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Programma di ricerca scientifica per una laguna “regolata”

Linea 2.1

Qualità del sedimento lagunare a supporto della sua gestione sostenibile

D2.1.4.4

Risultati dei biomarkers presso la bocca di porto di Chioggia

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Sommario

Summary

The Venice lagoon is a vulnerable and heterogeneous coastal environment subject to various pressures from several stressors that can affect the biology of species inhabiting this complex habitat. Due to its environmental characteristics, such as the peculiar tidal exchanging, the lagoon of Venice is well suited to be an optimal farming area for edible bivalve species, like Manila clams (*Ruditapes philippinarum*) and Mediterranean mussel (*Mytilus galloprovincialis*), whose welfare, in turn, may be affected by the presence of stressors.

Farming activities in recent years have been affected by climate change, by heat waves and hypoxia events. Among the potential anthropogenic disturbances, the activation of the MoSE system, designed to protect the city of Venice and its lagoon from the high tides, requires an accurate monitoring plan able to investigate possible effects on the lagoon ecosystem and bivalve farming sites.

In this deliverable, we report for the first time the results obtained from the evaluation of the potential effects of the MoSE system in Manila clams and Mediterranean mussel farmed in the southern basin of the lagoon of Venice. After the annual spat release in May, the animals were seasonally sampled over the course of a year. The monitoring was carried out twice over two calendar years, before and after the inauguration of the MoSE system barriers in December 2020. In detail, immunological biomarkers were analysed in the haemolymph, while antioxidant defence, detoxification, oxidative stress damage and neurotoxicity biomarkers were analysed in gills and digestive gland.

For both clams and mussels, the results obtained highlighted a small influence of the farming sites on the analysed biomarkers, with no specific trends in most of the cases. No differences are expected between the various sites in terms of environmental parameters, pollutants and pathogens. Biomarkers analysed in gills and digestive gland highlighted different modulation of the organism defences between the two tissues in order to protect them from any damage.

Differences have been detected among sampling times and, even more markedly, between the two years. Differences in results between sampling times and sites are more pronounced in the first year than in the second one. Although this cannot be related to the MoSE alone, being the lagoon of Venice a very complex environment, our findings suggest that the environmental conditions were more homogeneous among sites and time in the second year.

Riassunto

La laguna di Venezia è un ambiente costiero vulnerabile ed eterogeneo soggetto a diverse pressioni da parte di molti fattori di stress che possono influenzare la biologia delle specie che abitano questo complesso habitat. Per le sue caratteristiche ambientali, la laguna di Venezia si presta bene ad essere un'area ottimale per l'allevamento di bivalvi commestibili, come vongole filippine (*Ruditapes philippinarum*) e cozze (*Mytilus galloprovincialis*), il cui benessere può essere influenzato dalla presenza di condizioni ambientali stressogene.

Le attività di venericoltura e mitilicoltura sono minacciate negli ultimi anni dai cambiamenti climatici, e in particolare dalle ondate di calore e fenomeni di ipossia che colpiscono in modo rilevante l'ambiente lagunare. Tra i potenziali disturbi antropogenici, l'attivazione del sistema MoSE, progettato per proteggere la città di Venezia e la sua laguna dalle alte maree, richiede un accurato piano di monitoraggio in grado di indagare i possibili effetti per l'ecosistema lagunare di Venezia e i siti di allevamento dei bivalvi.

In questo rapporto riassumiamo per la prima volta i risultati ottenuti dalla valutazione dei potenziali effetti del sistema MoSE su vongole filippine e cozze allevate nel bacino sud della laguna di Venezia. Dopo la semina annuale effettuata nel mese di maggio, gli animali sono stati campionati stagionalmente lungo il corso di un anno. Il monitoraggio è stato eseguito per due volte lungo due anni solari, prima e dopo l'inaugurazione delle barriere del sistema MoSE nel dicembre 2020. In dettaglio, negli animali sono stati analizzati biomarcatori immunologici nell'emolinfa, mentre biomarcatori di difesa antiossidante, detossificazione, danno da stress ossidativo e neurotossicità sono stati analizzati nelle branchie e nella ghiandola digestiva.

Sia per le vongole che per le cozze, i risultati ottenuti hanno evidenziato poche differenze tra i siti di allevamento nei vari biomarcatori analizzati, senza tendenze specifiche, nella maggior parte dei casi. Questo suggerisce un'elevata somiglianza dei parametri ambientali, di livelli di contaminazione e di patogeni tra i siti all'interno del bacino lagunare meridionale, anche tra quelli più vicini e più lontani allo sbocco al mare. I biomarcatori analizzati nelle branchie e nella ghiandola digestiva hanno evidenziato una diversa modulazione delle difese dell'organismo tra i due tessuti al fine di proteggerli da eventuali danni.

Sono state rilevate differenze tra i tempi di campionamento e, in modo ancora più marcato, tra i due anni. Infatti, le differenze nei risultati tra i tempi e i siti di campionamento sono più marcate nel primo anno rispetto al secondo. Anche se questo non può essere correlato al solo MoSE, in quanto la laguna di Venezia è un ambiente molto complesso, i nostri risultati suggeriscono che le condizioni ambientali fossero più omogenee tra i siti e tempi nel secondo anno.

1. Introduction

The Venice Lagoon is a heterogeneous ecosystem nourished by both fresh and saltwater inputs following the flood exchanging (Cucco and Umgiesser, 2006; Deheyn and Shaffer, 2007).

Tidal exchange is one of the main factors that influences the lagoon ecology playing a major role in nutrient supply and water renewal (Cucco and Umgiesser, 2006) with effects on the hydrodynamics and sediment transport (French and Stoddart, 1992; Perillo et al., 2009) and producing land-to-sea gradients of several environmental features and parameters.

In this context, the system of flood barriers named Experimental Electromechanical Module (MoSE) has been unofficially inaugurated in December 2020 to safeguard the city and the lagoon from high tides, to defend from sea storms as well as morphological deterioration processes. However, despite the awareness of the importance of protecting the area, operations for the MoSE system construction and the following barriers activation may represent another stress source for the lagoon in the long, medium and short term, requiring consequently a well-planned ecological risk assessment.

The Venice Lagoon also represents a high productive farming area of shellfish Manila clam (*Ruditapes philippinarum*) and Mediterranean mussels (*Mytilus galloprovincialis*), two species able to adapt to different conditions and to strong variations in environmental parameters that characterize this environment (Boscolo Brusà et al., 2013). However, seasonal variations, adverse climatic conditions, chemical-physical features of different farming sites, and anthropogenic activities (Bertolini et al., 2021; Matozzo et al., 2012, 2013) can influence the immunological and antioxidant defences of farmed animals, with potential negative consequences on the whole shellfish farming aquaculture industry. In recent years, natural and farmed populations of Manila clam experienced dramatic stocks decrease. While further investigations are necessary to clarify the cause of recent widespread mortality events, climate change (e.g., heat waves) and anthropogenic activities (e.g., emerging contaminants; MoSE) seem to play key roles as stressors affecting this species.

In this context, we investigated differences in the immune system and oxidative defences using a battery of biomarkers in clams and mussels collected in several areas placed at gradual distances from the Chioggia inlet in different seasons along two years of monitoring, pre- and post-MoSE functioning. Biomarkers were measured in the haemocytes/haemolymph, gills and digestive gland. Immunological biomarkers included total haemocyte count (THC), haemocyte diameter and volume, cell proliferation, cytotoxicity, lysozyme activity. Antioxidant defence biomarkers included activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Protein carbonyl content (PCC) was estimated as an oxidative damage biomarker. The detoxifying activity of glutathione-S-transferase (GST) was used as a proxy of contaminants presence in the environment. Lastly, measurement of the activity of acetylcholinesterase (AChE) was used as biomarker of neurotoxicity in gills.

2. Materials and methods

2.1 Area of interest and samples

As already described in the Deliverable 2.1.4.1 and in previous RTSs, two monitoring campaigns (May 2018-May 2019 1st year; May 2019-June 2020 2nd year) were carried out on Manila clam (*R. philippinarum*) and Mediterranean mussel (*M. galloprovincialis*), two species of relevant ecological and commercial importance. For both monitoring years, 5000 spats of Manila clam, supplied by Satmar Company (France), and natural spats of Mediterranean mussels were placed in different farming areas at gradual distances from the Chioggia inlet (south of the Lagoon) as reported in Fig.1.

Figure 1. Sampling sites of clams and mussels considered in the first and the second monitoring year.

The 8 SAU investigated site was added in the second monitoring campaign.

Five samples' collections were performed in both the first (2019-2020) and the second year (2020-2021). In detail, clams and mussels were harvested in July 2019/2020, October 2019/2020, February 2020/2021 and May 2020/2021 representing the four seasons along the year. Furthermore, spats of clams were collected before the transplantation in each site in May 2020.

2.2 Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At each sampling time, ten pools of haemolymph (from five animals each) from each experimental condition were prepared. 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 activity assay).

Gills and digestive gland from clams and mussels 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. The small

size of clams (SAU8 May 2021) and mussels (July 2019/2020, October 2019/2020 and CAM6 May 2021) did not allow the sample of haemolymph. Therefore, we decided to use the whole organisms for biomarker measurement.

2.3 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 (CFH). Pooled haemolymph (500 µL) from each experimental condition was centrifuged at 780 x g for 10 min, and the supernatant (=CFH) 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).

2.4 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) activity in gill SN (25 µL), following the colorimetric reaction between acetylthiocholine 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.

Oxidative damage in gills and digestive gland was measured through both protein carbonyl content (PCC) and the DNA strand breaks assays. 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.

2.5 Statistical analysis

Statistical differences in the biomarker responses depending on sampling time and site were investigated by a two-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. Due to the lack of the CAM6 haemolymph samples, results of immunological biomarkers in mussels harvested in the second year were statistically analysed through a Welch's t-test.

The differences between years and sites were visualized with a principal component analysis (PCA). Four PCAs were carried out highlighting differences in the response of immune system and oxidative stress of clams and mussels. To test if 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).

3. Results

3.1 Manila clams (*R. philippinarum*)

3.1.1 Haemocyte/haemolymph parameters

3.1.1.1 First year 2019/2020

Results showed a large variability in THC values between sampling times and sampling sites during the first year of the study (Figure 2 A). Differences in THC were observed in July and October 2019. In particular, VAR3 clams had considerably fewer haemocytes than clams in other sites for the first four months. The values of the SAU4 site showed an increase over time from July 2019 to February 2020, then settling at values like those of VAR1 and VAR2 for February and May 2020.

