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Multidisciplinary long-term survey of Manila clam grown in farming sites subjected to different environmental conditions

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Abstract

In recent years recurrent bivalve mass mortalities considerably increased around the world, causing the collapse of natural and farmed populations. Venice Lagoon has historically represented one of the major production areas of the Manila clam *Ruditapes philippinarum* in Europe. However, in the last 20 years a 75% decrease in the annual production has been experienced. While climate change and anthropogenic interventions may have played a key role in natural and farmed stocks reductions, to date no studies investigated at multiple levels the environmental stressors affecting farmed Manila clam.

In this work we carried out a long-term monitoring campaign on Manila clam reared in four farming sites located at different distances from the southern Venice Lagoon inlet, integrating (meta)genomic approaches (i.e. RNA-seq; microbiota characterization), biometric measurements and chemical-physical parameters. Our study allowed to characterize the molecular mechanisms adopted by this species to cope with the different environmental conditions characterizing farming sites and to propose hypotheses to explain mortality events observed in recent years. Among the most important findings, the disruption of clam's immune response, the spread of *Vibrio spp.*, and the up-regulation of molecular pathways involved in xenobiotic metabolism suggested major environmental stressors affecting clams farmed in sites placed close to Chioggia's inlet, where highest mortality was also observed.

Overall, our study provides knowledge-based tools for managing Manila clam farming on-growing areas. In addition, the collected data is a snapshot of the time immediately before the commissioning of MoSE, a system of mobile barriers aimed at protecting Venice from high tides, and will represent a baseline for future studies on the effects of MoSE on clams farming and more in general on the ecology of the Venice lagoon.

Keyword: Host-microbiota interactions; Bivalve; Venice lagoon; anthropogenic impacts; Metagenomics; Transcriptomics.

1. Introduction

Bivalve farming is one of the fastest growing food industries, having increased from nearly 1 million tons in 1950 to 16.1 million tons in 2015 (FAO, 2018). However, in recent years recurrent bivalve mass mortalities have considerably increased affecting farming areas all over the world, but also causing collapse of natural populations (Burdon et al. 2014; McFarland et al. 2016; Ortega et al. 2016; Milan et al. 2019; Tan & Ransangan, 2019). Mortality events are often associated with environmental conditions induced by climate change drivers (e.g. ocean warming, ocean acidification, salinity changes, heat waves) (Shi et al. 2016; Su et al. 2019; Soon & Zheng, 2020) and anthropogenic activities (e.g. the presence of toxic substances) (Visciano et al. 2015; Milan et al. 2019) that may also promote the proliferation and spread of opportunistic pathogens (Zannella et al. 2017, Green et al. 2019).

Here, we present the case of the Manila clam (*Ruditapes philippinarum*), a bivalve mollusk with a worldwide distribution that inhabits sandy-mud bottoms. This species was originally present in Asia, but in consideration of its rapid growth rate and resistance to harsh environmental conditions, it has been introduced in other continents. In Europe, it was first imported in 1972 and natural reproduction of introduced individuals favored rapid geographical expansion into the wild, particularly in Italy, France, and Spain. Following its introduction in the Venice Lagoon in 1983, the Manila clam has spread in estuarine areas along the Adriatic coast at a rate of 30 km/year (Breber et al. 2002), confirming its remarkable ability to adapt to a wide range of environmental conditions that makes it an excellent model species for the study of adaptation to environmental changes and for biomonitoring of impacted marine areas (Pranovi et al., 2006; Matozzo et al., 2010; Wang et al., 2010; Boscolo Brusà et al., 2013; Milan et al., 2015;). Currently, the Manila clam represents one of the major cultured bivalve species worldwide and the most important species for commercial clam landings in Europe (STECF 2018). Until a few years ago, the Venice Lagoon represented one of the major production areas in Europe. However, mainly due to the lack of

recruitment from natural seed and because of mortality events affecting several farming sites, this area experienced a dramatic decrease in annual production from 40,000 tons produced in 2000 to 3,000 tons in 2019 (Veneto Agricoltura report).

The Venice Lagoon represents a vulnerable ecosystem subject to significant spatiotemporal variations of biotic and abiotic factors as well as extensive anthropogenic interventions (Deheyn and Shaffer, 2007). Recently, several studies investigated Manila clam populations inhabiting different Venice lagoon areas, identifying the disruption of key molecular pathways (e.g. xenobiotic metabolism, apopotosis, energy metabolism, inflammatory and immune response among others) and the recurrent presence of opportunistic pathogens (i.e. *Arcobacter spp.*) in clams from the polluted site of Porto Marghera (Milan et al. 2013; Milan et al. 2015; Milan et al., 2018; Iannello et al. 2021). All these evidences highlighted that chemical stressors may affect directly and indirectly the health of this sessile and highly sedentary organisms, farmed and naturally inhabiting this ecosystem.

In this study we focussed on monitoring clams reared at four farming sites located in the Southern part of the Venice Lagoon. All experimental animals originated from the same batch of hatchery-produced clams, ensuring genetic, life-history stage, and size homogeneity of clam populations at the beginning of the monitoring campaign, when clams were seeded in the different sites. Whole-transcriptome profiles of the host and 16S rRNA gene amplicon sequencing were applied to characterize host-associated microbiota across different sites and seasons. These data were integrated with several biometric measurements and chemical-physical parameters to understand the molecular mechanisms adopted by this species to cope with the different environmental conditions that characterize the monitored farming sites and to explain the mortality events observed in recent years. Overall, our study provides knowledge-based tools for managing and preserving farmed and natural Manila clam stocks. In addition, the collected data is a snapshot of the time immediately before the commissioning of MoSE (Modulo Sperimentale Elettromagnetico), a system of mobile barriers aimed at protecting the city of Venice from high tides, and will represent the baseline for

future studies on the effects of MoSE on Manila clam farming and more in general on the ecology of the Venice lagoon.

2. Materials and methods

2.1 Experimental sites, sampling plan, and biometric parameters

Twenty thousand individuals of Manila clam (approximately 0.8 cm length) were supplied by the Satmar Company (France) in August 2018 and maintained in flupsies (located close to site 1VAR, Fig. 1) until May 2019 (2.68±0.29 cm) to achieve the minimum growing shell length that minimizes subsequent losses during the fattening phase due predation mostly by fish (e.g. *Sparus aurata*) and crabs (e.g. *Carcinus maenas* and *C. aestuarii*).

Then, they were randomly partitioned in four groups and placed in four farming sites at progressively increasing distance from the Chioggia inlet. Farming sites were named 1VAR, 2VAR, 3VAR, and 4SAU reflecting the names of the two farming cooperatives that managed them and the geographical position (from the outermost to the innermost site; Figure 1, Supplementary File 1). Clams were seeded at a density usually applied by farmers in the four farming sites after the removal of bivalves and empty shells that were naturally present in the field. Unfortunately, due to technical limitations, for site 3VAR not all clam shells could be removed from the sediment. Therefore, mortality could not be calculated for 3VAR (see below). Due to the high predation risk in 1VAR and 2VAR, clams were protected with nets similar to those routinely employed by farmers in these areas. The investigated farming areas are classified as marine delta (1VAR, 2VAR), open lagoon (3VAR) and enclosed lagoon (4SAU) (http://www.atlantedellalaguna.it/). Existing data (Scarton et al. 2019) indicate well-defined gradients for several chemical physical characteristics (e.g. salinity, water residence time, chlorophyll) along the experimental area, starting from the inlet (1 VAR and 2 VAR) to the inner part of the Venice lagoon (3VAR and 4SAU). Considering that site 3VAR showed intermediate environmental features between 1VAR-2VAR and 4SAU, and it

historically represents one of the most productive sites, we considered it as the reference site for microbiota and gene expression analyses (see below). To cover an entire productive cycle, the first sampling of clam's specimens was performed in May 2019 ("T0", immediately before clam seeding) and at four time points throughout a one-year period (July 2019, "T1"; October 2019, "T2"; February 2020, "T3"; and May 2020, "T4"). On each sampling date, clams were randomly collected using a manual rake and then transferred to the laboratory. Biometric parameters and condition index (CI) were recorded at each sampling time and digestive glands and gills were collected for transcriptome analyses (RNA-sequencing) and microbiota characterization (16S rRNA Amplicon Sequencing). Soft tissues of clams for each site and sampling point were pooled for chemical analyses (i.e. persistent organic pollutants and metals). In addition, sediment from each sampling area was collected for metal content detection and grain-size analysis. Biometric parameters including shell length, shell dry weight and soft tissue dry weight were determined in approximately 100 clams for each sampling site/season. Total weight (wet soft tissue + wet shells weight) was determined in all farming sites at T3 and T4. Growth rate (GR), and CI were calculated as follows:

$$GR = \frac{(mean\ length\ time\ (Tn) - mean\ length\ time\ (Tn-1)}{number\ of\ days\ (Tn-(Tn-1))}\ x\ 100$$

$$CI = \frac{soft\ tissue\ dry\ weight}{shell\ dry\ weight}\ x\ 100$$

Significant differences in shell length, CI, and soft tissue dry weight were detected through the post hoc statistical Tukey's HSD Test (FDR<0.05).

Percentage mortality was obtained at 1VAR, 2VAR and 4SAU as

$$Mortality = \frac{\text{Number of empty shells} * \text{ or dead individuals}}{300\ \text{randomly sampled clams}}\ x\ 100$$

** 2 half valves were counted as 1 dead invidual*

Seasonal mortality occurring at 1VAR, 2VAR, and 4SAU sites in time intervals between two sampling points was calculated as increase in the percentage of dead individuals compared to previous sampling time. In case the percentage of dead individual were equal or lower to the percentage obtained in the previous sampling time, due to random sampling, 0% was reported. Total mortality (%) was also calculated for each investigated site as here reported considering the whole productivity cycle:

$$Total\ mortality = \frac{total\ number\ of\ emty\ shells\ or\ dead\ individuals}{\text{total number of randomly sampled individuals}}\ x\ 100$$

Additional details about biometric characterizations are reported in Supplementary File 2.

2.2 Monitoring of environmental parameters and chemical analyses

Two multi-parametric probes located between 1VAR and 2VAR (Probe – north) and close to 4SAU (Probe – south) (see Figure 1) measured water temperature (°C), turbidity, dissolved oxygen (ppm), and saturation (%) in continuous throughout the year. From the recorded environmental data, we derived mean, standard deviation and range (maximum and minimum values) for all environmental parameters in every season (summer: 15th July 2019- 20th September 2019, autumn: 21th September 2019-20th December 2019; winter:21th December 2019-20th March 2020, spring: 21th March 2020-27th May 2020). Because the installation of the probes was in July, data recording started in correspondence of the T1 sampling time. Unfortunately, the salinity and chlorophyll probes at the two sites gave some unreliable readings, thus to explore differences in primary production (food availability) between the two sites, respiration and production (pmax) were calculated from dissolved oxygen data recorded in the water column at the two sites (Ciavatta et al. 2008; Bertolini et al. 2021). Concerning salinity, general characteristics of salinity (‰) in the area, referring to

2001-2003, were obtained from Atlante della laguna (http://cigno.atlantedellalaguna.it/layers/atlante2006%3ASalinitaPrimavMELa120012003R). In addition, to report more recent salinity data, data collected by multi-parametric probes from June 2020 to July 2021 were also reported. Sediments particle-size analysis was also carried out by laser diffraction (Mastersizer 3000, Malvern) in the range 0.1-1000 μm.

