# Venezia2021

# Programma di ricerca scientifica per una laguna “regolata”

**Linea 2.1**

***Qualità del sedimento lagunare a supporto della sua gestione sostenibile***

D2.1.4.5

*Risultati delle analisi ecotossicologiche mediante genomica e delle comunità microbiche presso la bocca di porto di Chioggia*

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# Sommario

## Summary

The Venice lagoon is a vulnerable and heterogeneous costal environment susceptible to different pressures from several stressors that may influence the biology of species inhabiting this complex habitat.

Because of its environmental characteristics, like the tidal exchanging, the Lagoon of Venice is ideal as farming area for edible bivalve species, like Manila clams (*Ruditapes philippinarum*) and Mediterranean mussel (*Mytilus galloprovincialis*).

Despite Manila clam and mussels show high adaptability and resistance, the deterioration of the environment due to climate change and anthropic disturbance source threaten the wild and aquacultured populations. These factors led to dramatic decrease in the annual Manila clam production in the Venice lagoon, from 40,000 tons in 2000 down to 3,000 tons in 2019. While more and more frequent and intense heat waves are significantly impacting natural Manila clam stocks,the recent activation of the MoSE system to protect the city of Venice and its lagoon from high tides may lead to new challenges for natural and farmed bivalve populations. MoSE

In this deliverable we report for the first time the results obtained from the evaluation of the potential effects of MoSE system in Manila clams and Mediterranean mussel farmed in the southern lagoon. In detail, transcriptomic analyses (RNA-seq) and microbiota characterization (16S) have been applied to animals grown in different farming sites along two years, before and after the inauguration of the barriers of the MoSE system in December 2020.

Results of genes profiling showed significant differences in both clams and mussels between the two investigated years, mainly related to immune response, apoptosis, metabolic and proliferation process.

With regard to microbial communities, over-representation of potentially pathogenic taxa, like *Vibrio* spp. was pointed out mostly in the first year and in the outmost site.

Overall, this survey represents the first monitoring plan aiming to assess the possible effects of MoSE system on the health of farmed molluscs. However, due to the complexity of the Venice lagoon that may subject to variations in chemical-physical parameters between years, results reported in this report cannot be related just to MoSE functioning. Accordingly, a longer-term monitoring is recommended to assess the real repercussion of climate change and MoSE on the ecosystem and farming activities along the time.

## Riassunto

La laguna di Venezia è un ambiente costiero vulnerabile ed eterogeneo soggetto alla pressione di diversi fattori di stress che possono influenzare la biologia delle specie che abitano questo complesso habitat. Per le sue caratteristiche ambientali, come lo scambio delle maree, la Laguna ben si presta ad essere un'ottima zona di allevamento per specie commestibili di bivalvi, come la vongola filippina *Ruditapes philippinarum* e il mitilo mediterraneo *Mytilus galloprovincialis*, il cui benessere può tuttavia risentire della presenza di fattori di stress. Oltre ai cambiamenti climatici, ed in particolari alle ondate di calore che hanno colpito pesantemente le attività produttive negli ultimi anni, l'attivazione del sistema MoSE volto a 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 e per i siti di allevamento di bivalvi.

In questa deliverable sono stati monitorati per la prima volta i potenziali effetti del sistema MoSE nelle vongole di Manila e nelle cozze mediterranee. In dettaglio, analisi trascrittomiche (RNA-seq) e microbiologiche (16S) sono state applicate ad animali allevati in diversi siti di allevamento nel corso di due anni, prima e dopo l'inaugurazione delle barriere del sistema MoSE avvenuta nel dicembre 2020.

I risultati delle analisi trascrittomiche hanno mostrato differenze significative sia nelle vongole che nelle cozze tra i due anni studiati, principalmente legate alla risposta immunitaria, alla regolazione dell'apoptosi, a diversi processi metabolici e alla proliferazione. Per quanto riguarda le comunità microbiche, nelle vongole è stata osservata la sovra-rappresentazione di taxa potenzialmente patogeni, come *Vibrio* spp. nel primo anno e nel sito più esterno.

Complessivamente, considerata la complessità della laguna di Venezia e le variazioni annuali nei diversi parametri chimico-fisici, i risultati riportati non possono essere ricondotti unicamente all'entrata in funzione del MoSE. Di conseguenza, si raccomanda la continuazione di tali attività di monitoraggio per valutare i reali effetti a lungo termine dei cambiamenti climatici e del MoSE sulle attività di allevamento di molluschi bivalvi.