Cell diameter showed an opposite trend to that of THC during the first year (Figure 2 B). The greatest variability in the values of this parameter was detected in July and October 2019. The clams of the VAR1, VAR2 and SAU4 sites showed similar values, while the VAR3 clams showed higher values than the other sites, except in July 2019.

Likewise, cell volume showed higher values in the VAR3 group during the first sampling year (Figure 2 C).

The cytotoxicity assay (expressed in DO optical density at 490nm) showed few differences among sites in July and October 2019, while differences were highlighted in February and May 2020 (Figure 2 D). In these two periods of the year, the values of VAR1 samples were higher when compared to the other sites and in particular with respect to VAR3 and SAU4.

Cell proliferation values (DO 450nm/THC*10^{-7}) showed an activation of compensatory mechanisms for the low THC values, particularly for sites VAR3 and SAU4 in July 2019 compared to all the other sites and times. Cell proliferation in animals from VAR3 was high also from October 2019 to February 2020 (Figure 2 E).

Lysozyme activity values showed differences between sites at the sampling times in July-October 2019 as well as in July-October 2020 and February 2021 (Figure 2 F). The VAR3 clams showed always the highest values of lysozyme activity compared to the other sites.

Considering all parameters related to the haemocyte parameters of clams sampled between July 2019 and May 2021, the site with the highest stressful conditions was VAR3, where low THC values, high cell diameter and volume, and high proliferation and lytic activity (lysozyme) values were found. Water and sediment analyses will serve to clarify whether the values found in our analyses correlate with high bacterial and/or contaminant concentrations in this site.

	THC	Diameter	Volume	LDH	XTT	Lysozyme
Site	$F_{(3,144)}$=63.264 p<0.001	$F_{(3,144)}$= 10.828 p<0.001	$F_{(3,144)}$= 9.876 p<0.001	$F_{(3,144)}$= 37.083 p<0.001	$F_{(3,144)}$= 45.502 p<0.001	$F_{(3,143)}$= 4.127 p= 0.008
Time	$F_{(3,144)}$=84.835 p<0.001	$F_{(3,144)}$= 2.807 p= 0.042	$F_{(3,144)}$= 2.569 p= 0.057	$F_{(3,144)}$= 79.548 p<0.001	$F_{(3,144)}$= 60.342 p<0.001	$F_{(3,143)}$= 6.932 p<0.001
Site: Time	$F_{(9,144)}$=25.585 p<0.001	$F_{(9,144)}$= 1.693 p= 0.096	$F_{(9,144)}$= 1.514 p= 0.148	$F_{(9,144)}$= 27.742 p<0.001	$F_{(9,144)}$= 24.571 p<0.001	$F_{(9,143)}$= 2.227 p= 0.023

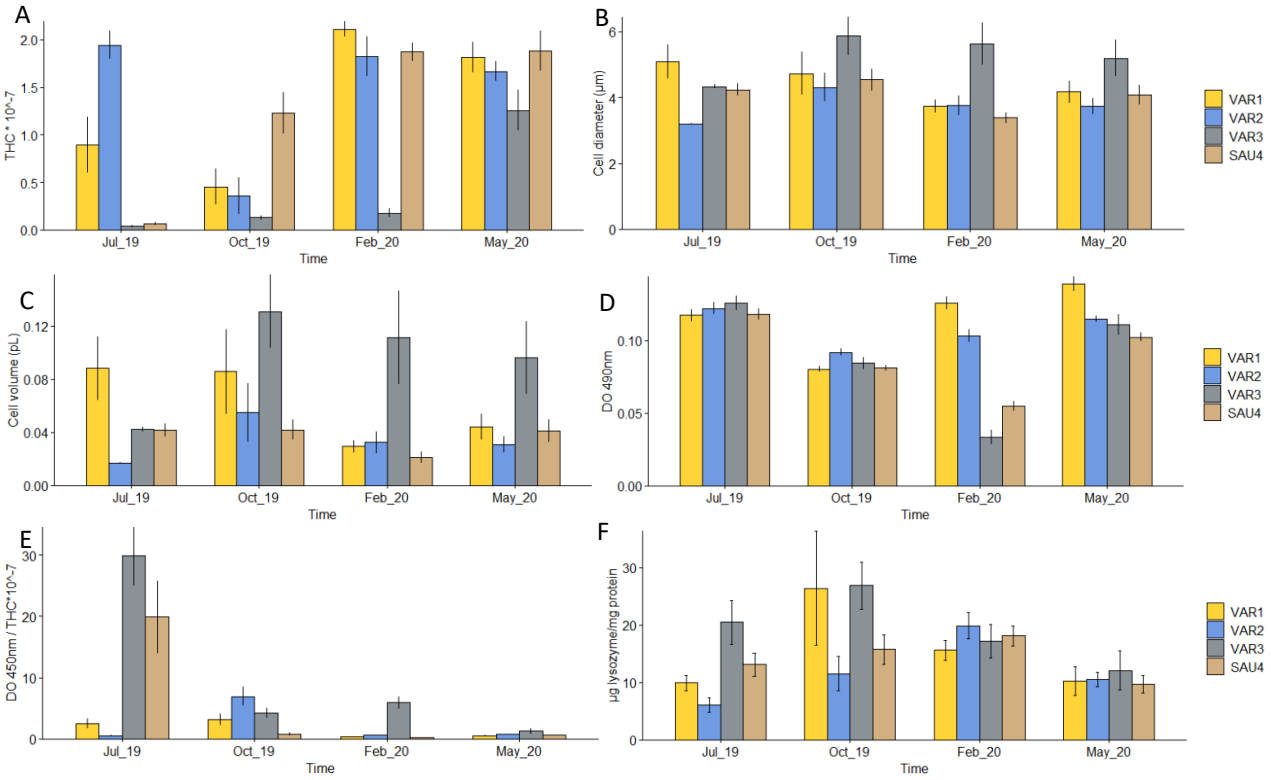

Figure 2. Results of cell biomarkers analysed in haemocytes/haemolymph of clams from the 4 farming areas (VAR1, VAR2, VAR3, SAU4) collected in July 2019, October 2019, February 2020 and May 2020. A: THC (total haemocyte count); B: haemocyte diameter; C: haemocyte volume; D: LDH activity; E: cell proliferation; F: lysozyme activity. Results of the Two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.1.1.2 Second year 2020/2021

Regarding the second sampling year, the statistical analyses highlighted differences among sampling sites, sampling time, and the interaction between site and time in almost all cases (Figure 3). However, a clear pattern of variation is not recognizable in the plots.

An increase of THC values was present in animals sampled in October 2020 (Figure 3 A). The values of cell diameter and volume were similar among sampling sites, with a trend of general decrease from July 2020 to May 2021 (Figure 3 B, C). Higher cytotoxicity (LDH assay) was measured in July 2020 and May 2021 (Figure 3 D), similarly to the first year. Nonetheless, a clear distinction among sites was not observed. The proliferation results (Figure 3 E) followed a similar pattern of that of LDH and THC assay, with higher values in July 2020 and May 2021. Lastly, lysozyme activity showed a decreasing trend over time (Figure 3 F).

	THC	**Diameter**	**Volume**	**LDH**	**XTT**	**Lysozyme**
Site	$F_{(4,171)}$= 0.963 p= 0.429	$F_{(4,171)}$= 3.084 p= 0.018	$F_{(4,171)}$= 3.207 p= 0.014	$F_{(4,171)}$= 7.202 p<0.001	$F_{(4,171)}$= 3.841 p= 0.005	$F_{(4,171)}$= 11.341 p<0.001
Time	$F_{(3,171)}$= 32.454 p<0.001	$F_{(3,171)}$= 15.581 p<0.001	$F_{(3,171)}$= 15.312 p<0.001	$F_{(3,171)}$= 106.357 p<0.001	$F_{(3,171)}$= 90.698 p<0.001	$F_{(3,171)}$= 26.636 p<0.001
Site: Time	$F_{(11,171)}$= 2.046 p= 0.027	$F_{(11,171)}$= 4.426 p<0.001	$F_{(11,171)}$= 4.255 p<0.001	$F_{(11,171)}$= 6.255 p<0.001	$F_{(11,171)}$= 7.753 p<0.001	$F_{(11,171)}$= 3.719 p<0.001