Levels of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), Polycyclic Aromatic Hydrocarbons (PAHs) and metals were measured at each sampling time/farming site. In detail, pooled soft tissues from approximately 100 individuals per site were subjected to mass spectrometry (MS) analysis to measure the concentrations of PCDDs/Fs (Method EPA 1613B, 1994), polychlorinated biphenyls and dioxin-like PCBs (PCBs and PCBs-DL; Method EPA 1668C, 2010), and HCB (MPI003, Internal method). IPA (MPI002 + EPA8L270E 2013). Likewise, levels of metals (As, Cd, Co, Cr, Cu, Ni, Pb, Sn, V, Zn, Hg, Al, Fe, Mn) were measured in clam soft tissues and in the sediment from each site/season using microwave-assisted acid digestion followed by ICP-MS analysis. Details about sample treatment and analytical methods are reported in Supplementary File 2.

2.3 Microbiota characterization and bioinformatics analyses

Digestive gland (DG) and gill (GI) samples from clams were individually collected and stored separately at -80°C in RNA later. For each tissue, total RNA was extracted from 5 pools (composed by 5 randomly chosen individuals) for each investigated site/season including T0 with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA extracted from DG was used for both RNA-Seq (see below) and 16S amplicon sequencing, while RNA from GI was used only for the latter. Quality and concentration of extracted RNA were assessed through Agilent 2100 Expert system (RIN value) and Qubit. For microbiome analysis, 1 μg of RNA was reverse transcribed to cDNA using the Superscript IV Kit (Invitrogen, Life Technologies, Monza, Italy). The cDNA was sent to BMR Genomics (Padova, Italy) for library construction using reverse and forward primers (10 μM) that specifically target the V3-V4 gene region of bacterial 16S rRNA, as described by Milan et al.

(2018). Libraries were sequenced with Illumina MiSeq (2x300). Microbiome sequencing generated 11,061,114 million reads, approximately 65,000 reads on average per pool (sequences available in the NCBI Sequence Read Archive https://www.ncbi.nlm.nih.gov/sra BioProject PRJNA744368). Raw reads were uploaded in QIIME 2 (Bolyen *et al.,* 2019) and primer sequences were removed using cutadapt. DADA2 (Callahan *et al.,* 2016) was used to filter low quality sequences and to merge forward and reverse reads (R1 and R2) obtaining high-quality representative sequences. After the quality-filter steps, read merging and removal of chimeric fragments, a total of 7,625,695 reads were retained, yielding 8,362 features. Representative sequence alignment was performed using MAFFT software (Katoh and Standley, 2013) and classified using the Python library Scikit-Learn. Taxa assignment was carried out using the SILVA database (132 update release) trained for used V3-V4 primers. To normalize our analysis, all samples were rarefied to 21'752 reads. The statistical analysis was performed by CALYPSO software (Zakrzewski *et al.*,2017), using the features table and the taxonomy produced in QIIME2. All samples, within each sampling time, were organized by Principal Coordinate Analysis (PCoA; Bray-Curtis distance) at OTU level, separately for gills and digestive gland. In addition, PCoA was also performed within each sampling time to pinpoint differences among farming sites. Shannon's diversity Index was also calculated at species level. ANOVA was carried out to identify different taxa between experimental groups at species and genus level (FDR<0.05). In details: *i)* seasonal changes in microbiota composition within each investigated site were investigated comparing differing sampling times at genus level (i.e. T0vsT1; T1vsT2; T2vsT3; T3vsT4); *ii)* pairwise comparisons between investigated sites were performed within each sampling time both at the species and genus level considering 3VAR as reference site (i.e. 1VARvs3VAR; 2VARvs3VAR; 4SAUvs3VAR); *iii)* in addition, considering that 1VAR and 4SAU showed the most important variations of physicochemical parameters, pairwise comparisons between these sites were also performed.

2.4 Transcriptomic analysis and bioinformatic analysis

Library preparation for gene expression analysis was performed using QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina starting from the same extracted RNA from the digestive gland used for microbiota analyses (5 pools for each investigated site/sampling time). The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a single-end 75 bp setup obtaining a total 562,907,955 reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PJNA744368) with an average yield of 6,622,446.53 reads across all samples (raw reads). Gene expression profiles were explored at three different levels*: i)* Principal Component Analysis (PCA) as unsupervised method performed considering all samples within each season; *ii)* pairwise comparisons within each sampling season between 3VAR (reference site) and the other locations separately, and between 1VAR and 4SAU as for microbiota analyses to identify differentially expressed genes; *iii)* functional analyses of molecular pathways through enrichment analyses.

In detail, the quality of the input reads was assessed with FastQC/v0.11.9 (https://www.bioinformatics.babraham.ac.uk/project s/fastqc/) and low-quality reads and residual adaptors were then removed with the program BBDuk (program specific options were taken from the Lexogen's website at: https://www.lexogen.com/quantseq-data-analysis/) of the suite BBTools (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/). Mapping was carried out using the high-quality reads and a reference transcriptome from the digestive gland (Iannello et al. 2021) by Kallisto/v0.46.1 (Bray et al. 2016) with default settings and finally the "abundance_estimates_to_matrix.pl" script from the Trinity suite (Haas et al. 2013) was used to generate the count table. Raw read counts were then imported into R/v3.6.0 (R Core Team 2014) and filtered: contigs with less than 5 reads in at least 24 libraries (out of 40), which would contribute to background noise (Peruzza et al. 2020, Pradhan et al. 2020), were removed. Filtered reads were then normalized using the RUVs function (with parameter "k = 9") from the RUVSeq/v1.18 library (Gerstner et al. 2016; Verma et al. 2020). Normalized counts were then used to perform pairwise comparisons with edgeR/v3.26.0 (Robinson et al., 2010; FDR < 0.05). Details

and the annotation of each contig is reported in Iannello et al. (2021). Zebrafish Ensembl IDs matching differentially expressed contigs were then used for enrichment analysis on differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003; Huang et al., 2008) and considering Gene Ontology Biological Process (BP), Molecular Function (MF) databases and KEGG pathways (KP) database. To investigate enriched GO and KP between our groups, DAVID analysis was performed considering up-regulated genes of each area separately, with the following settings, gene count 2 and ease 0.05.

3. Results

3.1 Physicochemical data

Farming sites showed similar average temperatures and dissolved oxygen. However, significant higher daily thermal excursions have been detected in northern sites compared to 4SAU from October to February (see supplementary file 1). Despite the high standard deviations of collected data, 4SAU also shows higher turbidity (NTU) and slightly higher respiration and production compared to other sites.

According to public data (e.g. http://cigno.atlantedellalaguna.it/layers/atlante2006%3ASalinitaPrimavMELa120012003R), 1VAR and 2VAR sites have significant higher salinity (32-35‰) than sites located in the innermost part of the southern part of the Venice lagoon. This trend was also confirmed by Zirino and colleagues analyzing data collected between 2000 and 2009 (Zirino et al. 2014), and by values recorded by our multi-parametric probes from June 2020 to May 2021, which indicated several peaks of salinity (>36‰) close to the Chioggia inlet (Supplementary File 1). The innermost part of the southern Venice lagoon shows higher water residence time (16-20 days) than the other sites (from 24h to less than 8 days) (http://cigno.atlantedellalaguna.it/maps/9/view). Sediment granulometry revealed a different sediment grain size distribution between 1VAR and 2VAR: 1VAR and 3VAR have significantly larger amounts of sandy fraction (on average, 78% and 81% respectively); 4SAU and

2VAR have higher silt content (56% and 50%, respectively), compared to 1VAR (19%) and 3VAR (12%), with 4SAU showing also the highest clay amount (13%) (Supplementary File 1).

3.2 Chemical data

Data on bioaccumulation and chemical characterization of sediments are reported in Supplementary file 1 and Supplementary file 3. Several of the investigated organic chemicals were below the detection limit across all sites/seasons. PCDD/Fs, PCBs, and metals (Pb, Hg, Cd) in the soft tissue were far below the limits imposed for the safety of food consumption (European Commission Regulation No 1259/2011; Commission regulation No. 1881/2006). PCBs and PCB-DL bioaccumulation varied between sites depending on sampling seasons suggesting that the levels of these compounds are almost similar in all investigated sites. Similarly, no relevant bioaccumulation of metals in clams was found in all farming areas, although seasonal fluctuations were observed. Overall, metal levels detected in the sediment were in compliance with Environmental Quality Standards (European Directive 2013/39/EU, Italian Legislative Decree 172/2015) except for Cr in some samples, an element reported to be naturally enriched in this area (Donazzolo et al., 1984).

3.3 Biometric parameters and mortality

Biometric parameters and mortality detected at each investigated site are reported in Table 1 and represented in Figure 2. After one year, clams reached the commercial size (shell length ≥4 cm) in all farming areas. However, differences in mortality and other biological parameters were observed between farming sites. At the last sampling time, the best performances for total weight, shell length and soft tissue dry weight of clams were obtained in 1VAR and 4SAU. 1VAR showed the lowest growth rate in summer (T1) and autumn (T2). Conversely, a faster growth of clams in 1VAR compared to the other sites was observed in winter (T3) and spring (T4), reaching also the highest condition index at the last sampling time. Overall, growth rate and condition index showed a seasonal pattern similar across all investigated sites, with higher values reached in the warmer period (T1 and T4) compared to autumn and winter.

Noteworthy, 4SAU showed the highest seasonal mortality in early summer (17% between May and July 2019; T1). In the following periods mortality decreased in 4SAU, reaching 10% in late summer (July-October), while additional mortalities were not observed in the last six months. A different trend has been observed in 1VAR and 2VAR showing peaks of mortality in late summer (July-October) and winter (October-February), respectively. As described in 4SAU, in the last period of the study (February-May 2021) when clams already reached the commercial size (>37 mm) no mortality was observed. Considering the whole monitoring period, the highest mortality was observed in 1VAR and 2VAR (23% and 21% of seeded clams, respectively), while 15% mortality was observed in 4SAU.

3.4 Microbiota characterization

Comparisons between the microbiota of gills and digestive gland showed a different structure in bacterial communities in all investigated sites (p-value <0.001; R >0.98; data not shown). Accordingly, clam microbiota has been characterized separately for digestive gland (DG) and gills (GI) through PCoA and pairwise comparisons (ANOVA). Results obtained by PCoA are summarized in Figure 3, while significant differences in taxa composition obtained for each comparison are reported in Table 2 and Supplementary File 4. For both tissues, PCoA showed separations in clusters reflecting the sampling seasons (Figure 3A-B).

