (mean ± standard error) exposed for 3 and 14 days to sediments from the different sites (1, 2, 3, 4, 5, 6) *during* Novembre 2020 (Exp1) and March 2021 (Exp2). SOD activity; CAT activity; GR activity; AChE activity; BChE activity; protein carbonyl content (PCC). Results of the three-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

5.4 Digestive gland biomarkers

The oxidative stress-related enzymes showed a different modulation of their activity between the two exposure experiments (Figure 4). SOD activity was significantly lower in the first experiment, in particular at T14. CAT activity was significantly lower in the first experiment; however, differences were also present between the sampling days (3 and 14 days). CAT activity of clams exposed to sediments 2 and 3 increased with time, while the activity lowered with time in clams exposed to sediments 4, 5 and 6.

GR activity and the oxidative damage were significantly lower in the second experiment.

The two detoxification enzymes showed an opposite pattern of variation, with higher GST levels in the first exposure experiment and higher GLIOX levels in the second one.

GST activity remained stable during the first exposure, while it lowered with time in the second one. GLIOX activity increased with time between T3 and T14 for most sediments, except #6, in the November 2020 exposure, while it remained stable in the March 2021 exposure (Figure 4).

Factors	SOD	CAT	GR	GST	GLIOX	PCC
Experiment	$F_{(1,96)}$=67.344 p<0.001	$F_{(1,96)}$=2535,465 p<0.001	$F_{(1,96)}$=393,656 p<0.001	$F_{(1,96)}$=226.439 p<0.001	$F_{(1,96)}$=35.418 p<0.001	$F_{(1,96)}$=48.217 p<0.001
Site	$F_{(5,96)}$=3.080 p=0.013	$F_{(5,96)}$=11.314 p<0.001	$F_{(5,96)}$=5.867 p<0.001	$F_{(5,96)}$=6.537 p<0.001	$F_{(5,96)}$=2.562 p=0.032	$F_{(5,96)}$=3.483 p=0.006
Day	$F_{(1,96)}$=35.983 p<0.001	$F_{(1,96)}$=24.667 p<0.001	$F_{(1,96)}$=13.379 p<0.001	$F_{(1,96)}$=52.892 p<0.001	$F_{(1,96)}$=12.826 p<0.001	$F_{(1,96)}$=0.690 p=0.408
Experiment:Site	$F_{(5,96)}$=2.017 p=0.083	$F_{(5,96)}$=4.198 p=0.002	$F_{(5,96)}$=3.937 p=0.003	$F_{(5,96)}$=2.334 p=0.048	$F_{(5,96)}$=1.674 p=0.148	$F_{(5,96)}$=3.926 p=0.003
Experiment:Day	$F_{(1,96)}$=61.736 p<0.001	$F_{(1,96)}$=0.952 p=0.332	$F_{(1,96)}$=9.303 p=0.003	$F_{(1,96)}$=5.864 p=0.017	$F_{(1,96)}$=3.446 p=0.066	$F_{(1,96)}$=3.137 p=0.080
Site:Day	$F_{(5,96)}$=3.299 p=0.009	$F_{(5,96)}$=25.653 p<0.001	$F_{(5,96)}$=1.699 p=0.142	$F_{(5,96)}$=3.678 p=0.004	$F_{(5,96)}$=1.611 p=0.164	$F_{(5,96)}$=0.575 p=0.719
Experiment:Site:Day	$F_{(5,96)}$=1.483 p=0.203	$F_{(5,96)}$=18.764 p<0.001	$F_{(5,96)}$=0.961 p=0.445	$F_{(5,96)}$=2.229 p=0.058	$F_{(5,96)}$=4.313 p<0.001	$F_{(5,96)}$=2.267 p=0.054