# 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 influence 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; Rinaldo et al., 1999; Perillo et al., 2009) and producing also land-to-sea gradients of several environmental features and parameters.

In this context, the system of flood barriers named Experimental Electromechanical Module (MoSE) was unofficially inaugurated in December 2020 in order to safeguard the city and the lagoon from high tides, defend from sea storms as well as from morphological deterioration processes. Despite the awareness of the importance of protecting the area, construction of the MoSE system and its 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 (Alvarez-Borrengo and Alvarez-Borrengo, 1982; Bertolini et al., 2021) can influence the fitness of farmed animals as well as their associated microbial communities, with potential negative consequences on the whole shellfish farming aquaculture industry. In recent years, natural and farmed populations of Manila clam experienced a dramatic stocks decrease. While further investigations are crucial 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 a key role in the cumulative stress these species are subject to.

In this context, we applied advanced molecular analyses in order to monitor spatial-temporal changes 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. To reach such a goal we applied the RNA-sequencing and 16S rRNA Amplicon Sequencing techniques whose results will be also used within a Weigth of Evidence approach for the evaluation of MoSE impacts on shellfish farming sites in the Venice Lagoon.

# 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. In 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. Investigated farming sites of clams and mussels considered in the first and the second monitoring year.

The 8 SAU investigated site was added for the second monitoring campaign, however, because of the detected high mortality of clams along the year (probably due to the unsuitable characteristics of the sediment to clam's farming), samples from this farming area were not analysed.

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

During each sampling time, animals were used to determine the mortality, biometric characteristics, bioaccumulation and other information already described in the previous reports.

Furthermore, aliquots of gills and digestive glands were collected and stored in RNA later at -80°C for molecular analyses.

## 2.2 Molecular analyses

Total RNA was extracted from 5 pools (each composed by 5 individuals) for each investigated site/season with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA extracted by digestive gland were used for both

gene expression (RNA-Seq) and microbiota analyses (16S). Quality and concentration of extracted RNA was assessed through Agilent 2100 Expert system (RIN value) and Qubit.

## 2.3 Transcriptomic analyses

Extracted RNA was used for library preparation using QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina starting from the same RNA extracted. The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a single-end 75 bp.

The first approach to study the gene expression profiles was the principal component analysis (PCA). Since the ultimate aim of the study was the evaluation of potential effects on bivalve health following the inauguration and activation of the MoSE system barriers, we considered all samples from each site (1VAR, 2VAR, 3VAR, 4SAU) collected in winter and spring in the first and in the second year (the latter reflects the two sampling times after the activation of the MoSE system).

Secondly, to facilitate data comprehension, following the evidences of environmental parameters (already reported in previous Deliverable 2.1.4.1, Milestone 2.1.4.1 and 2.1.4.2, VII Rapporto Tecnico Scientifico – periodo Luglio – Dicembre 2021), pairwise comparisons were performed between the outmost and the innermost sites.

Third, the enrichment analysis of genes was then done through the study of the Hallmark Gene Sets (Liberzon et al., 2015) on results from the pairwise comparisons.

The quality of the input reads (FastQC/v0.11.9) was check and low-quality reads and residual adaptors have beenremoved (suite BBTools of BBDuk program). Mapping was carried out using the high-quality reads and a reference *Ruditapes philippinarum* (Iannello et al. 2021) and *Mytilus galloprovincialis* transcriptome (Moreira et al., 2015). 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 the 60% of libraries, 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) and then normalized counts were used to perform pairwise comparisons with edgeR/v3.26.0 (Robinson et al., 2010). Genes with FDR < 0.05 and FC ≥ 2 were deemed differentially expressed. Functional annotation of the reference transcriptome was performed by Blastx similarity search on Swissprot (Uniprot), Homo sapiens protein Ensembl database, Danio rerio protein Ensembl database and *Crassostrea gigas* protein Ensembl database (Evalue < 0.0001).