At T1, several changes in the composition of microbial communities following the transfer of clams from flupsies (located close to 1VAR) to farming sites were observed (Figure 3C-D; Table 2A). Among them, increase abundance of the genus *Spirochaeta* was observed in all farming sites after seeding (Supplementary File 4). Both 1VAR and 2VAR showed the down-representation of several taxa compared to T0 (81.5% in common) as well as lower species richness in DG than the other sites (Supplementary File 5). Conversely, the acquisition of several new taxa after seeding has been suggested in 3VAR and 4SAU clams (Table 2A). It should be also noted the over-representation of the genus *Arcobacter* in th DG of clams seeded in 4SAU.

At T2 (late summer-autumn), few significantly differentially represented taxa were observed between farming sites (Table 2B; Supplementary file 4). Despite most seasonal transitions were observed in 3VAR and 4SAU (Table 2A), the genera *Arcobacter* and *Vibrio* increased in DG of 1VAR and 2VAR clams, respectively.

At T3, reflecting the winter season, 2VAR, 3VAR and 4SAU showed similar microbial composition in both tissues (Figure 3G-H; Table2B). Conversely, an over-representation of the genus *Vibrio* (DG and GI) and the species *Vibrio tapetis* (GI) was found in 1VAR when compared to other sites.

From February to May (T4) several changes in clams' microbial communities occurred at all farming sites, in particular at 3VAR and 4SAU (Table 2A). Among them, increased abundance in the genera *Litoricola, Alcanivorax* and *Flavobacteriales* was observed at all farming sites. Overall, 2VAR, 3VAR and 4SAU showed similar seasonal microbiota transitions (>40% of common significant taxa in at least 2 sites) and microbiota composition at T4 (Table 2B). However, spread of the genus *Arcobacter* was observed only in 4SAU. Among differentially represented taxa between sites at T4, an over-representation of the genus *Vibrio* and the species *Vibrio gigantis* was observed in both tissues of 1VAR, while the genera *Arcobacter* and *Spirochaeta* were over-represented in 3VAR and 4SAU. Over-representation of the genus *Vibrio* was found also in 2VAR in both GI and DG (p-value <0.05; FDR <0.2). Seasonal and site-specific trends of the most important taxa here discussed are represented in Supplementary File 6.

3.5 Gene expression analyses

As for microbiota analyses, samples were divided by PCA in different clusters according to sampling season (Figure 4). However, when performed within each sampling time, PCA showed clear discriminations between farming sites. In detail, while a cluster including samples from 1VAR was clearly differentiated from all other farming sites at T1, from T2 to the end of the monitoring period the two outermost sites (i.e. 1VAR and 2 VAR) were separated from 3VAR and 4SAU along the first component of the PCA.

Full lists of differentially expressed genes (DEGs) and enriched pathways obtained by pairwise comparisons are reported in Supplementary File 7, while a summary of the number of the most relevant DEGs is reported in Table 3 and summarized in Supplementary File 8. The highest number of DEGs was found comparing 1VAR to other sites. Conversely, minor transcriptional changes were observed between 3VAR and 4SAU in all sampling times.

Unexpectedly, clams farmed in 1VAR showed at all sampling times several up-regulated genes involved in xenobiotic metabolism when compared to both 3VAR and 4SAU. Among them, several *sulfotransferase family genes* (*ST1B1*; *ST1B2*; *ST1C4*), *cytochrome P450* (*CYP4A2; CYP2D26; CYP2U1; CYP3A24; CYP2J6; CYP2R1*), member of the superfamily of *ATP-binding cassette (ABC) transporters* (*multidrug resistance protein, MDR1* and *MDR5; ABCA8; ABCF1; ABCF3; ABCG8), glutathione S-transferase (GST1; GSTO1; GST1; MGST1; GST5; GST7)* and several genes coding for *Nose resistant to fluoxetine 6 (NRF6)* were found. This result was supported by DAVID analyses revealing the up-regulation of "Metabolism of xenobiotic by cytochrome p450", "Glutathione metabolism" and "Drug metabolism" (Table 3). Similar results were found in 2VAR when compared to 3VAR (see Table 3).

Several genes and pathways involved in the regulation of apoptosis and DNA repair were also found differentially regulated in 1VAR compared to the other sites at all sampling times. Among them, 1VAR showed the up-regulation of *Baculoviral IAP repeat-containing protein 7-A* (*BIRC7*), *Apoptosis regulator BAX* and *programmed cell death 6-interacting protein, IAP-like protein, Proapoptotic caspase adapter protein, Caspase 7 (CASP7)* and *Cell division cycle and apoptosis regulator protein 1* (*CCAR1*). In addition, enrichment analyses showed the up-regulation in 1VAR of "apoptotic process" and "regulation of cell death" when compared to 3VAR at T1, and the down-regulation of "*negative regulation of cell death*" at T2. At the same sampling time, 1VAR showed also the down-regulation of "oxidative phosphorylation" and "Metabolic process". At T3, 1VAR showed the up-regulation of putative *Growth Arrest and DNA Damage Inducible Gamma* (*GADD45G*), whose transcript levels are usually increased following exposures to DNA-damaging

agents. Similarly, *DNA repair protein XRCC4* was up-regulated in 2VAR compared to 3VAR at the same sampling time. At T4, the up-regulation of "cell death in response to oxidative stress" was found in 1VAR when compared to 4SAU.

Differential regulation of key processes related to protein turnover and stress response were also observed in 1VAR. At T1, the over-expression of *Cathepsin L* (*CTSL*), involved in intracellular protein catabolism, and *Peroxisome biogenesis factor 1*, essential for peroxisome biogenesis, were found when compared to 3VAR. At T3, an increase in protein turnover in samples from 1VAR was suggested by the over-expression of several ubiquitin-related proteins and by the up-regulation of "protein processing in endoplasmic reticulum", "protein export", "unfolded binding protein" and "response to stress". Similarly, at T4 the over-expression of *glutathione peroxidase* (*GPX*) and of several heat shock proteins (*HSP*), as well as the up-regulation of "protein folding", "response to stress", "protein processing in ER", "unfolded protein binding", "PPAR signaling pathway" and "regulation of cellular response to oxidative stress" suggested an increased protein turnover and stress responses in 1VAR and 2VAR compared to the other sites.

Transcriptional changes in genes coding for putative neurotransmitters or involved in synapse should be also highlighted in 1VAR and 2VAR. Among others, *acetylcholinesterase* (*ACHE*), *synaptosyn* (SYP), an integral membrane protein of small synaptic vesicle involved in the regulation of synaptic plasticity, and *Neuroligin 4 X-linked* (*NLGN4*) that belongs to a family of neuronal cell surface proteins exhibiting synaptogenic activity, were all up-regulated in 1VAR compared to 4SAU at T1 and T4. Similarly, in clams grown in 2VAR we identified the up-regulation of *ACHE* and *Excitatory amino acid trasporter 2* (*SLC1A2*, also known as glutamate transporter 1 *GLT-1*), which is the principal transporter that clears the excitatory neurotransmitter glutamate from the extracellular space of synapses in the central nervous system.

Among the most relevant differentially regulated processes among farming sites at all sampling time there were immune response and inflammation. Down-regulation in 3VAR of many inflammation and immune-related genes was found at T1 when compared to 1VAR-2VAR. Among

others, should be noted the down-regulation of *Phospholipase A2* (PLA2), *Complement-C1q/tumor necrosis factor-related proteins*, *Complement C1q-like protein 4 (C1QL4)* and of the BP/KEGG terms "cell adhesion", "wound healing", "tissue regeneration" and "phagosome". Similar results were obtained in 4SAU compared to 1VAR, suggesting an overall down-regulation of immune and inflammatory genes in the innermost sites 3VAR and 4SAU in summer (T1). However, an opposite scenario was observed at T2 and T3, with most down-regulated genes involved in immune response and inflammation observed in 1VAR and 2VAR when compared to the innermost sites. At the last sampling time (T4), several DEGs involved in inflammation and immune response were found in all pairwise comparisons, while enrichment analyses suggested similar enriched pathways/BP to those identified in summer (T1) in 1VAR and 2VAR, as the over-regulation of "wound healing", and "tissue regeneration". All results obtained through chemical-physical and biometric characterization, as well microbiota and gene expression analyses are summarized in Figure 5.

4. Discussion

4.1 Best performances for traits of commercial interest are obtained in the southern part of Venice lagoon

While the commercial size has been reached at the end of one-year survey in all farming sites, the best performances for most important commercial traits (i.e. shell size and total weight) were obtained at the two geographical extremes, namely 1VAR and 4SAU (Figure 2). However, seasonal variations on growth performance between farming sites have been also observed. Growth rate was higher in the innermost site 4SAU from May to October, while an opposite trend was observed from February to May, when clams farmed close to Chioggia inlet showed the fastest growth rate. The faster growth observed in southern areas (i.e. 3VAR and 4SAU) in summer could be related to the higher primary productivity of these sites. This is also confirmed by microbiota analyses, that highlighted in 3VAR and 4SAU the over-representation of several taxa widely associated to phytoplankton blooms, such as *Alcanivorax, Litoricola, Flavobacteriales* and *Flavobacterium spp.*

(Kegler et al. 2018; Yang et al. 2016; Zhou et al. 2018 Chernikova et al. 2020). Among the possible factors explaining the slower growth observed in clams placed close to Chioggia's inlet in summer the high salinity that characterized these sites could play an important role (Zirino et al., 2014; Supplementary File S1). Recent studies demonstrated that salinity explained 87% of the variability in growth in *Mytilus edulis* (Wing & Leichter 2011) and that fluctuations and/or high salinities can reduce growth rate and survival also in Manila clams (Hiebenthal et al. 2012; Guzmán-Agüero et al. 2013; Cao et al. 2015; Bae et al. 2021). In particular, when exposed to salinity values that are close to their tolerance limits, bivalves respond immediately by closing the valves and by reducing the filtration (Mcfarland et al. 2013; Wang et al. 2011), eventually reducing energy input (Lavaud et al. 2017; Solan et al. 2016). Considering that the recommended optimum salinity range for Manila clam farming is between 20 and 30 PSU (Coughlan et al. 2009), the higher salinity observed in farming sites placed close to Chioggia's inlet can also explain the down-regulation of "oxidative phosphorylation" and "ATP metabolic process" in 1VAR when compared to southern farming sites. Conversely, while the highest condition index observed in 1VAR at the end of the monitoring period is likely due to the different investment in shell making between the two sites (see Bertolini et al. 2021), a possible factor leading to highest growth rate of clams in 1VAR between February and May could be represented by the higher mean temperature during winter.