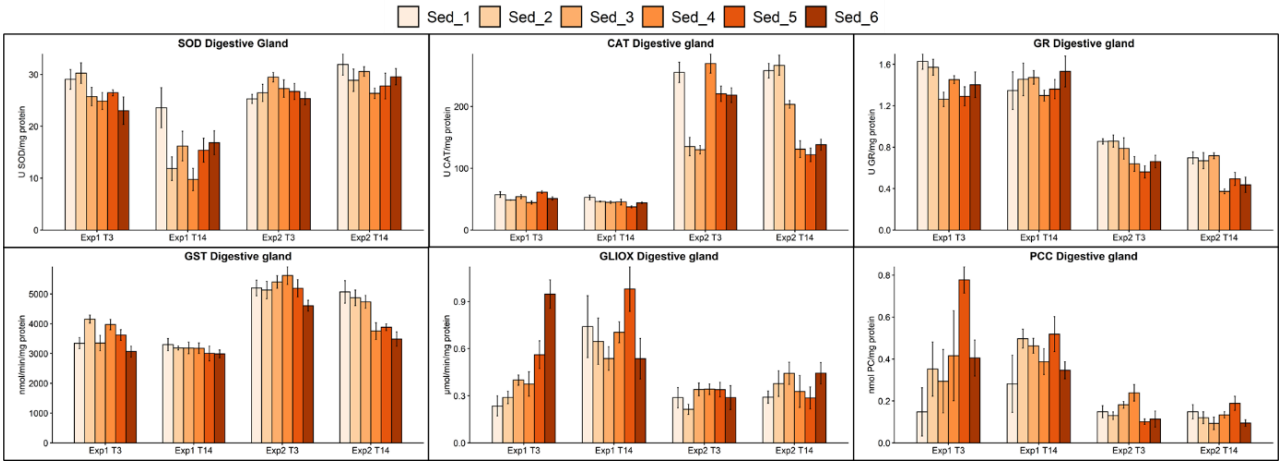

Figure 4. Results of digestive gland biomarkers measured in clams (mean ± standard error) exposed for 3 and 14 days to sediments from the different sites (1, 2, 3, 4, 5, 6) during *Novembre* 2020 (Exp1) and March 2021 (Exp2). SOD activity; CAT activity; GR activity; GST activity; GLIOX activity; protein carbonyl content (PCC). Results of the three-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

5.5 Multivariate analyses

The PERMANOVA and the PCA analyses confirmed the significant differences between the two experiments. The PCA showed that the two experiments are clearly separated along the PC1 (33.74% of the variance explained). Along the PC2 (10.06%), the two sampling times (T3 and T14) were slightly separated, although dots overlapped. It is noteworthy that the two sampling times (T3 and T14) are separated in an opposite way regarding the experiments (i.e. along the PC2) (Figure 5). This means that the biomarker response pattern varied in a different way between days of exposure when comparing the two experiments. For example, the SOD activity in gills was lower at T14 compared to T3 in the first experiment, but was higher at T14 compared to T3 in the second experiment. The specific variation of the other biomarkers has been described in the previous chapters. Among sites, within each experiment, a separation between exposure day is only slightly visible, although not clear. This suggested a slightly broader difference in the values of the biomarkers investigated in clams during the first experiment, when compared to the second one.

Factors	Df	F value	R^2	p value
Experiment	1	13.987	0.104	< 0.001
Site	5	1.616	0.060	0.126
Day	1	0.001	0.000	0.978
Experiment:Site	5	0.698	0.026	0.701
Experiment:Day	1	2.982	0.022	0.083
Site:Day	5	1.547	0.058	0.145
Experiment:Site:Day	5	0.471	0.018	0.850
Residuals	96		0.713	

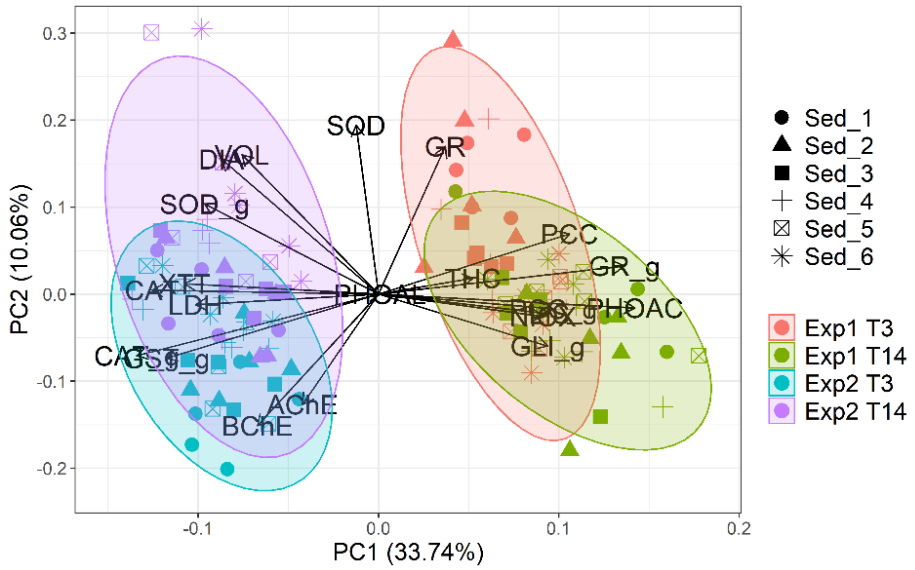

Figure 5. PERMANOVA and PCA analyses performed on the whole dataset of biomarkers analysed in haemolymph, gills and digestive gland of clams exposed for 3 and 14 days to *the* sediments sampled in Novembre 2020 (Exp1) and March 2021 (Exp2) from different sites (1, 2, 3, 4, 5, 6). Results of the PERMANOVA are reported in the table above the graph.