## 2.4 Microbiota analyses

For microbiota analyses, 1 µg of RNA was retrotranscribed to cDNA using the Superscript IV Kit (Invitrogen, Life Technologies, Monza, Italy). The cDNA was sent to BMR Genomics (Padova, Italy) for libraries construction using reverse and forward primers (10 µM) that specifically target the V3-V4 gene region of the bacterial 16S rRNA, as described by Milan et al. (2018). Libraries were then sequenced with an Illumina MiSeq (2x300) approach. Raw reads were uploaded in QIIME 2 (Quantitative insights into microbial ecology; Bolyen et al., 2019) and using cutadapt primer sequences were removed, later 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 chimaeric fragments were retained. Representative sequence alignment was performed using MAFFT software (Katoh & Standley, 2013) and then 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. The statistical analysis was performed as following. Raw reads produced by

microbiome sequencing were randomly checked using FastQC tool and then were uploaded in QIIME 2 (Bolyen et al., 2019). Primer sequences were firstly removed through cutadapt; trimmed reads were then processed with DADA2 (Callahan et al., 2016) to discard sequences with low quality or PCR artefacts and to merge forward and reverse reads producing an AVs table with high quality representative sequences. Within QIIME 2 pipeline, MAFFT software (Katoh and Standley, 2013) and Phyton library were used to align representative sequences and to assign taxonomic classification based on SILVA V3-V4 trained database (132 release) respectively. The obtained feature-table, associated with the taxa information, was uploaded in Microbiome Analyst to perform statistical analyses (Chong et al., 2020). The samples were organized through Principal Coordinate Analysis using Bray-Curtis distance at OTU level and alpha diversity boxplots were prepared using Chao1, Simpson and Shannon indexes. Pairwise comparison between the farthest and nearest site to the port in the same sampling time and between the same site in different sampling years was perfomed using DESeq2 method and FDR < 0.05 to identify taxa up or down represented in the investigated sites.

# 3 Results

## 3.1 Gene expression

### *3.1.1 Gene expression analyses in Manila clams (R. philippinarum)*

Considering that our previous studies highlighted that both gene expression profiles and microbial communities are severely influenced by seasonality (Milan et al. 2013; Milna et al. 2018), transcriptomic analyses were performed separately for each sampling season, focusing on variations occurring between the pre- and post-activation of MoSE system.

First, principal component analysis (PCA) was applied separately for winter (2020 and 2021) and spring (2020 and 2021) sampling seasons (Fig.2).

A clear separation between clams collected in the first and in the second year is noteworthy along the x-axis in both sampling seasons (PC1 = 12.82% in winter, PC1 = 14.36% in spring). Farming sites were also clearly separated in both seasons before the functioning of MoSE system, while these separations were less evident after MoSE functioning in year 2 (2021), in particular in February. In addition, clear separations between 1VAR-2VAR and 3VAR-4SAU widely observed during the first monitoring year (Milan et al. in submission) were confirmed also in June of the second monitoring year.

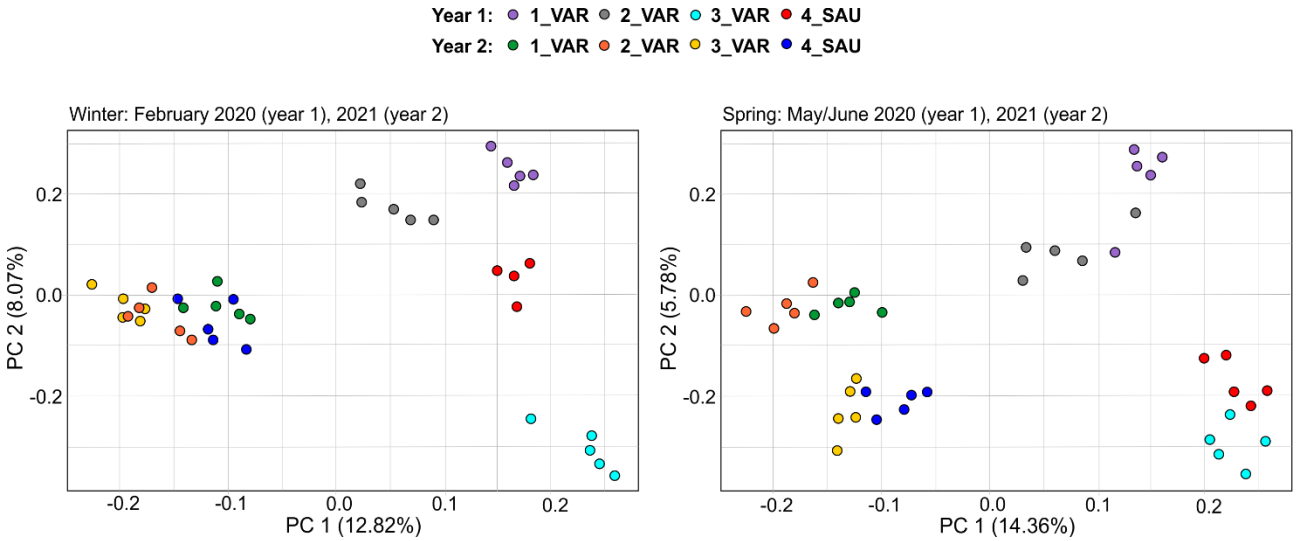


Figure 2. PCA of gene expression in clams from the 4 investigated farming areas (1VAR, 2VAR, 3VAR, 4SAU) collected in winter (left) and spring (right) in the year 1 and 2.