While 4SAU showed lower mortality considering the entire surveyed period, different mortality rates between sampling sites in different seasons have been also observed. After seeding, the highest mortality has been observed in 4SAU (>17%). Then, in this site, mortality decreased in late summer, while has not been observed from October to May. Considering that before seeding in farming sites clams were maintained in flupsies located close to Chioggia inlet, the high mortality rate observed in 4SAU in the first period could be explained by the difficult acclimation to southern environmental conditions (see section below). Among the possible factors explaining high mortality at T1, the predominance of fine-grained sediment should be also considered, particularly in the early life stages when organisms are most susceptible to external factors (Joo et al. 2021).

Overall, the obtained results can be summarized in a few points that might be useful for managing Manila clam: *i)* at the end of the productive cycle, the best performances for Manila clam commercial traits (i.e. size, weight) have been obtained at the two geographical extremes (1VAR and 4SAU), confirming the remarkable ability of this species to adapt to a wide range of environmental conditions; *ii)* with high water temperatures, summer is the most critical period for Manila clam, in which significant mortalities were observed in all farming sites; *iii)* transferring animals from flupsy/pre-fattening sites to areas having different environmental conditions (i.e. 4SAU) may lead to mortality peaks in the early stages after seeding. This observation can be certainly extended to other Manila clam farming areas outside the Venice lagoon presenting potentially similar environmental criticalities; *iv)* site located closed to Chioggia's inlet (1VAR) showed higher mortality than farming sites located in the innermost part of Venice lagoon (+8%). This difference can be due to biotic and abiotic stressors affecting these sites, as suggested by transcriptomic and microbiota analyses that will be discussed in depth in the next sections.

4.2 Microbiota characterization highlights different criticalities at farming sites

Our findings confirmed that seasonality strongly influences both microbial composition and transcriptional regulation, confirming the results already discussed in our previous studies (Milan et al. 2013; Milan et al. 2018). This is due to the remarkable seasonal fluctuations of different chemical-physical parameters in the Venice lagoon (e.g. Facca et al. 2009; Quero et al. 2017). Among the most relevant seasonal changes, productivity and temperature (the latter showing excursions higher than 25°C between summer and winter; see Supplementary file 1) have significant effects on bivalve physiology (Zippay and Helmuth, 2012; Shelmerdine et al., 2017). In addition, clam physiology is influenced by the energetically costly reproductive cycle, characterized by a period of sexual resting from October to January, followed by gametogenesis (from February to May) and spawning, which occurrs between the end of May and September (Rodríguez-Moscoso et al., 1996; Meneghetti et al. 2004; Delgado and Pérez-Camacho, 2007).

During our monitoring campaign, the most important seasonal transitions in clam's microbiota have been detected after seeding (May 2019-July2019) and in the last monitored period (February 2020-May 2020), in particular in clams grown at the innermost sites (3VAR and 4 SAU). Particular attention should also be paid to *Arcobacter* and *Vibrio* spp., widely described as infective agents and opportunistic pathogens involved in bivalve mass mortality events (Le Roux et al., 2016; Milan et al., 2018, 2019; Vezzulli et al., 2018; Zampieri et al., 2020; Alfaro et al., 2019; Lasa et al. 2020). Overall, our data clearly indicated an over-representation of *Vibrio spp.* in clams grown close to Chioggia's inlet, while *Arcobacter* spp. were mainly found in clams inhabiting the innermost site 4SAU. Over-representation of *Arcobacter* spp. was particularly evident in gills and digestive gland of clams transferred in 4SAU immediately after seeding, potentially playing a role in the high mortality observed in this site at the early experimental stage (17%). Despite *Arcobacter* has been widely described in several marine organisms as dolphins, seaweeds, crabs, mussels and oysters (Lima *et al.*, 2012, Givens *et al.*, 2013, Hoelzer *et al.*, 201,1 Romero *et al.*, 2002; Collado *et al.*, 2009), its dominance in unhealthy/moribund animals suggests a possible role as opportunistic pathogen able to amplify effects of other environmental stressors (Fan et al., 2013; Tanaka et al., 2004; Lasa et al. 2019; Richard et al. 2021; Lokmer & Wegner, 2015). In clams transferred in 4SAU, we can hypothesize that acclimation to the new environmental conditions, in concomitance with the breeding season, may have represented an important energetic extra effort, which led to an impairment of the immune response and a reduced ability to control core microbiota, possibly facilitating the spread of *Arcobacter*. This is particularly suggested by the down-regulation of *Complement C1q-like proteins, phospholipase A2* and other pathways involved in immune response and inflammation observed at T1, and by the up-regulation of *Myeloid differentiation primary response protein MyD88,* a protein that plays a central role in the Toll-like receptor (TLR) signaling pathway, one of the most important pathways for host immune responses against pathogen invasion (Tang et al. 2020). Seeding clams in the innermost farming site during winter may reduce the mortality here observed at the early stage. Clams' acclimation to the environmental conditions of

the innermost sites may be facilitated in winter by the lower water temperatures, the suspension of the reproductive cycle, and the weak differences in the composition of microbiota between farming sites, thus increasing survival probabilities.

Farming sites located close to Chioggia's inlet were subject to the significant over-representation of *Vibrio* spp., including *Vibrio tapetis*, the causative agent of Brown Ring Disease in *R. philippinarum*, characterized in the initial stages by a brown deposit in the inner edge of the clam's shell (Paillard et al., 1989; Borrego et al., 1996). Subsequently, the penetration into the extrapallial fluids and in tissue lesions may lead to clam's death (Paillard et al. 2004a; Allam et al., 2000, 2002). This pathogen, recently described also in the Venice Lagoon (Zampieri et al. 2020), is inhibited at temperatures higher than 21°C (Paillard et al. 2004b), explaining its spread in winter (T3). Accordingly, a role of *Vibrio* spp. and in particular of *V. tapetis* in clams' mortality observed in 1VAR and 2VAR in late summer and winter cannot be excluded. As for mortality occurring in the early stage at 4SAU, the mortality observed in 1VAR and 2VAR was accompanied by the significant down-regulation of several genes and pathways involved in immune system and inflammation (see Table 3 and Supplementary File 8). The disruption of clam's immune response and the spread of *Vibrio spp.* may be the consequence of environmental stressors affecting clams inhabiting these farming sites, a condition strongly suggested by gene expression profiling (discussed below).

Considering that each lagoon and estuarine areas has specific environmental peculiarities and specific stressors potentially influencing clam's phisiology and the spread of opportunistic pathogens, our findings cannot be generalised to other farming sites outside the Venice lagoon. However, to our opinion the microbiota characterization of Manila clams inhabiting different farming areas at different seasons accompanied by gene expression profiling may certainly provide useful information to define the best period for seeding activities and to predict the spread of opportunistic pathogens at specific sites, potentially playing an important role in mortality evens.

4.3 Gene expression analyses suggest criticalities in farming sites close to the Chioggia inlet

The up-regulation in 1VAR and 2VAR farming sites of several molecular pathways and genes playing key roles in xenobiotic metabolism and stress response represents, to our opinion, the most interesting and unexpected result. Chemical analyses focused on metals and the organic pollutants most impacting the Venice Lagoon did not reveal any significant contamination in these sites. However, gene expression profiles of clams from 1VAR and 2VAR were similar to those detected in clams inhabiting the polluted areas of the lagoon close to the industrial site of Porto Marghera (Milan et al. 2013; Milan et al. 2015; Milan et al. 2016; Iannello et al. 2021). Among them, the up-regulation of several genes belonging to sulfotransferase gene family, cytochrome P450, multidrug resistance proteins and glutathione S-transferases and KEGG pathways involved in xenobiotic metabolism are typical of bivalves exposed to chemical stress either in the wild or under controlled conditions (Iori et al. 2020; Bernardini et al. 2021). Similarly, several contigs coding for putative NRF6 were constantly found up-regulated at both farming sites. NRF6, playing a role in the uptake of a range of molecules including xenobiotic compounds from the intestine to surrounding tissues in the nematode *Caenorhabditis elegans* (Choy et al. 2006; Choy & Thomas, 1999), was found positively correlated with concentrations of several organic compounds in the Manila clam (Milan et al., 2013, 2015) and up-regulated in populations of *Chamelea gallina* affected by mass mortality probably due to chemical exposure (Milan et al. 2019). Additional evidence of potential exposure to chemical stress was provided by the disruption of AChE transcriptional regulation, a classical biomarker for a broad spectrum of organic environmental pollutants (Fu et al. 2018), and of molecular pathways involved in DNA repair, apoptosis and cell death regulation. Among them, particularly relevant appears the disruption of several IAP-like protein and *GADD45G*. IAP (inhibitors of apoptosis) proteins, already described in several invertebrate species including bivalves (Moreira et al. 2012; Morga et al. 2012, Zhang et al 2012), play a key role in regulating apoptosis by interacting with caspases (Deveraux et al. 1999; Kaufman et al. 2000, Zhao et al. 2012; Meng et al. 2013). Disruption of IAP genes, already described in bivalve species exposed to a wide range of stressors including emerging contaminants (Wang et al. 2019; Chi et al. 2019; Iori et al.,

2020; Bernardini et al. 2021), probably represent a generic response to chemical/environmental stress. Similarly, GADD45G was often found differentially expressed in Manila clam and mussels following exposure to different chemical stressors (Volland et al. 2015; de Boissel et al. 2017). GADD45G is a member of a group of genes encoding regulatory molecules that primarily protect cells to ensure survival under stressful conditions by arresting cell cycle, repairing DNA and, ultimately, activating apoptosis (Fornace et al., 1989, Hollander et al., 1999). Overall, disruptions of molecular pathways involved in apoptosis, cell cycle and DNA repair regulation in the outermost sites 1VAR and 2VAR were detected mainly in late summer and winter, which coincide with periods of higher mortality. Additional evidence about the activation of stress response in 1VAR was provided by the up-regulation of several genes involved in protein turnover, response to oxidative stress (e.g. GPX), heat shock proteins, peroxisomes and response to endoplasmic reticulum (ER) stress. Among the most important cellular functions, ER plays a key role in protein folding and assembly of multi-subunit complexes, extracting and degrading proteins that are not correctly folded or assembled into native complexes (Tsai et al. 2010). Simultaneous over-expression of GO/KEGG terms "protein processing in endoplasmic reticulum" and "unfolded protein binding" as well as the over-expression of several ubiquitin-related protein, suggest that environmental stressors occurring at 1VAR may lead to ER stress with possible accumulations of unfolded proteins and activation of "unfolded protein response" to restore ER homeostasis. When ER stress persists, cells could activate pathways leading to cell death (Ron et al. 2007).