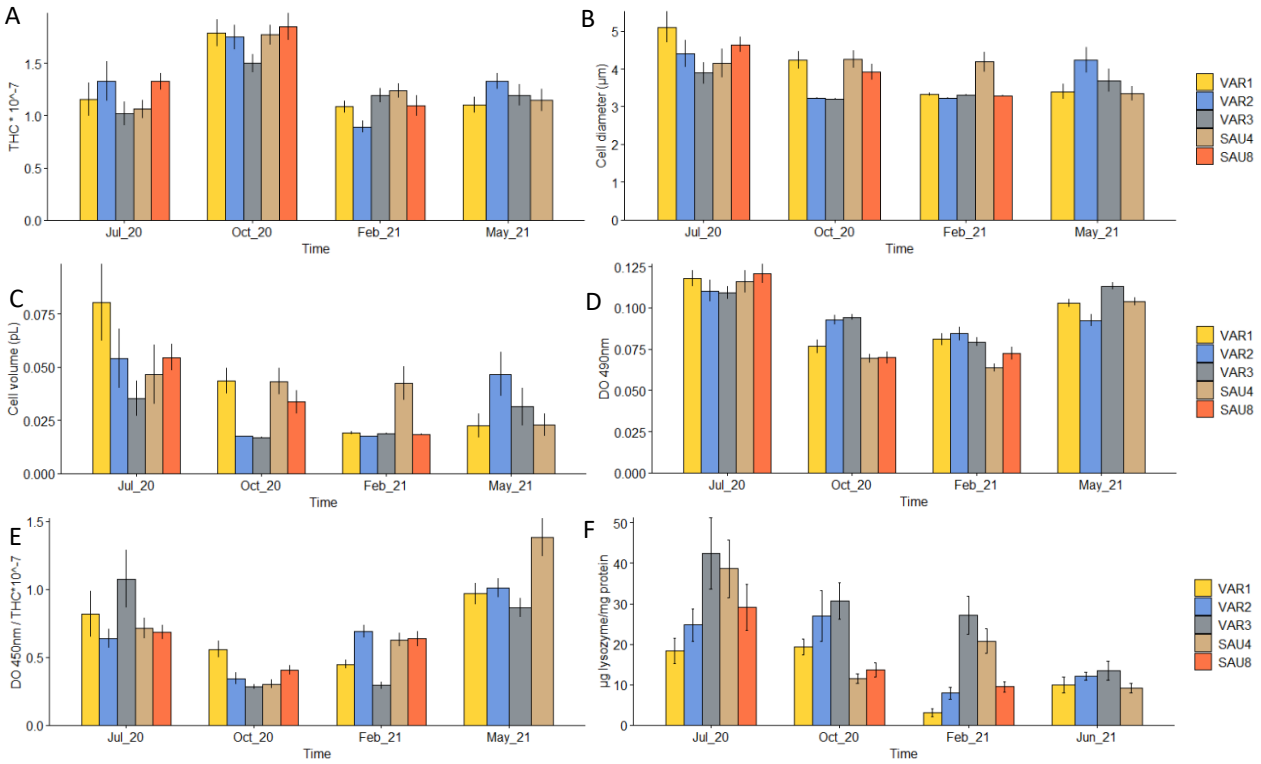

Figure 3 Results of cell biomarkers analysed in haemocytes/haemolymph of clams from the 5 farming areas (VAR1, VAR2, VAR3, SAU4, SAU8) collected in July 2020, October 2020, February 2021 and May 2021. A: THC (total haemocyte count); B: haemocyte diameter; C: haemocyte volume; D: LDH activity; E: cell proliferation; F: lysozyme activity. Results of the Two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.1.2 Gills and digestive gland biomarkers

3.1.2.1 First year 2019/2020

The results showed a variability in the activity of antioxidant enzymes analysed in the gills of bivalves, depending on the season or on the growth of the animals (Figure 4). During the first months of harvest, higher SOD activity was found, when compared to the other antioxidant enzymes (Figure 4 A). The activity of CAT and GR is higher in the following months and in the samples of 1 year after sowing (Figure 4 C, E). The levels of PCC (Figure 4 G) showed how the activity of the antioxidant enzymes CAT and GR seems to have a primary role in protecting tissue against oxidative stress on proteins (carbonyl groups). Indeed, the highest PCC values were found when the antioxidant defences of the animals were lower.

	SOD gills	**SOD gland**	**CAT gills**	**CAT gland**	**GR gills**	**GR gland**	**PCC gills**	**PCC gland**	**AChE gills**	**GST gland**
Site	$F_{(3,144)}$= 5.845 p<0.001	$F_{(3,144)}$= 0.828 p=0.481	$F_{(3,144)}$= 29.024 p<0.001	$F_{(3,144)}$= 20.554 p<0.001	$F_{(3,144)}$= 2.805 p= 0.042	$F_{(3,144)}$= 7.584 p<0.001	$F_{(3,144)}$= 1.880 p=0.136	$F_{(3,144)}$= 0.761 p=0.518	$F_{(3,143)}$= 4.961 p=0.003	$F_{(3,144)}$= 6.929 p<0.001
Time	$F_{(3,144)}$= 219.872 p<0.001	$F_{(3,144)}$= 131.875 p<0.001	$F_{(3,144)}$= 78.475 p<0.001	$F_{(3,144)}$= 227.288 p<0.001	$F_{(3,144)}$= 2247.526 p<0.001	$F_{(3,144)}$= 209.851 p<0.001	$F_{(3,144)}$= 42.881 p<0.001	$F_{(3,144)}$= 41.621 p<0.001	$F_{(3,143)}$= 34.285 p<0.001	$F_{(3,144)}$= 108.728 p<0.001
Site: Time	$F_{(9,144)}$= 10.898 p<0.001	$F_{(9,144)}$= 1.574 p=0.128	$F_{(9,144)}$= 21.863 p<0.001	$F_{(9,144)}$= 12.363 p<0.001	$F_{(9,144)}$= 4.581 p<0.001	$F_{(9,144)}$= 10.955 p<0.001	$F_{(9,144)}$= 4.160 p<0.001	$F_{(9,144)}$= 1.783 p=0.076	$F_{(9,143)}$= 3.327 p=0.001	$F_{(9,144)}$= 2.994 p=0.003

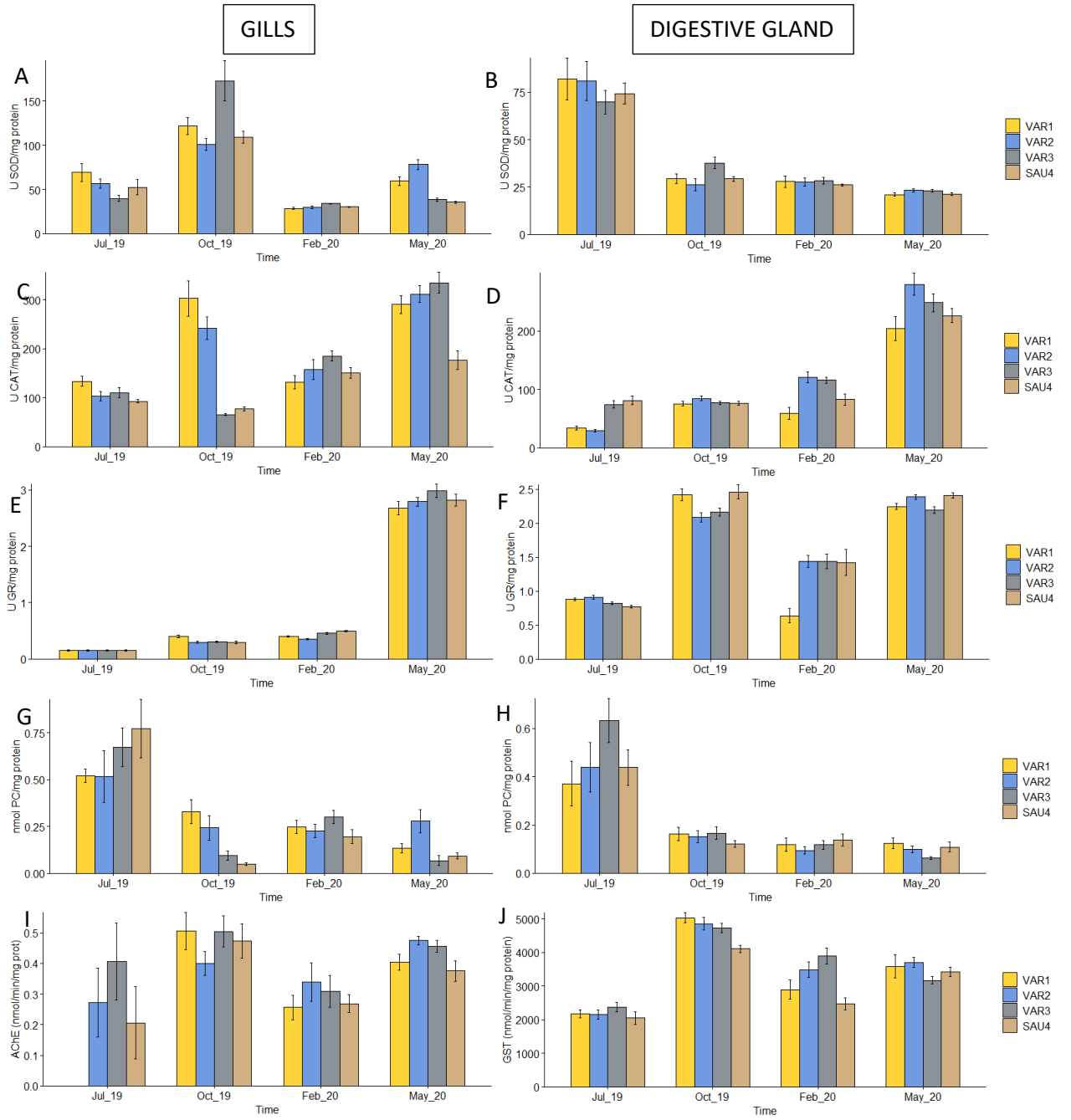

Figure 4. Results of antioxidant defence biomarkers analysed in the gills (A, C, E, G, I) and digestive gland (B, D, F, H, J) of clams from the 4 sampling sites (VAR1, VAR2, VAR3, SAU4) collected in July 2019, October 2019, February 2020 and May 2020. A-B: SOD activity; C-D: CAT activity; E-F: GR activity; G-H: PC content; I: AChE activity; J: GST activity. Results of the Two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

Except for CAT activity measured in animals sampled in October 2019 (higher in VAR1 and VAR2 compared to VAR3 and SAU4), there are no clear patterns of variation between sampling sites.

The inhibition of AChE activity is a good indicator of neurotoxicity. The activity of the enzyme was lower in the months of July 2019 and February 2020 (Figure 4 I). Information on contamination levels in water or sediment samples from the sampling sites could shed light on the observed differences.

About the antioxidant defences of the digestive gland of clams, there is a trend similar to the one seen in gills: a decreasing trend over time for the SOD activity and an increasing trend for CAT and GR activities

(Figure 4 B, C, F). Oxidative damage (PCC) also showed higher values in the first month of sampling than the others (Figure 4 H).

The detoxifying activity of the GST enzyme is linked to the presence of contaminants in the environment. In this case, there is an increase in GST activity between July and October 2019 with a subsequent adjustment to intermediate values in animals sampled in February and May 2020 (Figure 4 J). The trend is like that of the AChE seen in the gill. The presence of contaminants can therefore be suspected in the areas of interest. The results of the chemical analysis of water and sediments will help to better interpret the results obtained.

3.1.2.2 Second year 2020/2021

Except for CAT measured in the digestive gland and the PCC levels measured in both gills and digestive gland from bivalves, all the biomarker responses showed a significant effect of sampling site, sampling time and their interaction (Figure 5). Similarly to enzyme activities, it was difficult to reveal a clear pattern of variation. Among the sites, VAR1 stood out more often than other sites did. Indeed, molluscs from VAR1 presented high values of both SOD and CAT activity in gills in October 2020 and in digestive gland in February 2021. It also had high values of GR in both gills and digestive gland in July 2020.