Considering that 1VAR and 4SAU represent the geographical extremes among investigated sites, pairwise comparisons were performed considering these sampling sites *i)* comparing the first and second year within each farming site and for each season; *ii)* comparing 1VAR and 4SAU farming sites at each sampling times.

The number of differentially expressed genes obtained for each comparison are summarized in Figure 3. First, major transcriptional changes between the two years (pre vs post MoSE functioning) were observed in the outermost site 1VAR in both seasons, with higher number of up-regulated genes in year 2 compared to year 1 (Figure 3a).

Regarding the comparison between 1VAR and 4SAU, results obtained demonstrate less transcriptional changes between farming sites during the second year as already suggested by PCA analysis (Fig.3b).

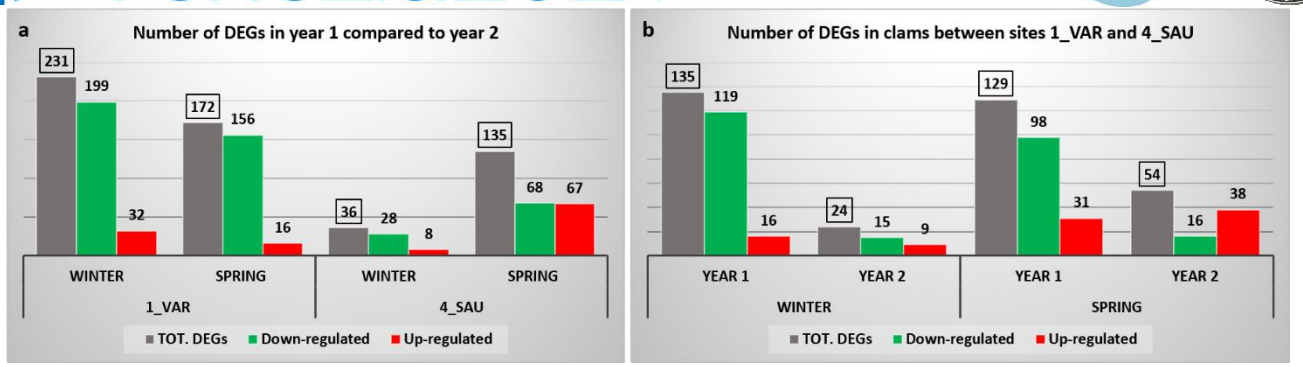


Figure 3. a) Number of differently expressed genes DEGs from the comparison between the year 1 and 2 within the same season in clams collected in 1VAR and 4SAU. Gray bars refer to the total number of DEGs, the green and the red bars represent the down – and the up- regulated genes in clams collected in the year 1 compared to the year 2. b) Number of differently expressed genes DEGs from the comparison between clams from 1VAR and 4SAU within the same season during the year 1 and 2. Gray bars refer the total number of DEGs, the green and the red bars represent the down – and the up- regulated genes in clams collected in 1VAR compared to 4SAU.

The lists of differently expressed genes detected through the first and second pairwise comparisons are reported in Supplementary File 1. GSEA were also performed to identify most important variations in molecular pathways regulations. This analysis, performed considering the HALLMARK gene sets, will be also functional for the application of the WoE approach to define environmental risks in farming sites.

First GSEA was applied to compare farming sites between different years (Table 1). Noteworthy, all significant molecular pathways were up-regulated in year 2 (post-MoSE) compared to year 1 (PRE-MoSE) at both farming sites. Among them, several pathways having key roles in immune response were found in 1VAR, in particular in winter. Conversely, up-regulation of pathways involved in signalling (estrogen response, TGF beta, and TNFA via NFKB) were observed in 4SAU in winter after functioning of MoSE system compare to the previous year. Similarly, over-expression of several metabolic pathways, including oxidative phosporilation and xenobiotic metabolism, were found in spring in clams grown in 4SAU during second year as confirmed also by the up-regulation of genes like *Cytochrome P450*, *Heat shock 70 kDa protein 12A*, *Sulfotransferase family cytosolic 1B member 1*, *Multidrug resistance-associated protein,* DNA damage response, peroxisome and protein secretion. Overall, while connections with other biometric and chemical-physical parameters (described in D2.1.4.1) will be soon established, these data indicate increased exposure to environmental stressors during the second monitoring year.