It should be also noted that high salinity levels observed at farming sites close to Chioggia's inlet may also represent an important stressor for clams. This could explain part of the transcriptional changes related to stress response highlighted in clams farmed at 1VAR. By means of RNA-seq a recent study demonstrated that the liver of the marine medaka *Oryzias melastigma* plays a crucial role in the acclimation to hypo- and hyper-tonic environment (Liang et al. 2021). In particular, exposures to hypertonic environment led to the up-regulation of "protein processing in endoplasmic reticulum", "aminoacyl-tRNA biosynthesis", "glycine, serine, and threonine metabolism" and "drug

metabolism and cytochrome P450", quite completely overlapping transcriptional changes detected in clams farmed close to Chioggia's inlet. Authors proposed that salinity is able to affect protein synthesis and processing in liver tissue, with the consequent activation of endoplasmic reticulum stress that may play a protective role. Additional evidence about the potential role of salinity in the transcriptional regulation of many molecular pathways in the outmost site was provided by changes in amino acid metabolism. To adapt to osmotic stress, osmoconforming animals such as bivalves are able to adjust cell volume by intracellular free amino acids (FAA), which are produced from protein degradation (Pourmozzafar et al. 2020). In particular, following salinity increase, cellular proteins could be broken down to increase cytosol osmolarity through the accumulation of intracellular FAA preventing cell shrinking (Tlkington et al 2015; Haider et al. 2018). Accordingly, the increase of several aminoacids (including glycine) observed in bivalve species exposed to hyperosmotic conditions (Henry and Magnum 2020) may explain the up-regulation of "amino acids activation" and "glycine, serine and threonine metabolism" in 1VAR farming site. To conclude, observation of transcriptional changes in genes involved in detoxification were already proposed in *Crassostrea gigas* and in the teleost *Sarotherodon melanotheron* exposed to hyper-saline waters (Meng et al. 2013; Tine et al. 2003).

Overall, transcriptomic analyses strongly suggested major environmental stressors affecting clams farmed in sites placed close to Chioggia's inlet. While the up-regulation of several genes involved in the chemical response was not explained by bioaccumulation and sediment chemical analyses, we cannot exclude exposure of clams to other non-investigated emerging contaminants, possibly related to the maintenance of MOSE system (i.e antifouling chemicals). Being close to Chioggia's inlet and considering that with every tidal cycle one-third of the waters in the lagoon is changed through the three inlets, a second possibility could be that clams farmed near the inlet are more exposed, with the outgoing tides, to chemicals coming from the industrial center of Marghera and/or the urban center of Venice and Chioggia. Further investigations about these two hypotheses are needed. As a third possibility, not necessarily alternative to the others, but possibly synergic with

higher salinity, is thermal daily excursions that may also play a role as an environmental stressor explaining part of the transcriptional changes reported in this work. Regardless of the causes, the sub-optimal environmental conditions of these farming sites may facilitate the bloom of *Vibrio spp.*, which are able to spread following stressful environmental conditions eventually causing clam mortalities (Vezzulli et al., 2010). These considerations can be also extended to other lagoons subject to salinity gradient. Among them, the lagoon of Marano representing one of the most important clam's productive areas in North Adriatic Sea, with salinity gradient from 24 to 36 PSU. While other peculiarities of specific sites should be also considered (e.g. the proximity to urban areas; food quality and availability), farming areas located close to the inlets, beeing subject to higher salinity (Sladonja et al. 2011) can lead to stressful conditions for farmed clams.

5. Conclusions

In the present study, a single batch of Manila clam supplied by a hatchery was monitored for one year in four farming sites subject to different environmental conditions within the Venice lagoon. On one hand, the applied multidisciplinary approach allowed us to characterize the investigated farming sites identifying possible criticalities due to environmental stessors affecting clam stocks. On the other hand, molecular analyses provided new knowledge on host-microbiota interactions and on molecular mechanisms adopted by this species to cope with environmental stressors. Most importantly, the integration of chemical-physical data with biometry, mortality and molecular data allowed us to gather useful information for the management of Manila clam. Considering the threat of climate change and the progressively increased use of MOSE system to regulate the water flows in the Venice lagoon, all data collected here are of great value for the long-term monitoring of the lagoon and will help to identify potential impacts on farmed and natural Manila clam populations.

Data Availability Statement

16S Sequence data and RNAseq data sequencing files are available in NCBI Sequence Read Archive (SRA; https ://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA744368).

Conflict of interests

The data of this study are original, and no part of this manuscript has been published or submitted for publication elsewhere. The authors declare no competing financial interests.

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Author Contributions

Massimo Milan, Tomaso Patarnello, Luca Bargelloni designed and coordinated the research. Massimo Milan, Valerio Matozzo, Maurizio Varagnolo and Elena Semenzin coordinated experimental activities. Ilaria Bernardini, Giulia Dalla Rovere, Morgan Smits, Camilla Bertolini, Luciano Boffo, Andrea Sambo, Cristina Breggion and Jacopo Fabrello performed sampling activities and biometrics. Ilaria Bernardini, Giulia Dalla Rovere, Luca Peruzza, Alice Manuzzi performed bioinformatic analyses for gene expression profiling and microbiota characterization.

Loretta Gallocchio, Claudio Carrer, Francesco Sorrentino, Cinzia Bettiol, Lodi Giulia Carolina, Semenzin Elena performed chemical analyses. Roberto Pastres and Camilla Bertolini characterized chemical physical parameters. Massimo Milan, Luca Peruzza, Ilaria Bernardini, Cinzia Bettiol, Giulia Dalla Rovere and Camilla Bertolini wrote the manuscript. Luca Bargelloni, Valerio Matozzo, Cinzia Bettiol and Tomaso Patarnello revised the manuscript.

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Figure

Figure 1. Map of the Venice lagoon indicating the locations of the investigated farming sites *(1VAR, 2VAR, 3VAR, 4SAU and multiparametric probes (Probe –north and Probe –south) used throughout the study. Geographic coordinates of each site are reported in Supplementary file 1*

Figure 2*. Biometric parameters and mortality of Manila clam harvested in the four farming sites. Letters indicate significant differences between investigated site within each sampling time (Tukey's HSD test; FDR <0.05). Mortality for each period (% of dead clams out of 300 randomly sampled individuals or shells) is reported as increased mortality (%) compared to the previous period (see details in methods and supplementary File 2). Total mortality (%) has*

been obtained considering the total number of dead individuals (or shells) on the whole productive cycle, divided by the total number of randomly sampled clams/shells.

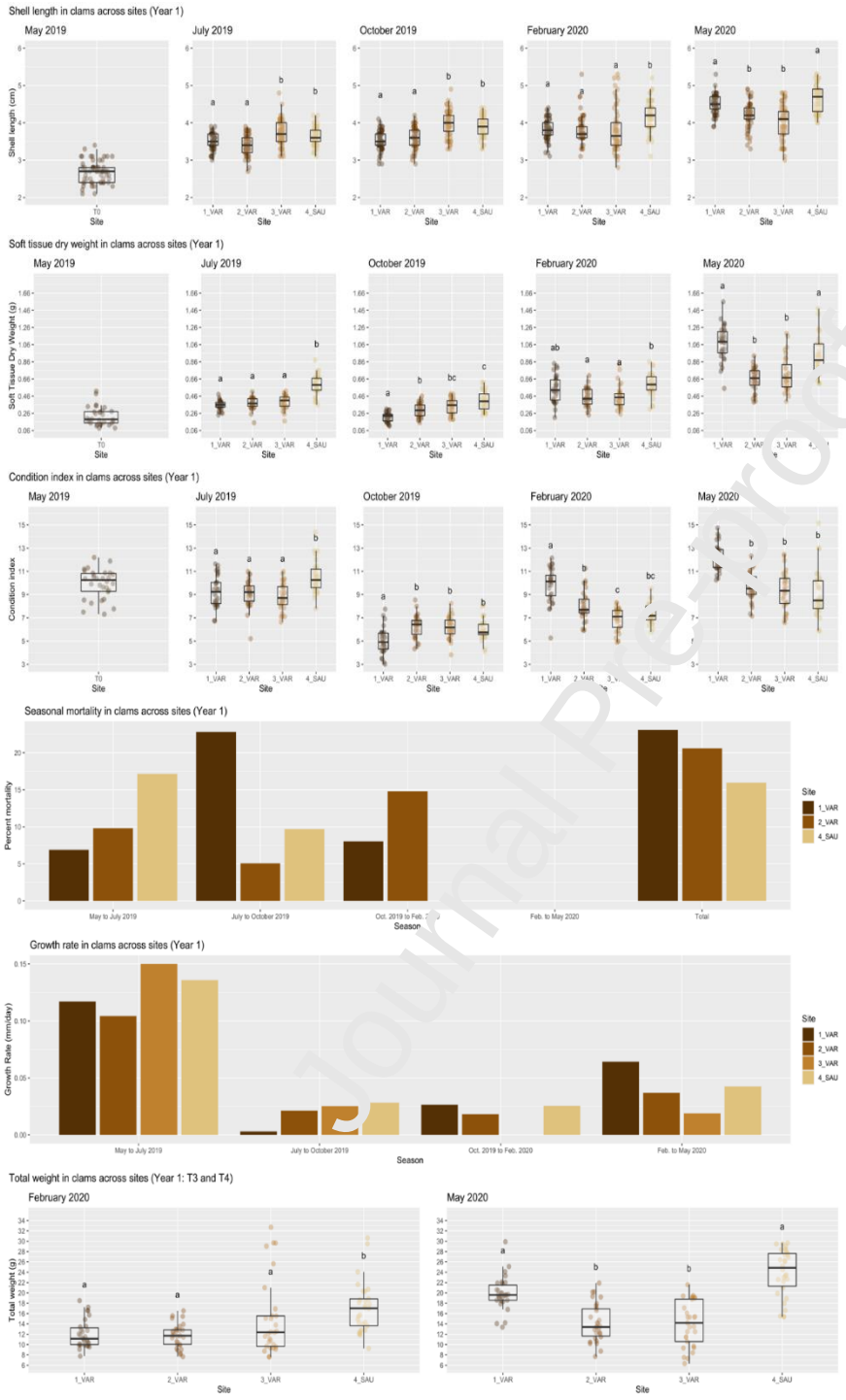


Figure 3. *Principal Coordinates Analysis (PCoA) obtained considering microbiota of digestive gland and gills of clams. A and B) Different colors indicate sampling time. C-L) different colors indicate sampling sites.*