Low values of AChE in all the sites, but in VAR2, in May 2021 suggested the presence of neurotoxic compounds in the environment. Considering values obtained in animals from the other sampling sites and times, they had similar values.

	SOD gills	**SOD gland**	**CAT gills**	**CAT gland**	**GR gills**	**GR gland**	**PCC gills**	**PCC gland**	**AChE gills**	**GST gland**
Site	$F_{(4,180)}$= 5.639 p<0.001	$F_{(4,180)}$= 7.030 p<0.001	$F_{(4,180)}$= 5.796 p<0.001	$F_{(4,180)}$= 0.828 p=0.509	$F_{(4,180)}$= 2.939 p=0.022	$F_{(4,180)}$= 48.815 p<0.001	$F_{(4,179)}$= 0.705 p=0.589	$F_{(4,180)}$= 2.364 p=0.055	$F_{(4,180)}$= 28.475 p<0.001	$F_{(4,180)}$= 56.393 p<0.001
Time	$F_{(3, 180)}$= 7.283 p<0.001	$F_{(3, 180)}$= 51.355 p<0.001	$F_{(3, 180)}$= 31.060 p<0.001	$F_{(3, 180)}$= 30.653 p<0.001	$F_{(3, 180)}$= 864.775 p<0.001	$F_{(3, 180)}$= 2686.604 p<0.001	$F_{(3, 179)}$= 14.610 p<0.001	$F_{(3, 180)}$= 38.674 p<0.001	$F_{(3, 180)}$= 110.366 p<0.001	$F_{(3, 180)}$= 226.310 p<0.001
Site: Time	$F_{(11, 180)}$= 12.200 p<0.001	$F_{(11, 180)}$= 6.647 p<0.001	$F_{(11, 180)}$= 6.253 p<0.001	$F_{(11, 180)}$= 9.960 p<0.001	$F_{(11, 180)}$= 19.520 p<0.001	$F_{(11, 180)}$= 41.850 p<0.001	$F_{(11, 179)}$= 2.237 p=0.014	$F_{(11, 180)}$= 1.887 p=0.044	$F_{(11, 180)}$= 12.000 p<0.001	$F_{(11, 180)}$= 7.762 p<0.001

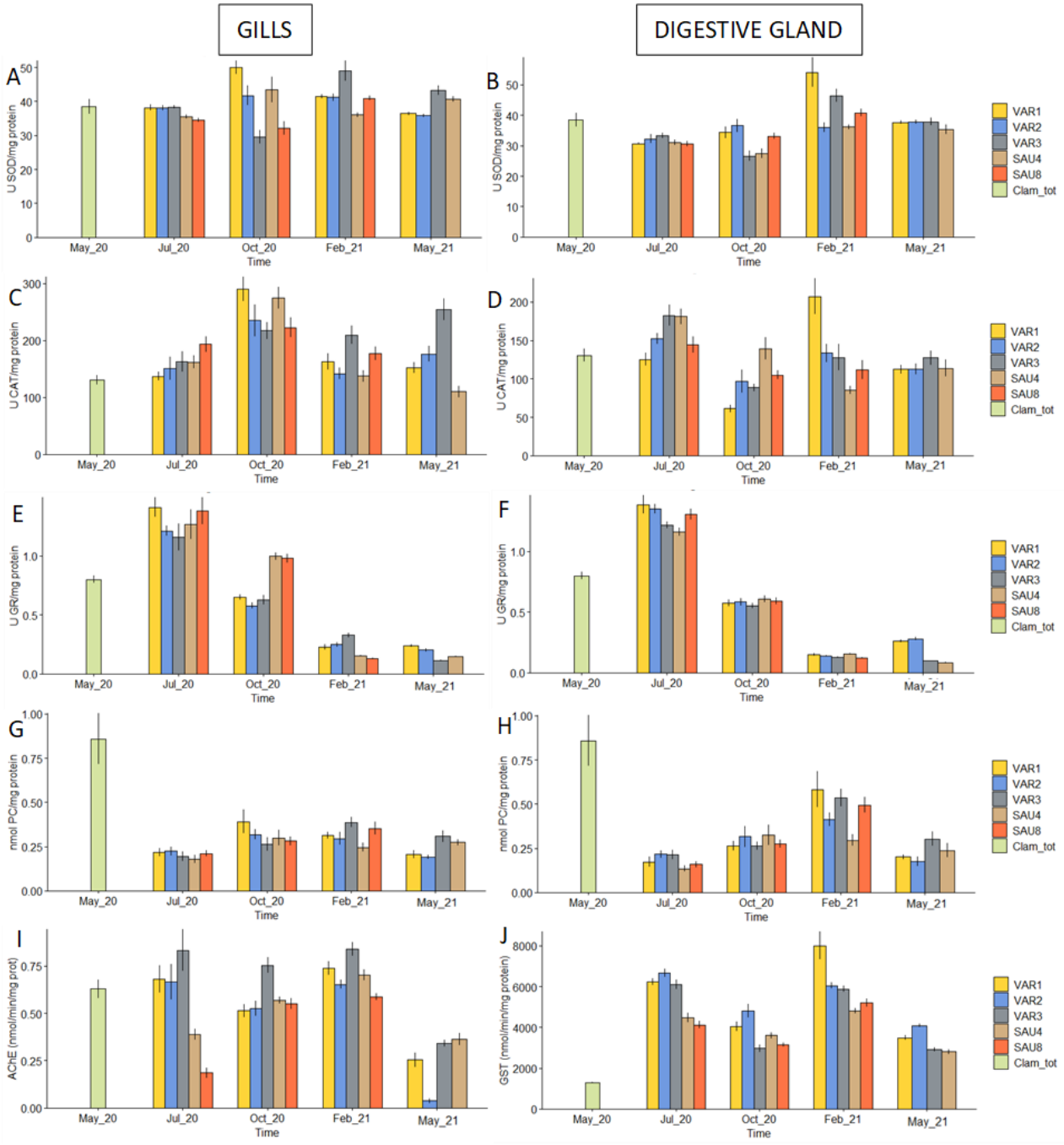

Figure 5. Results of antioxidant defence biomarkers analysed in the gills (A, C, E, G, I), digestive gland (B, D, F, H, J) or whole body (Clam_tot) of clams from the 5 sampling sites (VAR1, VAR2, VAR3, SAU4, SAU8) collected in May 2020 (spat), July 2020, October 2020, February 2021 and May 2021. A-B: SOD activity; C-D: CAT activity; E-F: GR activity; G-H: PC content; I: AChE activity; J: GST activity. Results of the Two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.1.3 Multivariate analyses

3.1.3.1 First year 2019/2020

The PERMANOVA confirmed that significant differences were present between sampling times and sites. The PCA showed that, regardless the sampling time, VAR3 is among the sites with lowest THC, and highest values of cell diameter, volumes and proliferation. Although the clusters are overlapping towards the centre of the plot, the most separated are the ones from the first sampling year, July and October 2019 in particular (Figure

6). This suggested a broader difference in the values of the cellular biomarkers investigated in clams during the first sampling year, when compared to the second one.

	Df	F value	p value
Time	3	118.032	< 0.001
Site	3	7.370	< 0.001
Time:Site	9	3.595	< 0.001
Residuals	144		

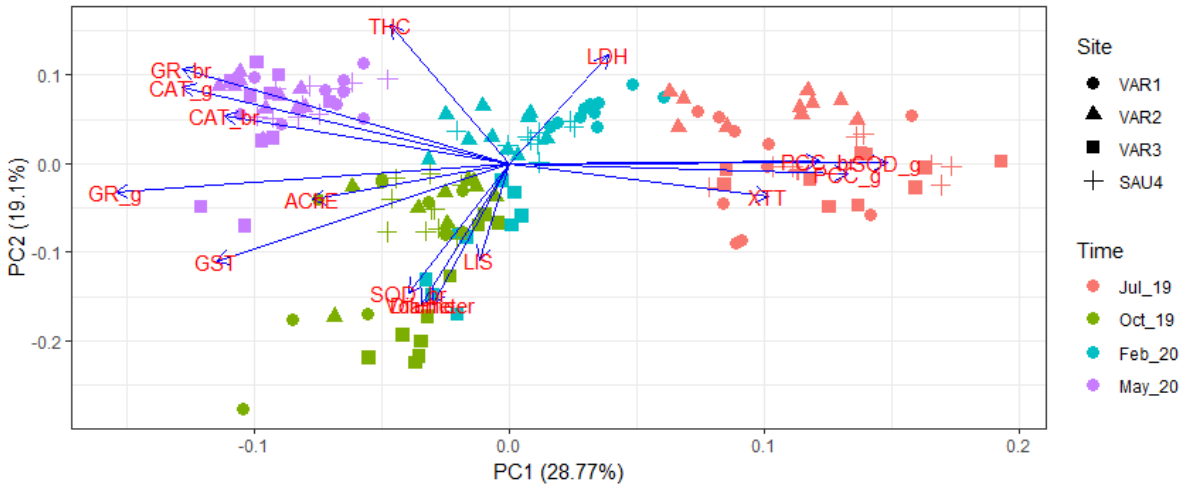

Figure 6. PERMANOVA and PCA analyses performed on the whole dataset of the first year in clams from the 4 investigated farming areas (VAR1, VAR2, VAR3, SAU4) collected in July 2019, October 2019, February 2020 and May 2020. Results of the PERMANOVA are reported in the table above the graph.