Table 1 Significant Hallmark categories (p-adjusted<0.2) obtained by the comparison of clams from the same farming area (1VAR and 4SAU) collected in winter and spring between the year 1 and year 2. Green indicates up-regulate pathways at year 2. Red indicates up-regulated pathways in year 1.

| HALLMARK categories - Year 1 vs year 2 (p-adjusted<0.2) | | | |
|---|---|---|---|
| Winter | | | |
| 1VAR | | 4SAU | |
| Pathway | process | Pathway | process |
| HALLMARK_COAGULATION | IMMUNE | HALLMARK_APICAL_JUNCTION | CELLULAR COMPONENT |
| HALLMARK_INTERFERON_RESPONSE | IMMUNE | HALLMARK_ESTROGEN_RESPONSE | SIGNALING |

| | | | |
|---|---|---|---|
| HALLMARK_APICAL_SURFACE | CELLULAR COMPONENT | HALLMARK_TGF_BETA_SIGNALING | SIGNALING |
| HALLMARK_COMPLEMENT | IMMUNE | HALLMARK_TNFA_SIGNALING_VIA_NFKB | SIGNALING |
| HALLMARK_INFLAMMATORY_RESPONSE | IMMUNE | | |
| HALLMARK_NOTCH_SIGNALING | SIGNALING | | |
| Spring | | | |
| 1VAR | | 4SAU | |
| Pathway | process | Pathway | process |
| HALLMARK_INTERFERON_RESPONSE | IMMUNE | HALLMARK_OXIDATIVE_PHOSPHORYLATION | METABOLIC |
| HALLMARK_APICAL_JUNCTION | CELLULAR COMPONENT | HALLMARK_BILE_ACID_METABOLISM | METABOLIC |
| | | HALLMARK_ADIPOGENESIS | DEVELOPMENT |
| | | HALLMARK_PROTEIN_SECRETION | STRESS RESPONSE |
| | | HALLMARK_PEROXISOME | CELLULAR COMPONENT |
| | | HALLMARK_XENOBIOTIC_METABOLISM | METABOLIC |
| | | HALLMARK_FATTY_ACID_METABOLISM | METABOLIC |
| | | HALLMARK_UV_RESPONSE_UP | DNA DAMAGE |

### *3.1.2 Gene expression analyses in Mediterranean mussel (M. galloprovincialis)*

Gene expression analyses of mussels grown in 5SCA and 6CAM farming sites were performed with the same approach proposed for Manila clam.

First of all, the PCA (Fig.4) showed clear separation along the x-axis (21.1%) for the two sampling seasons, while y-axis clearly separated year 1 from year 2. In addition, excluding winter of year 1, farming sites resulted also separated within each sampling time.