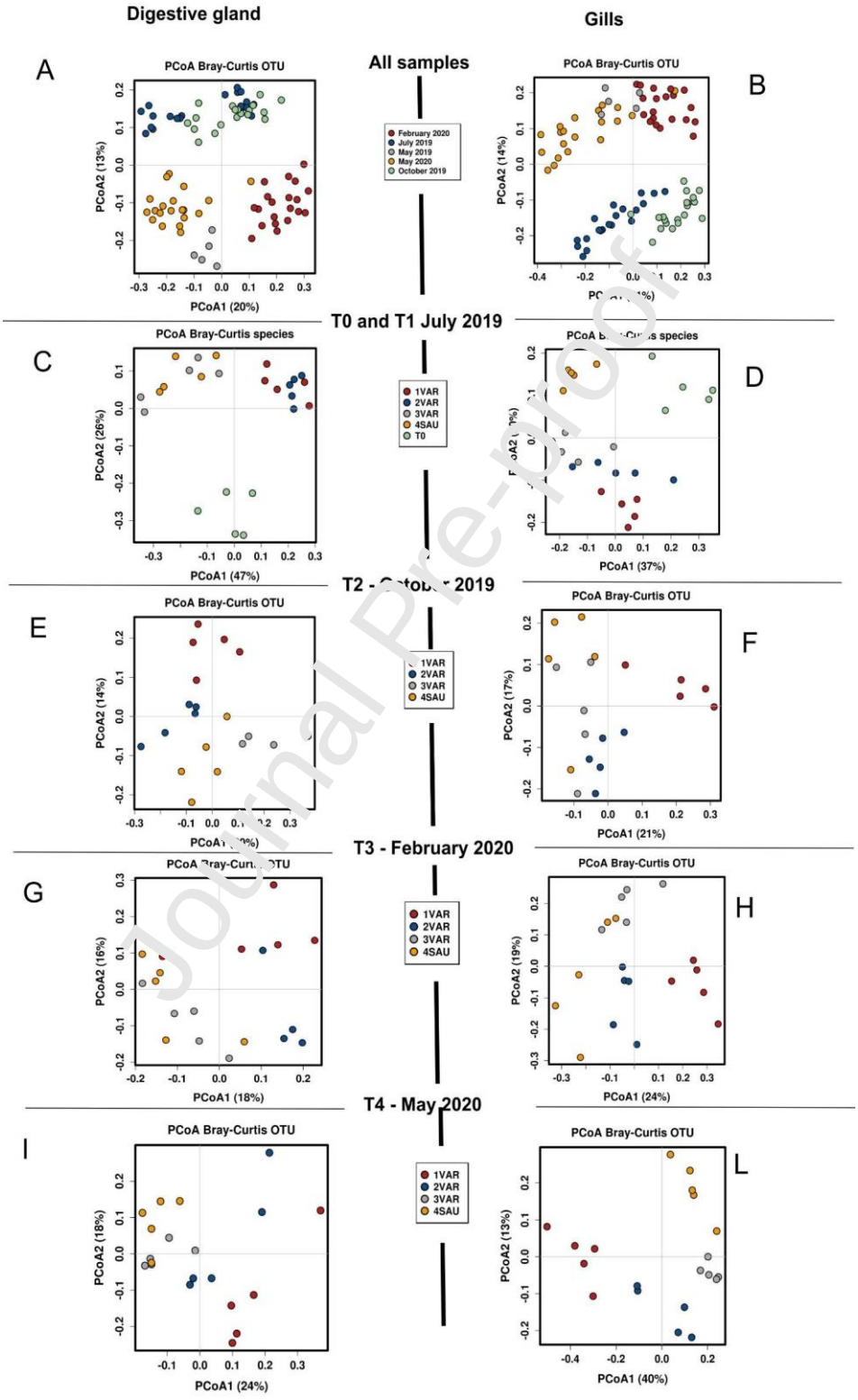


Figure 4. *Principal Component Analysis (PCA) obtained considering RNAseq data of clam's digestive gland. A) all samples were reported: different shapes indicate sampling time/seasons while colors indicate sampling sites. B) PCA performed separately for each sampling time. Colors indicate sampling sites.*

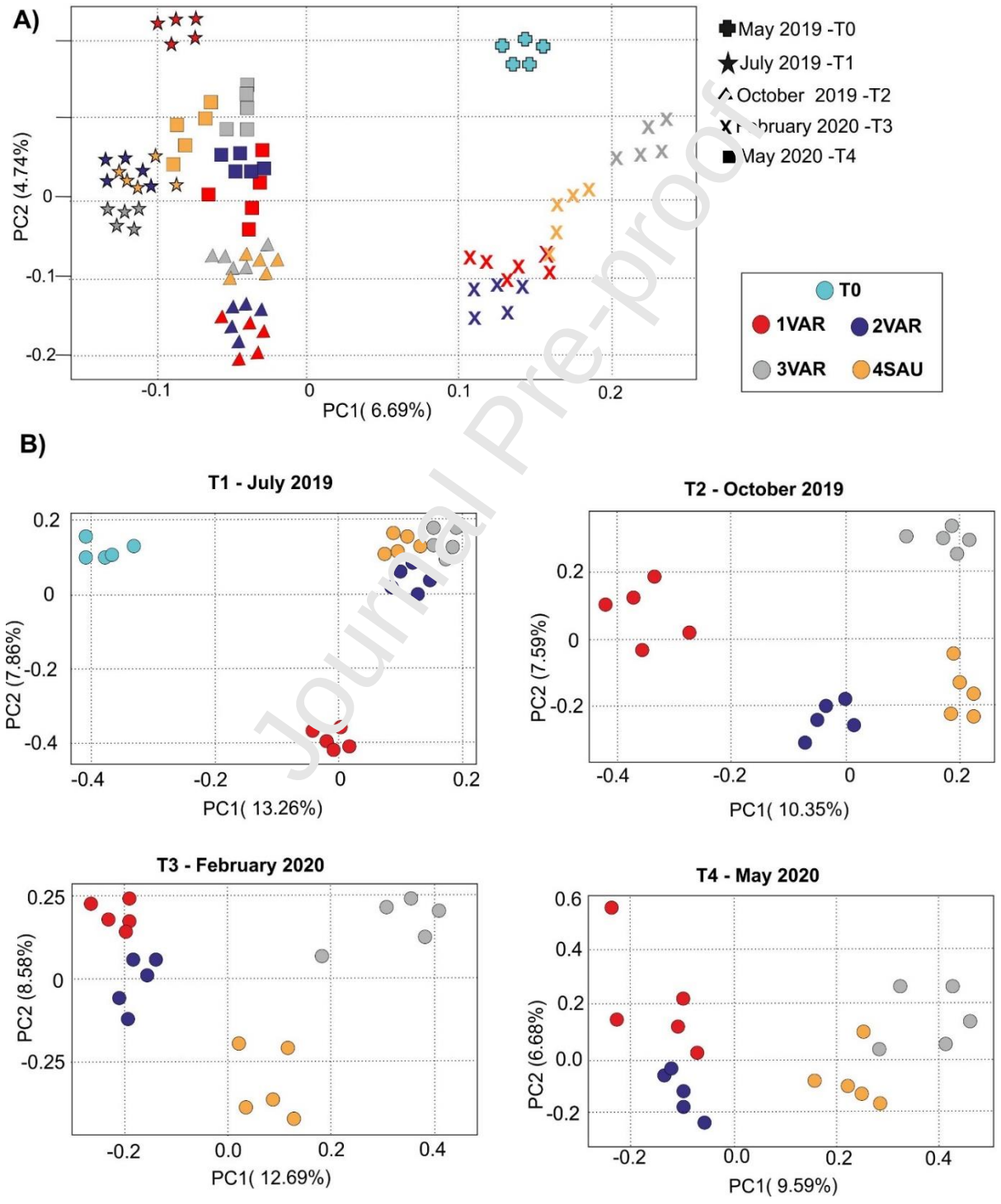
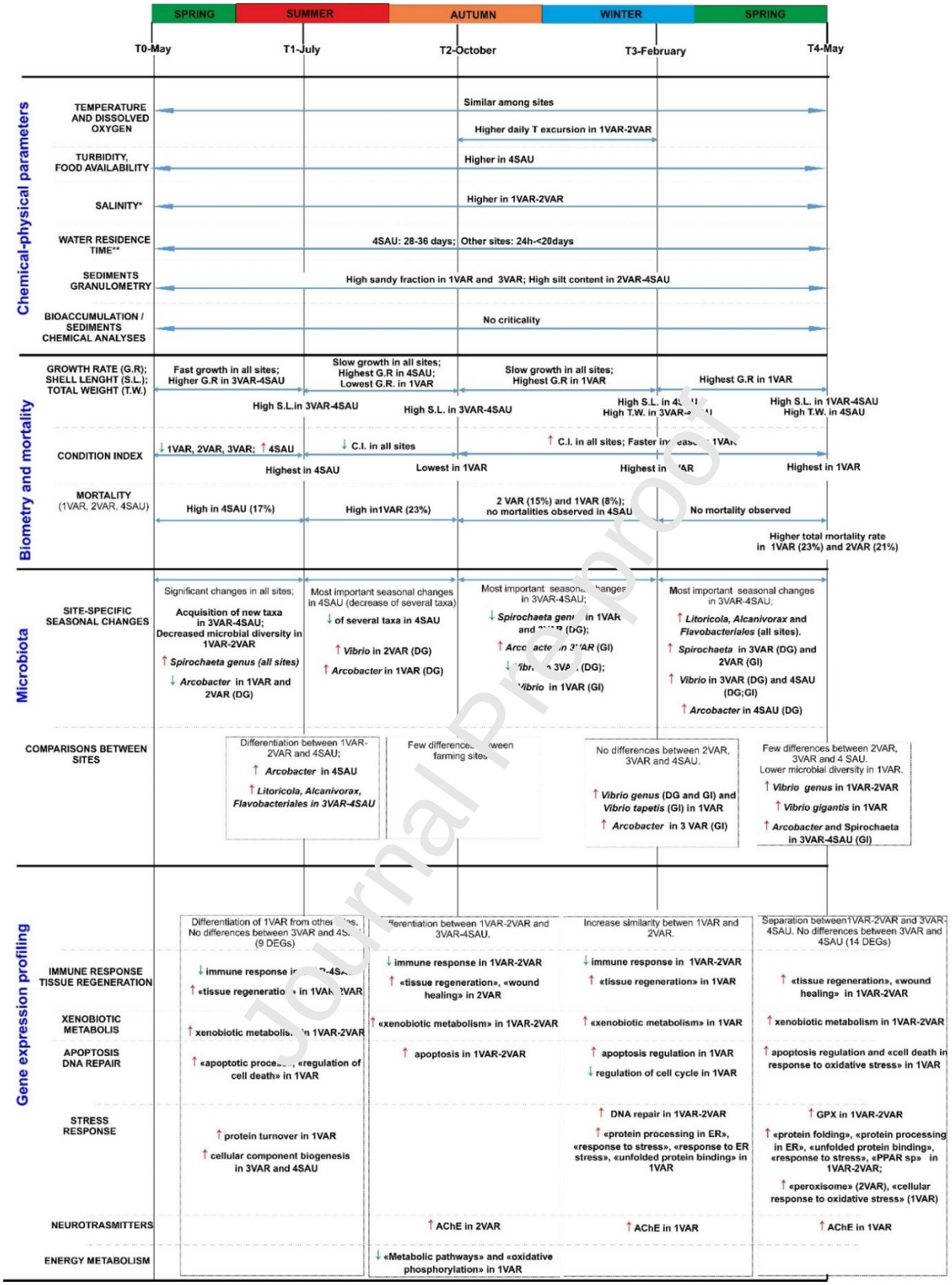