5.6 Water analyses

From the analysis of the seawater samples from the exposure tanks, different values of the parameters investigated were observed among the various sediments, with a similar pattern of variation in both experiments (Figure 6). However, the pattern does not always match between experiments.

In the first experiment, there is a gradual decrease in the concentration of nitrite, phosphate and ammonia over time, while an increase is observed in the case of sulphides. The sixth sediment, when compared to the others, showed intermediate conditions regarding the concentration of nitrites, phosphates and sulphides, while it showed the lowest and most constant concentrations of ammonia. The fifth sediment showed a higher concentration among the various analyses and differed more in the values of dissolved nitrites.

In the second experiment, nitrite concentration gradually increased during the exposure, while phosphate and ammonia decreased. Sulphide's content showed high variability along the exposure.

In general, results showed that higher values of nitrite, phosphate, ammonia and sulphides were present in the second experiment compared to the first one. This could reflects a seasonal-based natural variation of the nutrients' content in seawater. However, a differential contribution of clams' metabolism among sites may be present, given the statistical difference of the factor "Site" (Figure 6).

The sixth sediment, when compared to the others, showed intermediate conditions regarding the concentration of nitrites, phosphates and sulphides, while it presents the lowest and most constant concentrations of ammonia.

	NO_2	PO_4	NH_3	Sulphides
Experiment	$F_{(1,84)}$=513.507 p<0.001	$F_{(1,84)}$=821.294 p<0.001	$F_{(1,84)}$=41763.948 p<0.001	$F_{(1,84)}$=786.903 p<0.001
Site	$F_{(5,84)}$=19.599 p<0.001	$F_{(5,84)}$=10.835 p<0.001	$F_{(5,84)}$=1379.901 p<0.001	$F_{(5,84)}$=8.323 p<0.001
Day	$F_{(6,84)}$=1.585 p=0.162	$F_{(6,84)}$=64.381 p<0.001	$F_{(6,84)}$=90.421 p<0.001	$F_{(6,84)}$=5.641 p<0.001
Experiment:Site	$F_{(5,84)}$=30.282 p<0.001	$F_{(5,84)}$=15.358 p<0.001	$F_{(5,84)}$=202.898 p<0.001	$F_{(5,84)}$=7.022 p<0.001
Experiment:Day	$F_{(6,84)}$=10.837 p<0.001	$F_{(6,84)}$=3.754 p=0.002	$F_{(6,84)}$=9.600 p<0.001	$F_{(6,84)}$=8.878 p<0.001
Site:Day	$F_{(30,84)}$=1.593 p=0.050	$F_{(30,84)}$=1.157 p=0.296	$F_{(30,84)}$=2.318 p=0.001	$F_{(30,84)}$=3.286 p<0.001
Experiment:Site:Day	$F_{(30,84)}$=1.094 p=0.364	$F_{(30,84)}$=1.515 p=0.072	$F_{(30,84)}$=4.733 p<0.001	$F_{(30,84)}$=3.589 p<0.001

	NO2		PO4		NH3		Sulphides	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
Sed_1	A	A	A	AB	A	A	A	AB
Sed_2	B	AB	B	A	B	BC	A	AB
Sed_3	AB	AB	B	A	C	B	A	C
Sed_4	A	AB	B	C	D	C	A	AC
Sed_5	C	B	B	ABC	D	BC	A	B
Sed_6	A	C	B	BC	E	D	A	AB