3.1.3.2 Second year 2020/2021

	Df	F value	p value
Time	4	167.870	< 0.001
Site	4	46.341	< 0.001
Time:Site	11	8.274	< 0.001
Residuals	180		

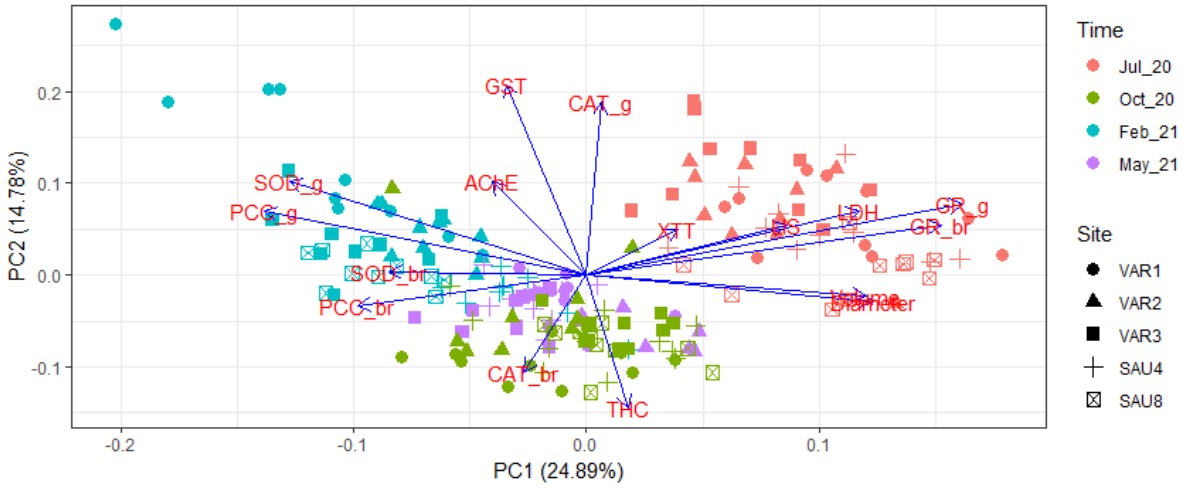

Figure 7. PERMANOVA and PCA analyses performed on the whole dataset of second year in clams from the 5 investigated farming areas (VAR1, VAR2, VAR3, SAU4, SAU8) collected in July 2020, October 2020, February 2021 and May 2021. Results of the PERMANOVA are reported in the table above the graph.

The PERMANOVA carried out on the whole dataset of the second year confirmed that significant differences were present between sampling times and sites depending on to the interaction between the two factors. The PCA showed a separation among all the sampling times (Figure 7). However, the separation among clusters is less clear compared to the first-year case.

3.1.3.3 Both years

PCA analysis carried out with the two-year dataset (Figure 8) showed that sampling times of the first year are more clearly separated compared to the sampling times of the second year. This suggested a broader difference in the values of the antioxidant biomarkers investigated in the clams during the first sampling year, when compared to the second one. The environmental conditions might be more similar among farming areas in the second year, when compared to the first one.

	Df	F value	p value
Time	8	165.470	< 0.001
Site	4	46.640	< 0.001
Time:Site	23	7.737	< 0.001
Residuals	324		

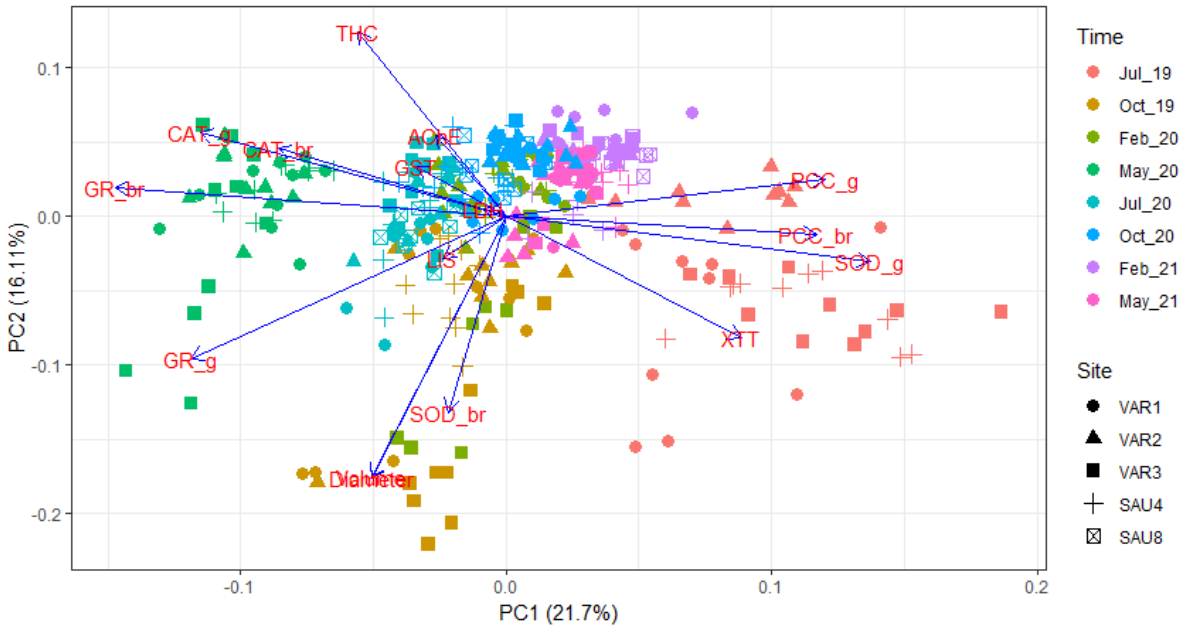

Figure 8. PERMANOVA and PCA performed on the whole dataset of clams from the 5 investigated farming areas (VAR1, VAR2, VAR3, SAU4, SAU8) collected in July 2019/2020, October 2019/2020, February 2020/2021 and May 2020/2021. Results of the PERMANOVA are reported in the table above the graph.

3.2 Mussels (*M. galloprovincialis*)

3.2.1 Haemocyte/haemolymph parameters

3.2.1.1 First year 2019/2020

The THC and LDH results of the first year were affected by the sampling site, while sampling time statistically altered all cell parameters. In addition, the site and time interaction influenced the THC and the proliferation index. Specifically, we observed an increase in haemolymph population in May 2020 respect to February

2020 (Figure 9 A). Similarly, the lysozyme activity was higher in May 2020 (Figure 9 F). On the contrary, the volume, the cytotoxicity parameter (LDH) and cell proliferation (XTT) showed a reduction in May 2020, as reported in Figure 9 C, D and E respectively.

	THC	Diameter	Volume	LDH	XTT	Lysozyme
Site	$F_{(1,36)}$= 8.341 p= 0.007	$F_{(1,36)}$= 0.882 p= 0.354	$F_{(1,36)}$= 0.086 p= 0.771	$F_{(1,36)}$= 4.637 p= 0.038	$F_{(1,36)}$= 2.855 p= 0.100	$F_{(1,35)}$= 1.962 p= 0.170
Time	$F_{(1,36)}$= 539.489 p<0.001	$F_{(1,36)}$= 9.439 p= 0.004	$F_{(1,36)}$= 13.642 p<0.001	$F_{(1,36)}$= 107.948 p<0.001	$F_{(1,36)}$= 463.526 p<0.001	$F_{(1,35)}$= 46.226 p<0.001
Site: Time	$F_{(1,36)}$= 8.685 p= 0.006	$F_{(1,36)}$= 0.938 p= 0.339	$F_{(1,36)}$= 0.631 p= 0.432	$F_{(1,36)}$= 1.157 p= 0.289	$F_{(1,36)}$= 13.757 p<0.001	$F_{(1,35)}$= 0.626 p= 0.434

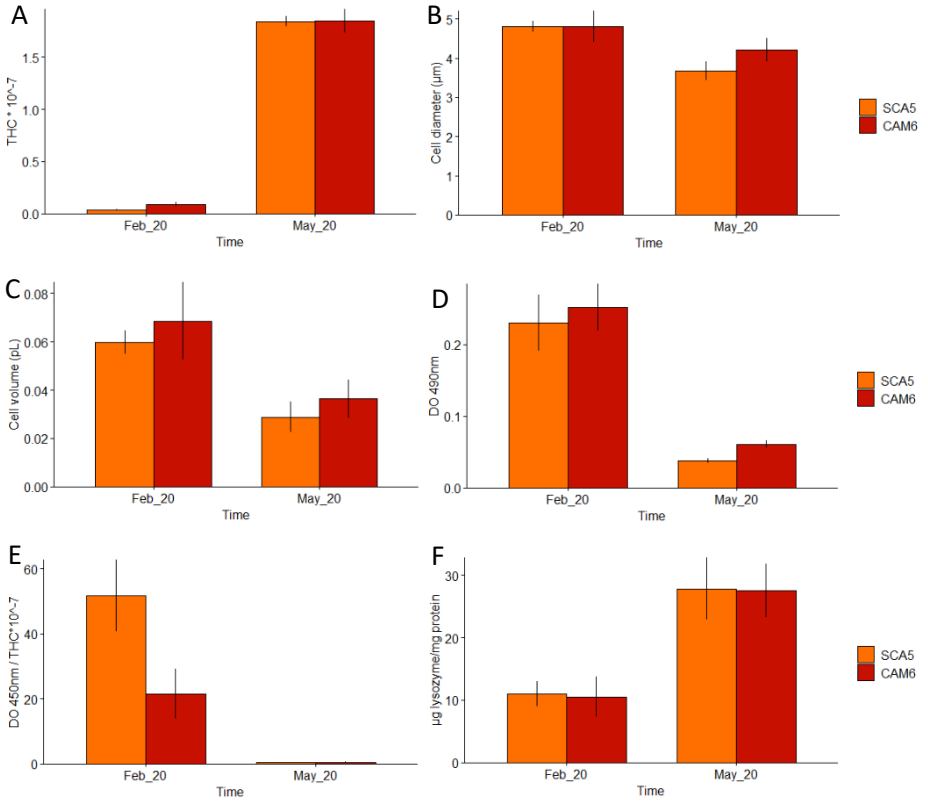

Figure 9. Results of cell biomarkers analysed in haemocytes/haemolymph of mussels from the 2 investigated farming areas (SCA5 and CAM6) collected in February 2020 and May 2020. A: THC; B: haemocyte diameter; C: haemocyte volume; D: LDH; E: XTT; F: lysozyme. Results of the two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.2.1.2 Second year 2020/2021

During the second year, all cell parameters were higher in May 2021 rather than February 2021, except for THC that showed a decrease (Figure 10). Indeed, sampling time statistically affected almost all cell parameters in site SCA5 between the two sampling times, with an increase in time (Figure 10). Comparing the two sampling sites, only cell proliferation was influenced, even if some cell parameters were higher in CAM6 animals, with respect to SCA5 animals, as reported in Figure 10 D, E and F.