Figure 5. *Summary of the results obtained during the monitoring year. Most important results for biometry, microbiota characterization and gene expression profiling are reported. * Salinity data referred to data collected in 2020-2021 and other studies/archives (Zirino et al. 2014; Atlante della Laguna). ** Residence time is based on data reported in Atlante della laguna.*



Table

Table 1. *Biometric parameters obtained from clams grown in different farming sites.*

Shell Lenght (cm)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	2.68±0.29	3.50±0.21	3.53±0.26	3.82±0.28	**4.50±0.26**
2_VAR		3.41±0.27	3.62±0.29	3.82±0.36	**4.21±0.35**
3_VAR		3.73±0.34	3.98±0.35	3.81±0.58	**4.01±0.46**
4_SAU		3.63±0.25	3.91±0.28	4.19±0.39	**4.64±0.36**
Total weight (g)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	n.d.	n.d.	n.d.	12.08±2.41	**19.81±3.04**
2_VAR				11.46±2.06	**13.88±3.21**
3_VAR				14.24±7.12	**13.46±4.39**
4_SAU				16.18±4.04	**23.34±4.64**
Shell dry weight (g)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	2.22±0.86	3.86±0.61	4.24±0.7	5.49±1.1	**8.83±1.37**
2_VAR		4.12±0.62	4.74±1.02	5.61±1.08	**6.79±1.13**
3_VAR		4.52±1.03	5.63±1.51	6.9±2.9	**7.86±2.27**
4_SAU		5.63±0.98	6.71±1.3	8.06±4.59	**10.48±2.93**
Soft tissue dry weight (g)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	0.23±0.11	0.35±0.05	0.21±0.06	0.54±0.15	**1.08±0.21**
2_VAR		0.37±0.07	0.3±0.08	0.45±0.11	**0.66±0.14**
3_VAR		0.39±0.08	0.34±0.09	0.46±0.13	**0.72±0.2**
4_SAU		0.59±0.13	0.4±0.12	0.59±0.13	**0.92±0.23**
Growth Rate					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	-	0.117	0.003	0.026	**0.064**
2_VAR	-	0.104	0.021	0.018	**0.037**
3_VAR	-	0.15	0.025	0	**0.019**
4_SAU	-	0.136	0.028	0.025	**0.042**
Condition Index					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	9.93±1.26	9.26±1.34	5.03±1.12	9.81±1.48	**12.19±1.17**
2_VAR		9.09±1.17	6.27±0.99	8.01±1.29	**9.67±1.25**
3_VAR		8.83±1.16	6.19±0.94	6.88±1.01	**9.31±1.64**
4_SAU		10.5±1.39	5.98±0.91	7.36±0.84	**9.07±2.07**
Seasonal and total mortality (%)					
	May 2019 - Jul 2019	**Jul 2019 - Oct 2019**	**Oct 2019 - Feb 2020**	**Feb 2020 - May 2021**	**Total (%)**
1_VAR	6.90%	22.82%	8.03%	0.00%	23.07%
2_VAR	9.80%	5.08%	14.79%	0.00%	20.58%
4_SAU	17.14%	9.69%	0.00%	0.00%	15.95%

Table 2. *Number of significant taxa obtained for each pairwise comparisons at genus level (FDR <0.05). Table A reports the number of differentially represented taxa obtained comparing seasonal transitions within each investigated site. Table B reports the number of differentially represented taxa between investigate sites at each sampling time.* ↑ indicate the number of over-represented taxa at the corresponding sampling time (table A) or sampling site (table B).

A) Pairwise comparison between sampling time (seasonal transition in microbiota)

		May vs July (T0vsT1)		July vs October (T1vsT2)		October vs February (T2vsT3)		February vs May (T3vsT4)	
		↑ May	↑ July	↑ July	↑ October	↑ October	↑February	↑ February	↑May
1VAR	DG	**22**	**2**	**2**	**2**	**3**	**1**	**1**	**13**
	GI	13	6	1	1	2	4	3	3
2VAR	DG	**23**	**2**	**0**	**5**	**1**	**0**	**0**	**7**
	GI	7	5	2	0	0	0	2	21
3VAR	DG	**16**	**14**	**3**	**1**	**8**	**1**	**2**	**23**
	GI	10	15	8	0	6	12	3	39
4SAU	DG	**14**	**14**	**15**	**1**	**6**	**2**	**4**	**29**
	GI	6	10	18	2	2	3	2	23

B) Pairwise comparison between sites

		1VAR vs 4SAU		3VAR vs 1VAR		3VAR vs 2VAR		3VAR vs 4SAU	
		↑1 VAR	↑4 SAU	↑3VAR	↑1VAR	↑3VAR	↑2VAR	↑3VAR	↑4SAU
T1	DG	**1**	**22**	**17**	**1**	**18**	**4**	**0**	**0**
	GI	**2**	**19**	**13**	**0**	**1**	**0**	**3**	**4**
T2	DG	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**1**
	GI	**1**	**0**	**0**	**1**	**2**	**0**	**0**	**0**
T3	DG	**1**	**2**	**3**	**2**	**0**	**0**	**0**	**0**
	GI	**4**	**1**	**2**	**3**	**0**	**0**	**1**	**0**
T4	DG	**5**	**10**	**0**	**2**	**0**	**0**	**2**	**1**
	GI	**3**	**21**	**52**	**6**	**1**	**0**	**0**	**0**

Table 3. *Number of differentially expressed genes (DEGs) obtained for each pairwise comparison at each sampling time. Number of DEGs detected for most relevant biological processes/molecular pathways are also indicated. In addition enriched terms are also reported including the number of DEGs belonging to each pathway/BP. Full lists of DEGs/enriched pathways are reported in Supplementary File 7 and summarized in Supplementary File 8.*

Comparison	T1-July 2019	T2-October 2019	T3-February 2020	T4-May 2020
3VAR vs 1VAR	**N° DEGs: 230; ↑1VAR: 110; ↑3VAR: 120** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 4; ↑3VAR: 0)** GO/KEGG: response to chemical (6) **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 8; ↑3VAR: 0)** GO/KEGG: cell adhesion (7); wound healing (4); tissue regeneration (3); Phagosome (4); **Apoptosis/Cell death/Cell cycle (N°DEGs: ↑1VAR: 2; ↑3VAR: 0)** GO/KEGG: apoptotic process (4), regulation of cell death (4) **Protein turnover/stress response (N°DEGs: ↑1VAR: 2; ↑3VAR: 2)**	**N° DEGs: 475; ↑1VAR: 180; ↑3VAR: 295** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 20; ↑3VAR: 0)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 1; ↑3VAR: 19)** **Apoptosis/Cell death/Cell cycle (N°DEGs: ↑1VAR: 4; ↑3VAR: 2)** GO/KEGG: negative regulation of cell death (4); **Protein turnover/stress response (N°DEGs: ↑1VAR: 10; ↑3VAR: 3)** **Energy metabolism/Metabolism** GO/KEGG: Metabolic pathways (18); Oxidative phosphorylation (6); Degradation of aromatic compounds (2)	**N° DEGs: 472; ↑1VAR: 235; ↑3VAR: 240** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 5; ↑3VAR: 1)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 5; ↑3VAR: 7)** GO/KEGG: immune system process (8) **Apoptosis/Cell death/Cell cycle/DNA repair (N°DEGs: ↑1VAR: 6; ↑3VAR: 1)** GO/KEGG: regulation of cell cycle (5) **Protein turnover/stress response/ cellular macromolecule biosynthetic process (N°DEGs: ↑1VAR: 10; ↑3VAR: 37)** GO/KEGG: Protein processing in endoplasmic reticulum (8); Protein export (3); **Neurotransmitter/synapse (N°DEGs: ↑1VAR: 1; ↑3VAR: 0)**	**N° DEGs: 372; ↑1VAR: 231; ↑3VAR: 141** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 10; ↑3VAR: 0)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 2; ↑3VAR: 7)** GO/KEGG: tissue regeneration (6); wound healing (6); **Apoptosis/Cell death/Cell cycle/DNA repair (N°DEGs: ↑1VAR: 2; ↑3VAR: 0)** **Protein turnover/stress response/ cellular macromolecule biosynthetic process (N°DEGs: ↑1VAR: 11; ↑3VAR: 0)** GO/KEGG: protein folding (9); response to stress (16); Protein processing in endoplasmic reticulum (8); unfolded protein binding (5); **Neurotransmitter/synapse (N°DEGs: ↑1VAR: 1; ↑3VAR: 0)**
3VAR vs 2 VAR	**N° DEGs: 87; ↑2VAR: 59; ↑3VAR:29** **Xenobiotic metabolism (N°DEGs: ↑2VAR: 5; ↑3VAR: 0)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑2VAR: 4; ↑3VAR: 0)**	**N° DEGs: 350; ↑2VAR: 160; ↑3VAR:190** **Xenobiotic metabolism (N°DEGs: ↑2VAR: 13; 3VAR: 2)** GO/KEGG: glutathione transferase activity (4); Metabolism of xenobiotics by cytochrome P450 (5); Glutathione metabolism (5)	**N° DEGs: 159; ↑2VAR: 90; ↑3VAR: 69** **Xenobiotic metabolism (N°DEGs: ↑2VAR: 2; 3VAR: 2)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑2VAR: 0; 3VAR: 4)**	**N° DEGs: 234; ↑2VAR: 165; ↑3VAR: 222** **Xenobiotic metabolism (N°DEGs: ↑2VAR: 5; 3VAR: 2)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑2VAR: 5; 3VAR: 4)** GO/KEGG: tissue regeneration (6); wound healing