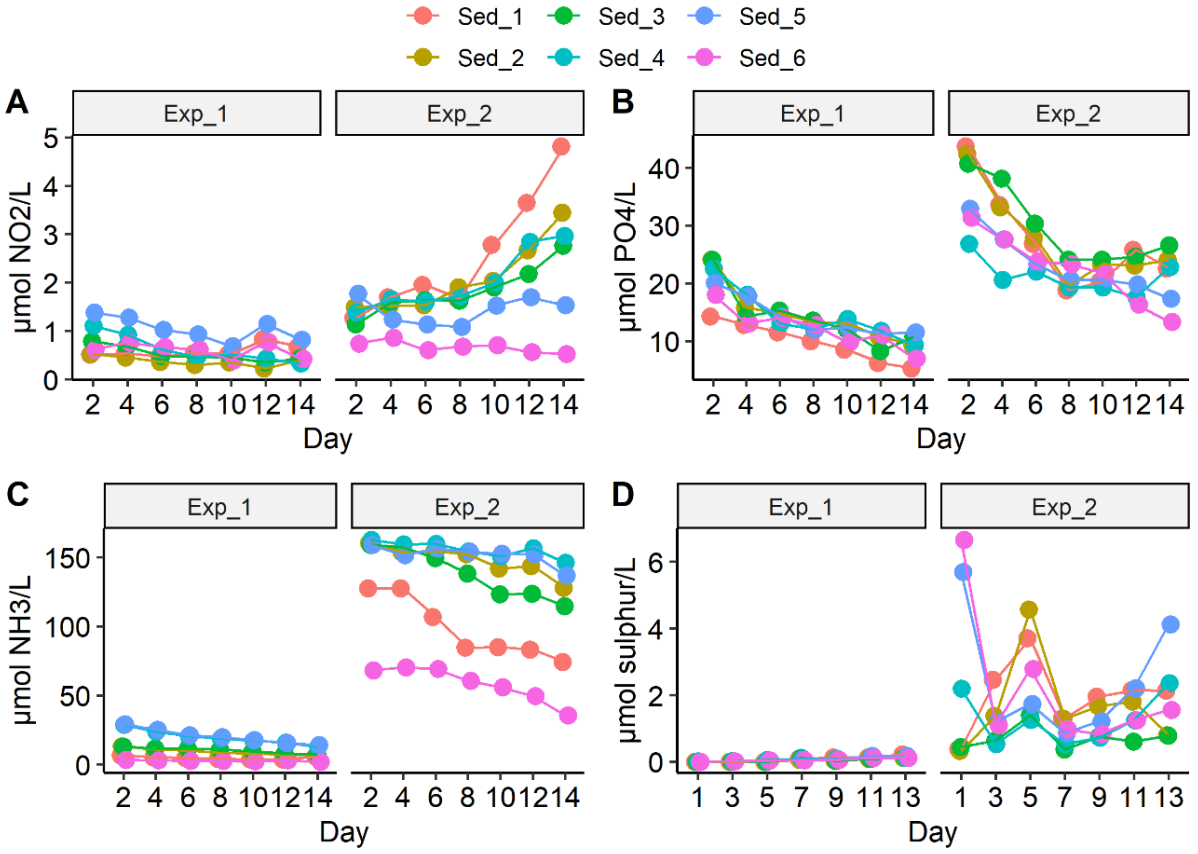

Figure 6. Variation pattern of concentration of nitrites, phosphates, ammonia and sulphides in seawater samples during the exposures of clams carried out with sediments sampled in Novembre 2020 (Exp1) and March 2021 (Exp2) from different sites (1, 2, 3, 4, 5, 6). A: nitrite content; B: *phosphate* content; C: ammonia content; D: sulphur content. Average values are reported. Results of the three-way ANOVA analysis are reported in the table above the graph. Significant post-hoc comparisons among sediments are highlighted in the second table, different letters indicate statistical significance.

6 Conclusions

In this deliverable, the results of a battery of biomarkers indicative of cell damage, oxidative stress and damage, neurotoxicity and detoxification capability of clams exposed to sediments sampled in 6 sites in the Venice lagoon are reported. Five sediments have been sampled along a transect between the city of Venice and the industrial area of Porto Marghera. A sixth sediment, namely reference site, was sampled in an area with presumably low contamination level. Two exposure experiments have been carried out with sediments sampled in different time. Clams were exposed to sediments in winter (November 2020) and spring (March 2021).

The results of haemolymph, gills and digestive gland of the animals as well as the seawater analyses, suggest that the biochemical responses of clams have been modified in the two experiments with potential influence of either the reproductive phase of the animal or the contamination pattern.

Site-based variations of the biomarker's response were present; however, no clear pattern could be identified. Oddly, some of the responses varied in the same way in presumably contaminated sites as in the control (site 6) sediment. Many biomarkers were influenced by the exposure duration showing both an increase and decrease over time for the different tissues and biomarkers. This could either indicate an increase in toxicity due to longer exposure time or the fact that the animals acclimate to the contamination level. Indications of lower immune system function and oxidative stress were evident, even if no clear pattern of variation among sites was determined, suggesting that similar chemical impacts were present.

Chemical analyses of the sediments will provide useful information on the contamination pattern that will help to understand the results of the present deliverable.

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