	THC	Diameter	Volume	LDH	XTT	Lysozyme
SCA5_feb:SCA5_may	$t_{(10)}$= 3.988 p= 0.002	$t_{(10)}$= 5.408 p<0.001	$t_{(9)}$= 4.649 p= 0.001	$t_{(13)}$= 0.551 p= 0.591	$t_{(18)}$= 14.102 p<0.001	$t_{(17)}$= 1.979 p= 0.064
SCA5_feb:CAM6_feb	$t_{(16)}$= 0.396 p= 0.698	$t_{(18)}$= 0.298 p= 0.769	$t_{(18)}$= 0.414 p= 0.684	$t_{(18)}$= 1.281 p= 0.217	$t_{(12)}$= 2.443 p= 0.030	$t_{(15)}$= 0.585 p= 0.567
SCA5_may:CAM6_feb	$t_{(10)}$= 2.244 p= 0.050	$t_{(11)}$= 5.497 p<0.001	$t_{(10)}$= 4.735 p<0.001	$t_{(14)}$= 1.095 p= 0.293	$t_{(12)}$= 5.639 p<0.001	$t_{(17)}$= 1.040 p= 0.313

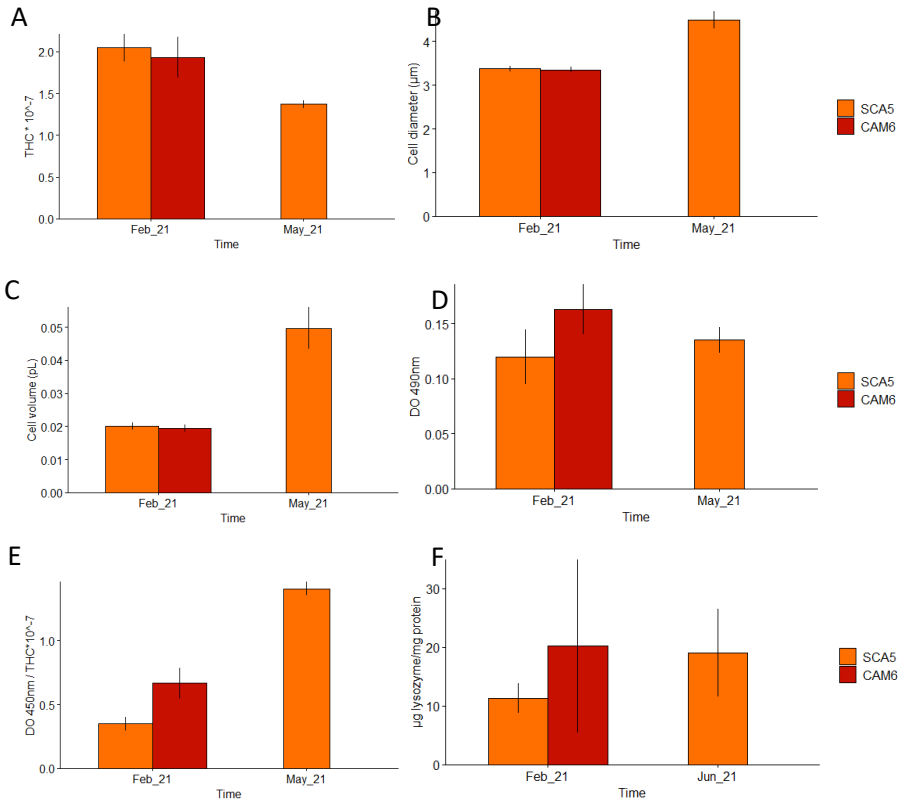

Figure 10. Results of cell biomarkers analysed in haemocytes/haemolymph of mussels from the 2 farming areas (SCA5 and CAM6) collected in February 2021 and May 2021. A: THC; B: haemocyte diameter; C: haemocyte volume; D: LDH; E: XTT; F: lysozyme. Results of the two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.3 Gill and digestive gland biomarkers

3.3.1.1 First year 2019/2020

During the first year, the factor "sampling site" statistically influenced the activity of SOD and AChE in gills, as well as PCP content. In digestive gland, all antioxidant parameters were influenced by such factor, except for GST. Regarding the sampling time, such factor influenced all the measured parameters. In addition, site-time interaction influenced the activity of SOD in gills and CAT in both tissues, AChE and GR activity in digestive gland. We observed that SOD activity in gills was higher in CAM6 animals for each sampling times, with highest activity observed in mussels collected in February 2020 (Figure 11A). Similarly, all the other biological parameters showed a higher activity in CAM6 animals rather than the SCA5 animals (Figure 11). This trend was also observed when comparing values from *in toto* animals between the two sampling sites, except for GST activity (Figure 11 A-I, and Figure 11J, respectively). Interestingly, we observed that the animals collected in February 2020 showed the highest SOD and CAT activity, as well as the highest PCC content in gills (Figure 11, A, C, G). As for the digestive gland, the highest SOD and GST activities and PCC

content were observed in mussels sampled in October 2019 (Figure 11, B, J, H). The CAM6 animals usually showed a higher enzyme activity rather than the SCA5 animals. Interestingly, the enzyme activity can be generally compared between *in toto* animals and the different tissues, beside of the CAT activity in *in toto* animals was very low respect to the CAT activity in specific tissues (gills and digestive gland) (Figure 11, C, D).

	SOD gills	**SOD gland**	**CAT gills**	**CAT gland**	**GR gills**	**GR gland**	**PCC gills**	**PCC gland**	**AChE gills**	**GST gland**
Site	$F_{(2,72)}$= 10.245 p<0.001	$F_{(2,72)}$= 4.118 p=0.020	$F_{(2,72)}$= 1.242 p=0.295	$F_{(2,72)}$= 23.507 p<0.001	$F_{(2,72)}$= 1.337 p=0.269	$F_{(2,72)}$= 6.149 p=0.003	$F_{(2,72)}$= 6.242 P=0.003	$F_{(2,72)}$= 4.805 p=0.011	$F_{(2,72)}$= 18.028 p<0.001	$F_{(2,72)}$= 0.084 p=0.920
Time	$F_{(2,72)}$= 39.723 p<0.001	$F_{(2,72)}$= 92.447 p<0.001	$F_{(2,72)}$= 113.942 p<0.001	$F_{(2,72)}$= 50.309 p<0.001	$F_{(2,72)}$= 21.511 p<0.001	$F_{(2,72)}$= 24.959 p<0.001	$F_{(2,72)}$= 12.397 p<0.001	$F_{(2,72)}$= 60.983 p<0.001	$F_{(2,72)}$= 10.147 p<0.001	$F_{(2,72)}$= 20.889 p<0.001
Site: Time	$F_{(2,72)}$= 9.077 p<0.001	$F_{(2,72)}$= 0.051 p=0.951	$F_{(2,72)}$= 3.915 p=0.024	$F_{(2,72)}$= 9.695 p<0.001	$F_{(2,72)}$= 0.862 p=0.427	$F_{(2,72)}$= 6.203 p=0.003	$F_{(2,72)}$= 2.472 p=0.092	$F_{(2,72)}$= 1.039 p=0.359	$F_{(2,72)}$= 5.525 p=0.006	$F_{(2,72)}$= 0.995 p=0.375

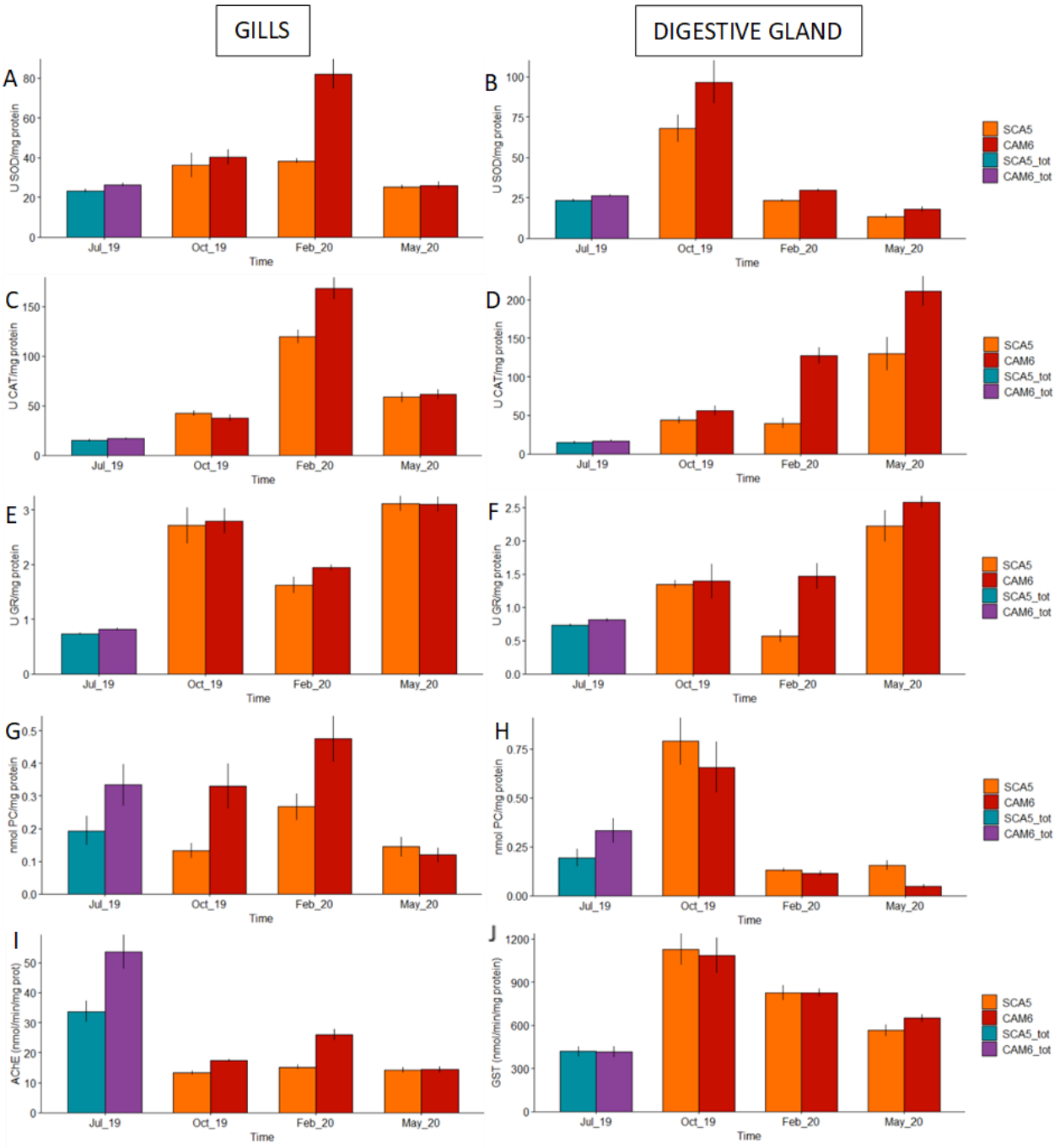

Figure 11. Results of antioxidant defence biomarkers analysed in gills (A, C, E, G, I) and digestive gland (B, D, F, H, J) of mussels from the 2 farming areas (SCA5 and CAM6) collected in July 2019, October 2019, February 2020 and May 2020. A-B: SOD activity; C-D: CAT activity; E-F: GR activity; G-H: PC content; I: AChE activity; J: GST activity. Results of the two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