	GO/KEGG: antigen processing and presentation (2) **Neurotransmitter/synapse (N°DEGs: ↑2VAR: 1; ↑3VAR: 0)**	**Apoptosis/Cell death/Cell cycle (N°DEGs: ↑2VAR: 1; ↑3VAR: 7)** GO/KEGG: negative regulation of apoptotic process (4); negative regulation of cell death (4); inhibition of cysteine-type endopeptidase activity involved in apoptotic process (2) **Immune response/inflammation/tissue regeneration (N°DEGs: ↑2VAR: 0; ↑3VAR: 4)** GO/KEGG: tissue regeneration (5); wound healing (5); **Protein turnover/stress response (N°DEGs: ↑2VAR: 3; ↑3VAR: 0)** **Neurotransmitter/synapse (N°DEGs: ↑2VAR: 1; ↑3VAR: 0)**	**Protein turnover/stress response (N°DEGs: ↑2VAR: 3; 3VAR: 9)** **Apoptosis/Cell cycle/DNA repair N°DEGs: ↑2VAR: 1; 3VAR: 1)**	(4) **Protein turnover/stress response/ cellular macromolecule biosynthetic process (N°DEGs: ↑2VAR: 9; 3VAR: 2)** GO/KEGG: protein folding (9); response to stress (12); unfolded protein binding (4); Protein processing in endoplasmic reticulum (7); peroxisome (4); PPAR signaling pathway (3); unfolded protein binding (4); **Neurotransmitter/synapse (N°DEGs: ↑2VAR: 1; 3VAR: 0)**
3VAR vs 4SAU	**N° DEGs:9; ↑4SAU: 5; ↑3VAR: 4**	**N° DEGs:58; ↑4SAU: 28; ↑3VAR: 30** **Immune response/inflammation/tissue regeneration** GO/KEGG: response to bacterium (2)	**N° DEGs:71; ↑4SAU: 57; ↑3VAR: 14** **Protein turnover/stress response (N°DEGs: ↑1VAR: 1; ↑4SAU: 0)** **Cell cycle (N°DEGs: ↑1VAR: 1; ↑4SAU: 0)**	**N° DEGs:14; ↑4SAU: 7; ↑3VAR: 7** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 0; ↑4SAU: 1)** **Protein turnover/stress response (N°DEGs: ↑1VAR: 1; ↑4SAU: 0)**
1VAR vs 4 SAU	**N° DEGs:144; ↑1VAR: 78; ↑4SAU: 66** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 8; ↑4SAU: 0)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 3; ↑4SAU: 1,** GO/KEGG: cell adhesion (5); Phagosome (3) **Apoptosis/Cell death metabolism (N°DEGs: ↑1VAR: 1; ↑4SAU: 0)** **Protein turnover/stress response (N°DEGs: ↑1VAR: 2; ↑4SAU: 1)** GO/KEGG: RNA biosynthetic process (6); Ribosome biogenesis (5) **Neurotransmitter/synapse (N°DEGs: ↑1VAR: 1; ↑4SAU: 0)**	**N° DEGs:444; ↑1VAR: 147; ↑4SAU: 297** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 22; ↑4SAU: 1)** GO/KEGG: Metabolism of xenobiotics by cytochrome P450 (3); Drug metabolism - cytochrome P450 (3) **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 0; ↑4SAU: 12)** GO/KEGG: cell adhesion (7); arachidonic acid metabolic process (2) **Apoptosis/Cell death/Cell cycle (N°DEGs: ↑1VAR: 1; ↑4SAU: 2)** GO/KEGG: regulation of cell differentiation (7) **Protein turnover/stress response (N°DEGs: ↑1VAR: 9; ↑4SAU: 4)** **Energy metabolism/Metabolism** GO/KEGG: Metabolic patways (1); Oxidative	**N° DEGs:363; ↑1VAR: 222; ↑4SAU: 141** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 3; ↑4SAU: 2)** **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 2; ↑4SAU: 14)** GO/KEGG: tissue regeneration (3); **Apoptosis/Cell death/Cell cycle/DNA repair (N°DEGs: ↑1VAR: 2; ↑4SAU: 0)** **Protein turnover/stress response repair (N°DEGs: ↑1VAR: 7; ↑4SAU: 29)** GO/KEGG: response to stress (10); response to endoplasmic reticulum stress (3); unfolded protein binding (3);	**N° DEGs:381; ↑1VAR: 226; ↑4SAU: 155** **Xenobiotic metabolism (N°DEGs: ↑1VAR: 17; ↑4SAU: 0)** GO/KEGG: Metabolism of xenobiotics by cytochrome P450 (4); Glutathione metabolism (4); **Immune response/inflammation/tissue regeneration (N°DEGs: ↑1VAR: 4; ↑4SAU: 7)** GO/KEGG: defense response to bacterium (2); tissue regeneration (4); Phagosome (7) **Apoptosis/Cell death/Cell cycle/DNA repair (N°DEGs: ↑1VAR: 2; ↑4SAU: 1)** GO/KEGG: cell death in response to oxidative stress (2) **Protein turnover/stress response (N°DEGs: ↑1VAR: 11; ↑4SAU: 3)** GO/KEGG: ubiquitin-protein transferase activity (5): protein ubiquitination (5); proteolysis (8);

		phosphorylation (6)		protein folding (10); unfolded protein binding (4); Protein processing in endoplasmic reticulum (12); response to endoplasmic reticulum stress (4); regulation of cellular response to oxidative stress (2); PPAR signaling pathway (5). **Neurotransmitter/synapse (N°DEGs: ↑1VAR: 3; ↑4SAU: 0)**

Author Contributions

Massimo Milan: conceptualization, funding acquisition; supervision; writing original draft; **Tomaso Patarnello**: conceptualization, funding acquisition, writing review & editing; **Luca Bargelloni**: conceptualization, writing original draft; **Valerio Matozzo**: conceptualization, writing review & editing, supervision; **Maurizio Varagnolo:** investigation, supervision, methodology; **Elena Semenzin**: conceptualization; **Luca Peruzza, Ilaria Bernardini, Giulia Dalla Rovere:** methodology, investigation, writing original draft; **Morgan Smits, Camilla Bertolini, Alice Manuzzi, Roberto Pastres, Luciano Boffo, Andrea Sambo, Cristina Breggion, Jacopo Fabrello, Loretta Gallocchio, Claudio Carrer, Francesco Sorrentino, Cinzia Bettiol, Lodi Giulia Carolina:** methodology, investigation.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1. *Biometric parameters obtained from clams grown in different farming sites.*

Shell Lenght (cm)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	2.68±0.29	3.50±0.21	3.53±0.26	3.82±0.28	**4.50±0.26**
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3_VAR		3.73±0.34	3.98±0.35	3.81±0.58	**4.01±0.46**
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2_VAR				11.46±2.06	**13.88±3.21**
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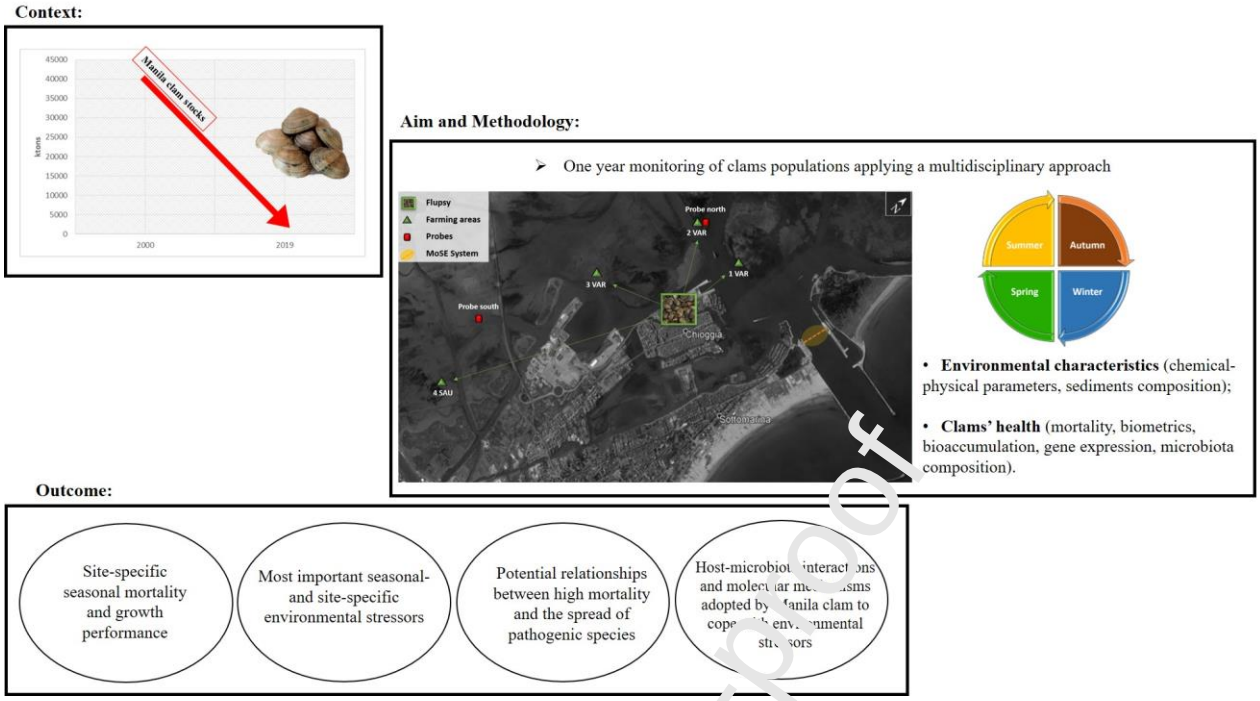
Shell dry weight (g)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	2.22±0.86	3.86±0.61	4.24±0.7	5.49±1.1	**8.83±1.37**
2_VAR		4.12±0.62	4.7±1.02	5.61±1.08	**6.79±1.13**
3_VAR		4.52±1.03	5.65±1.51	6.9±2.9	**7.86±2.27**
4_SAU		5.63±0.98	6.71±1.55	8.06±4.59	**10.48±2.93**
Soft tissue dry weight (g)					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	0.23±0.11	0.35±0.05	0.21±0.06	0.54±0.15	**1.08±0.21**
2_VAR		0.37±0.07	0.3±0.08	0.45±0.11	**0.66±0.14**
3_VAR		0.39±0.08	0.34±0.09	0.46±0.13	**0.72±0.2**
4_SAU		0.59±0.12	0.4±0.12	0.59±0.13	**0.92±0.23**
Growth Rate					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	-	0.117	0.003	0.026	**0.064**
2_VAR	-	0.104	0.021	0.018	**0.037**
3_VAR	-	0.15	0.025	0	**0.019**
4_SAU	-	0.136	0.028	0.025	**0.042**
Condition Index					
	May 2019	**Jul 2019**	**Oct 2019**	**Feb 2020**	**May 2020**
1_VAR	9.93±1.26	9.26±1.34	5.03±1.12	9.81±1.48	**12.19±1.17**
2_VAR		9.09±1.17	6.27±0.99	8.01±1.29	**9.67±1.25**
3_VAR		8.83±1.16	6.19±0.94	6.88±1.01	**9.31±1.64**
4_SAU		10.5±1.39	5.98±0.91	7.36±0.84	**9.07±2.07**
Seasonal and total mortality (%)					
	May 2019 - Jul 2019	**Jul 2019 - Oct 2019**	**Oct 2019 - Feb 2020**	**Feb 2020 - May 2021**	**Total (%)**
1_VAR	6.90%	22.82%	8.03%	0.00%	23.07%
2_VAR	9.80%	5.08%	14.79%	0.00%	20.58%
4_SAU	17.14%	9.69%	0.00%	0.00%	15.95%

Graphical abstract



Highlights

In recent years recurrent bivalve mass mortalities considerably increased around the world.

Criticalities affecting clam stocks in different sites within theVenice lagoon have been characterized through a multidisciplinary approach.

Microbiota compositions and gene expression profiling allowed the identification of most important seasonal- and site-specific environmental stressors.

Identification of host-microbiota interactions and molecular mechanisms adopted by Manila clam to cope with environmental stressors