1.1.1.1 Second year 2020/2021

During the second year, the factor "sampling site" statistically influenced the activity of SOD, CAT, AChE, GST and GR and PCC content in gills. Nonetheless, the factor "sampling time" influenced all the assayed parameters, with also a statistical effect of the site-time interaction on the GST activity and the PCC content in mussel digestive gland. Similarly to the first year the CAM6 animals showed a general higher enzyme activity level, while, comparing the different times, October 2020 showed the highest SOD and AChE activity and PC content in gills (Figure 12 A, G, I). In addition, a strong decrease of GR activity was observed in February

and May 2021 in gills, as reported in Figure 12 E. In the digestive gland we observed a strong increase of the PC content in February 2021, particularly in the CAM6 site indicating a higher oxidative damage amount (Figure 12H). These data were supported by the high CAT activity in that tissue, but not by the other antioxidant enzyme activities that are like the other sampling time.

	SOD gills	SOD gland	CAT gills	CAT gland	GR gills	GR gland	PCC gills	PCC gland	AChE gills	GST gland
Site	$F_{(3,72)}$= 19.249 p<0.001	$F_{(3,72)}$= 8.453 p<0.001	$F_{(3,72)}$= 3.755 p=0.015	$F_{(3,72)}$= 4.784 p=0.004	$F_{(3,72)}$= 17.174 p<0.001	$F_{(3,72)}$= 0.916 p=0.438	$F_{(3,72)}$= 5.627 p=0.002	$F_{(3,72)}$= 1.190 p=0.320	$F_{(3,72)}$= 77.930 p<0.001	$F_{(3,72)}$= 20.763 p<0.001
Time	$F_{(3,72)}$= 20.729 p<0.001	$F_{(3,72)}$= 24.848 p<0.001	$F_{(3,72)}$= 11.772 p<0.001	$F_{(3,72)}$= 41.100 p<0.001	$F_{(3,72)}$= 705.053 p<0.001	$F_{(3,72)}$= 385.441 p<0.001	$F_{(3,72)}$= 3.514 p=0.019	$F_{(3,72)}$= 15.402 p<0.001	$F_{(3,72)}$= 17.370 p<0.001	$F_{(3,72)}$= 18.619 p<0.001
Site: Time	$F_{(1,72)}$= 0.988 p=0.324	$F_{(1,72)}$= 3.371 p=0.070	$F_{(1,72)}$= 0.044 p=0.834	$F_{(1,72)}$= 0.707 p=0.403	$F_{(1,72)}$= 1.701 p=0.196	$F_{(1,72)}$= 0.041 p=0.841	$F_{(1,72)}$= 0.107 p=0.745	$F_{(1,72)}$= 19.233 p<0.001	$F_{(1,72)}$= 3.769 p=0.056	$F_{(1,72)}$= 14.310 p<0.001

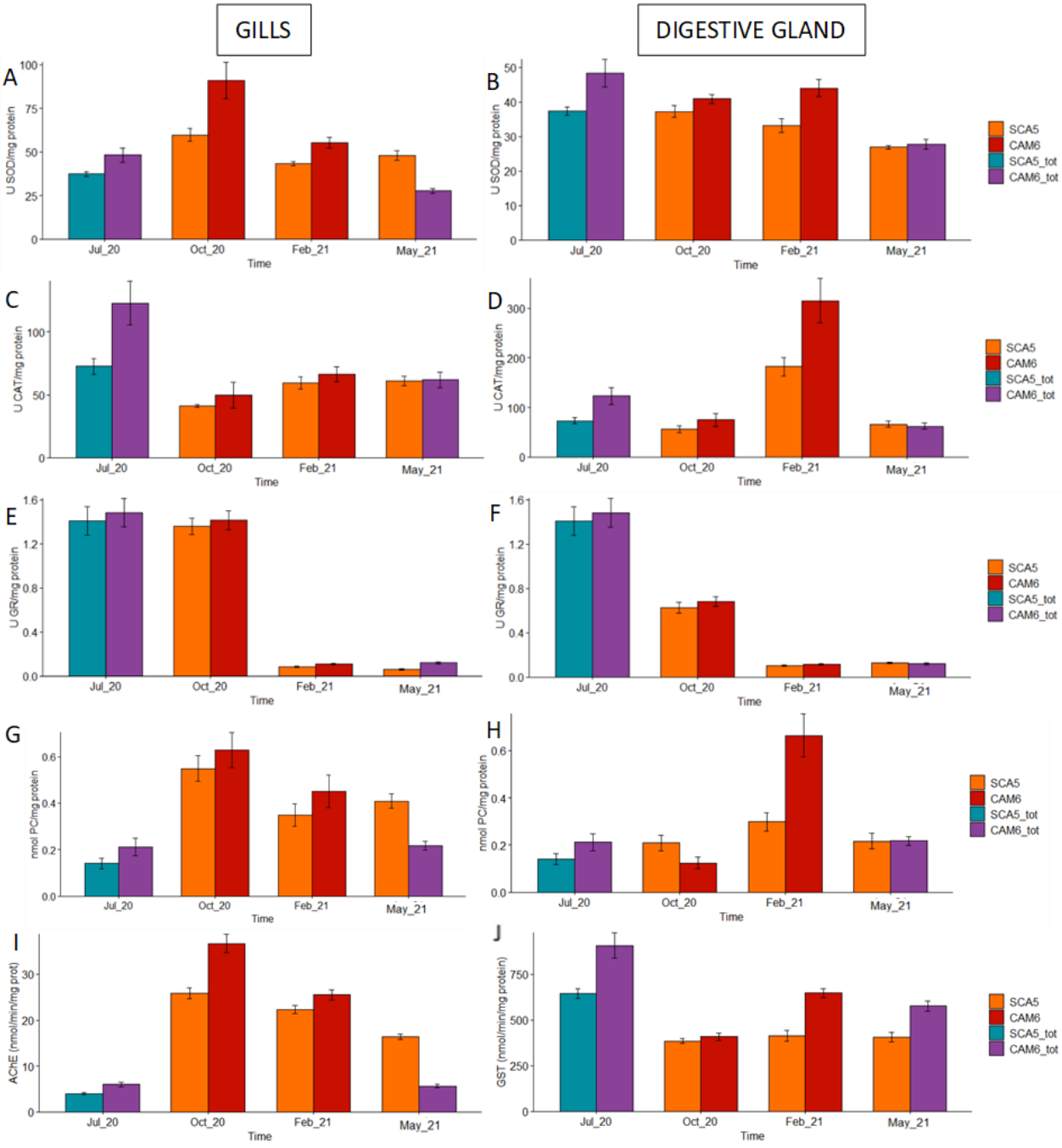

Figure 12. Results of antioxidant defence biomarkers analysed in the gills (A, C, E, G, I), digestive gland (B, D, F, H, J) or whole body (SCA5_tot and CAM6_tot) of mussels from the 2 farming areas (SCA5 and CAM6) collected in July 2020, October 2020, February 2021 and May 2021. A-B: SOD activity; C-D: CAT activity; E-F: GR activity; G-H: PC content; I: AChE activity; J: GST activity. Results of the two-way ANOVA analysis are reported in the table above the graph. Significant results are highlighted in red.

3.3.2 Multivariate analyses

Due to the lack of data on haemocyte/haemolymph parameters of mussels sampled in July and October of both years, the PCAs were carried out on the dataset that considered the sampling times February and May 2020/2021. Nonetheless, the PERMANOVA was performed on the whole dataset, including all the sampling times.

3.3.2.1 *First year 2019/2020*

The PERMANOVA carried out on data of the first year highlighted significant differences related to sampling times, but not to sampling sites (Figure 13).

In the PCA plot the two sampling times are clearly separated along the PC1 (50.33% of variance explained). However, differently compared to the PERMANOVA, in the PCA also the two sites were separated along the PC2 (13.85%). Within each sampling time, the two sites were characterized by different responses of the biomarkers investigated.

	Df	F value	p value
Time	3	42.030	< 0.001
Site	2	0.781	0.466
Time:Site	2	0.857	0.430
Residuals	72		

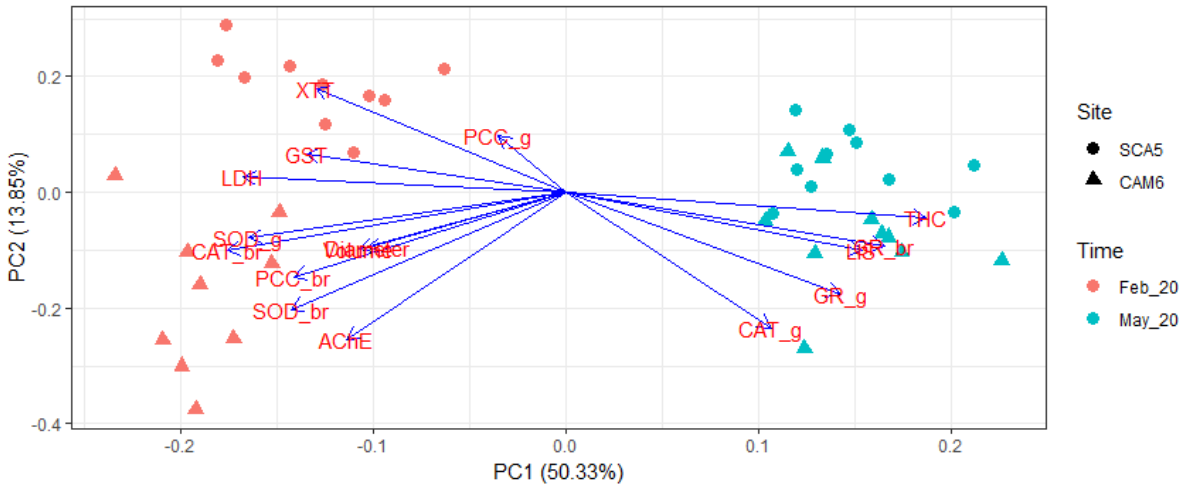

Figure 13. PERMANOVA and PCA performed on the whole dataset of first year in mussels from the 2 farming areas (SCA5, CAM6) collected in February 2020 and May 2020. Results of the PERMANOVA are reported in the table above the graph.

3.3.2.2 *Second year 2020/2021*

The PERMANOVA carried out on data of the second year of research activity highlighted significant differences due to sampling time, sampling site and the interaction between the two factors (Figure 14). In the PCA plot the two sampling times are clearly separated along the PC1 (36.84% of variance explained). Moreover, the two farming areas are separated along the PC2 (14.36%). The biomarkers that characterized the various clusters are different between the two sampling years. In this case, CAM6 clams sampled in February 2021 are the animals with the highest values on most of the biomarkers analysed (LDH, SOD, CAT, GR, PCC, AChE, GST; Figure 14).

	Df	F value	p value
Time	3	43.528	< 0.001
Site	3	15.587	< 0.001
Time:Site	1	9.117	0.001
Residuals	72		

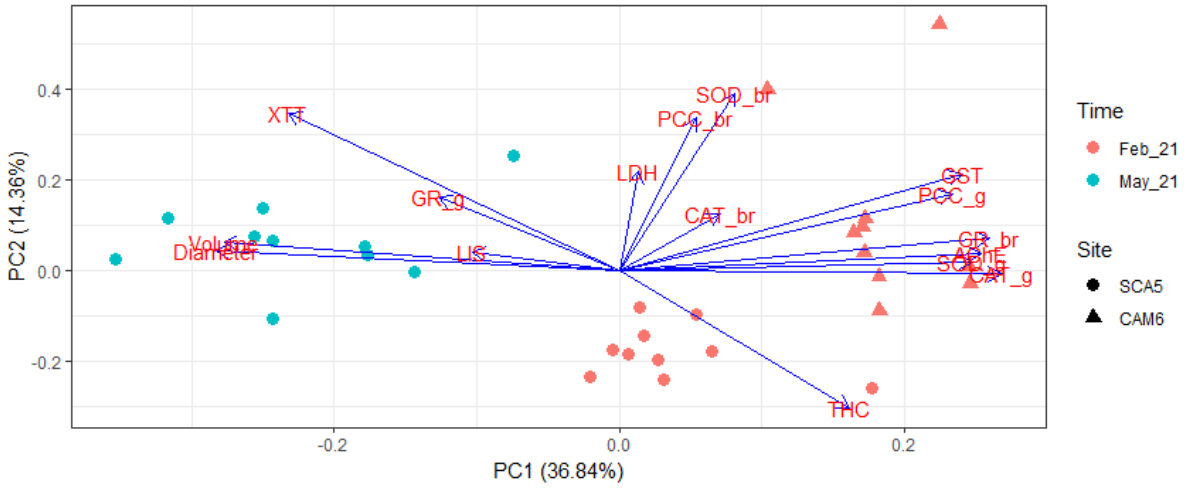

Figure 14. PERMANOVA and PCA performed on the whole dataset of second year in mussels from the 2 farming areas (SCA5, CAM6) collected in February 2021 and May 2021. Results of the PERMANOVA are reported in the table above the graph.

3.3.2.3 Both years

The PERMANOVA carried out on the whole dataset of both years highlighted significant differences due to sampling times, sampling sites and their interaction (Figure 15).

In the PCA plot, four clusters composed by the four sampling times are clearly separated. Along the PC1 (30.79%) and the PC2 (27.43%), the two clusters February 2020 and May 2020 were further apart than the other two clusters February 2021 and May 2021. Within each cluster, also a differentiation between sites was visible.

As for mussels, the results suggested that the environmental conditions were more similar between sites in the second year, after the inauguration of the MoSE.

	Df	F value	p value
Time	7	43.348	< 0.001
Site	3	5.919	< 0.001
Time:Site	5	3.164	0.007
Residuals	72		

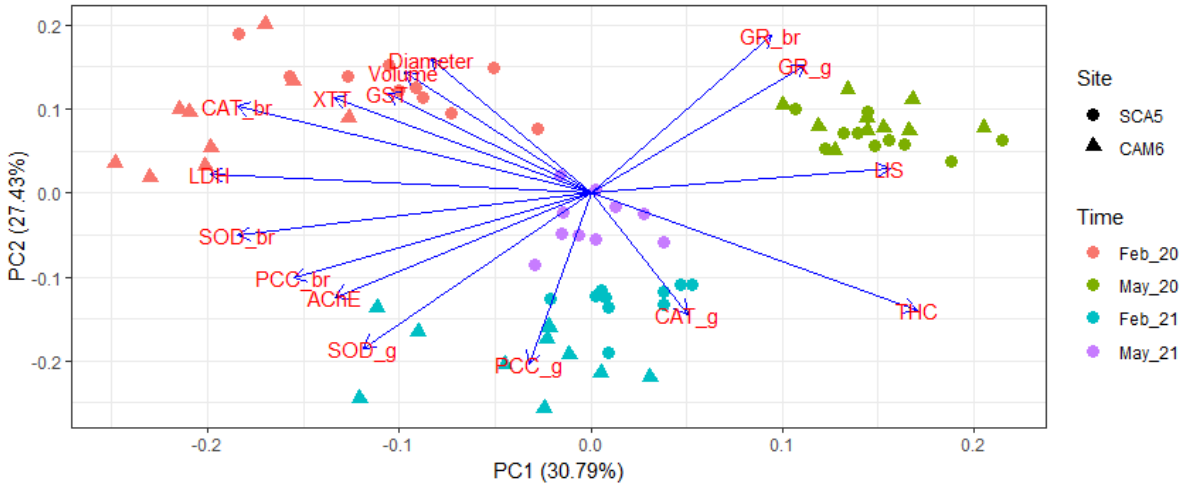

Figure 15. PERMANOVA and PCA performed on the whole dataset of mussels from the 2 farming areas (SCA5, CAM6) collected in February 2020/2021 and May 2020/2021. Results of the PERMANOVA are reported in the table above the graph.

4. Conclusion

In this deliverable, the results of haemocyte, antioxidant, oxidative damage, neurotoxicity and detoxification biomarkers evaluated in Manila clams and Mediterranean mussels farmed in the south Venice lagoon are reported. Four clams and two mussels farming sites were monitored for two consecutive years, reflecting periods pre- and post- the MoSE functioning (December 2020).

As for clams (*R. philippinarum*), results obtained highlighted small differences among sites, when compared to differences due to sampling times. Strong differences were present between the two years. In the PCA plot, the factors “sampling time” and “sampling sites” of the first year clearly separated biomarker responses. On the contrary, the results of the biomarkers analysed during the second year were less separated. Among the sites, VAR3 was the one that more often showed higher values in haemocyte biomarkers, which in turn could be correlated with pathogens’ presence. The results of the chemical analysis of water and sediments will shed light on the reasons for such responses and will help to better interpret the results obtained.

In mussels (*M. galloprovincialis*), the factor “sampling site” slightly influenced haemocyte biomarkers during the two years, while the factor “sampling time” strongly influenced the measured parameters, with a general increase through the first year and a general decrease through the second year. Similarly to clams, the enzymatic responses were different between the two tissues. Moreover, the CAM6 animals generally showed higher enzymatic activities in both tissues and in both years. Interestingly, during the first year, higher levels of antioxidant enzymes were observed in February 2020 in gills and October 2019 in digestive gland. On the contrary, in the second year (excluding the *in toto* animals) there was an opposite result, with generally higher activities in October 2020 in mussel gills and February 2021 in digestive gland.

It appears then crucial to continue with the monitoring program in order to shed light on the possible trends of clams and mussels’ response that could affect aquaculture production following MoSE activity.

References

Bertolini, C., Bernardini, I., Brigolin, D., Matozzo, V., Milan, M., Pastres, R., 2021. A bioenergetic model to address carbon sequestration potential of shellfish farming: example from *Ruditapes philippinarum* in the Venice lagoon. ICES Journal of Marine Science. 78 (6): 2082–2091, https://doi.org/10.1093/icesjms/fsab099

Boscolo Brusà, R., Cacciatore, F., Ponis, E., Molin, M., Delaney, E., 2013. Clam culture in the Venice lagoon: stock assessment of Manila clam (*Venerupis philippinarum*) populations at a nursery site and management proposals to increase clam farming sustainability. Aquatic Living Resources. 26. https://doi.org/10.1051/alr/2013042

Cucco, A., Umgiesser, G., 2006. Modeling the Venice Lagoon residence time. Ecological Modelling. 193, 34–51. https://doi.org/10.1016/j.ecolmodel.2005.07.043

Deheyn, D.D., Shaffer, L.R., 2007. Saving Venice: Engineering and ecology in the Venice lagoon. Technology in Society. 29, 205–213. https://doi.org/10.1016/j.techsoc.2007.01.014

French, J.R., Stoddart, D.R., 1992. Hydrodynamics of salt marsh creek systems: Implications for marsh morphological development and material exchange. Earth Surface Processes and Landforms. 17, 235–252. https://doi.org/10.1002/esp.3290170304

Matozzo, V., Chinellato, A., Munari, M., Finos, L., Bressan, M., Marin, M.G., 2012. First evidence of immunomodulation in bivalves under seawater acidification and increased temperature. PlosOne. 7(3): e33820. https://doi.org/10.1371/journal.pone.0033820

Matozzo, V., Chinellato, A., Munari, M., Bressan, M., Marin, M.G., 2013. Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis*? Marine Pollution Bulletin. 72 (1): 34-40. https://doi.org/10.1016/j.marpolbul.2013.05.004

Perillo, G.M.E., Wolanski, E., Cahoon, D.R., Brinson, M.M., (Eds.), 2009. Coastal Wetlands — An Integrated Ecosystem Approach, Elsevier Science, Amsterdam.