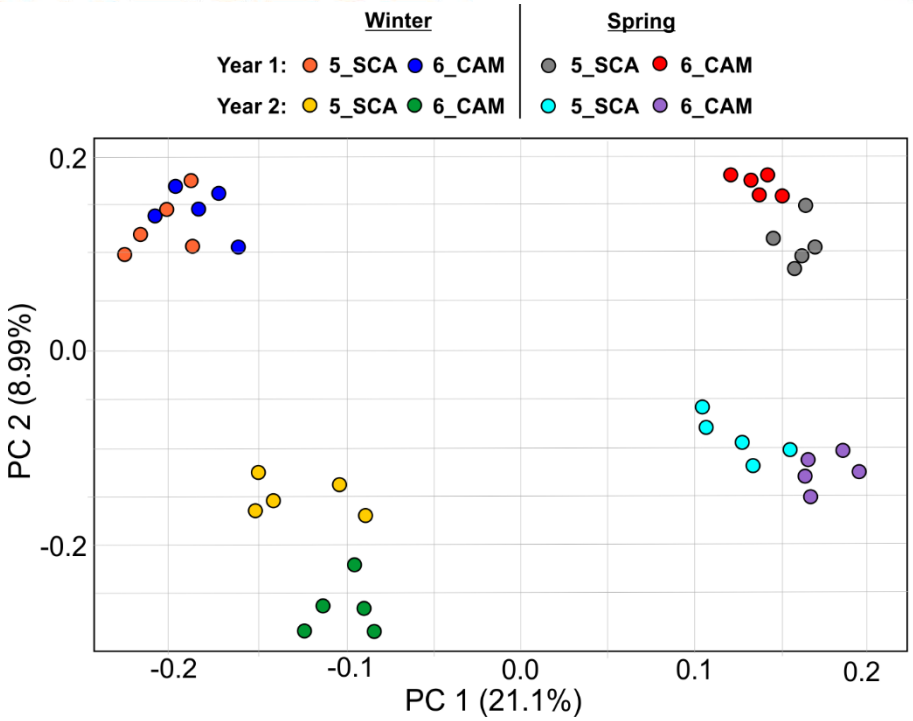


Figure 4. PCA of gene expression in mussels from the 2 investigated farming areas (5SCA, 6CAM) collected in winter and spring in the year 1 and 2.

As for clams, the pairwise comparisons analysis were performed *i)* to identify DEGs between year 1 and year 2 for each farming site/season; *ii)* to identify DEGs between farming sites for each sampling time.

Results obtained are summarized in Figure 5, while full lists and annotation of DEGs were reported in Supplementary File 2.

The highest number of DEGs between monitored years was observed in winter for both farming sites, with the most important variations observed in 6CAM at both sampling seasons.

Comparisons between farming sites showed similar number of DEGs at all sampling time, excluding spring of year 2, when few DEGs were obtained (49 DEGs).

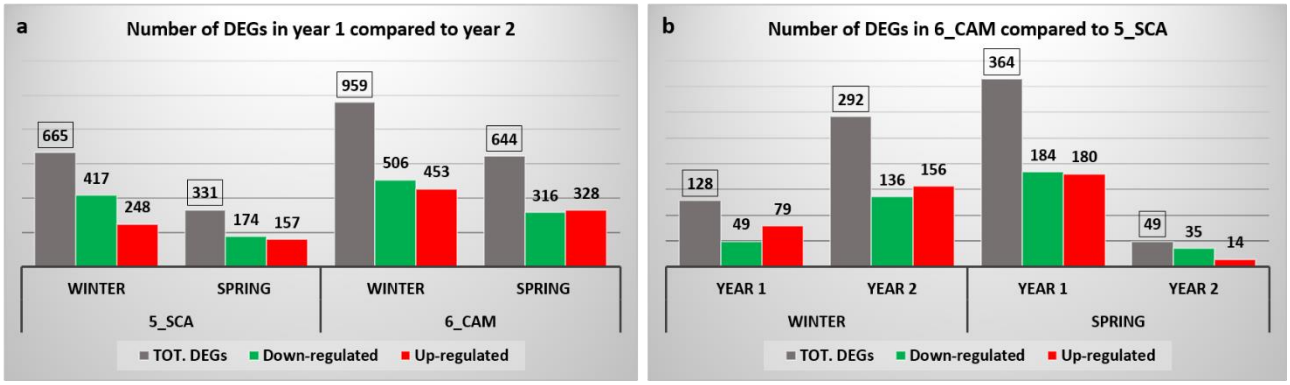


Figure 5. a) Number of differently expressed genes DEGs from the comparison between the year 1 and 2 within the same season in mussels collected in 5SCA and 6CAM. Gray bars refer the total number of DEGs, the green and the red bars represent the down – and the up- regulated genes in year 1 compare to year 2. b) Number of differently expressed genes DEGs from the comparison between clams from 6CAM and 5SCA within the same season during the year 1 and 2. Gray bars refer the total number of DEGs, the green and the red bars represent the down – and the up-regulated genes in mussels collected in 6CAM compared to 5SCA.

Of interest, GSEA applied to define changes between year 1 and year 2, shows results similar to those obtain for Manila clam. First, most of the significant molecular pathways were up-regulated in year 2 compared to year 1. In both farming sites, up-regulation of several pathways playing key roles in immune response (complement, interferon response, inflammatory response), stress response (hypoxia, protein secretion) and DNA damage were found up-regulated after MoSE functioning. In addition, up-regulation of many pathways involved in proliferation were observed in 5SCA collected in spring of the second monitoring year. Also in this case, completion of chemical analyses and correlation with chemical physical parameters (in validation) will facilitate the interpretation of the results obtained.

Table 2. Significant Hallmark pathways (p-adjusted<0.2) obtained by the comparison of mussels within the same farming area (5SCA and 6CAM) between the year 1 and year 2. Green indicates up-regulated pathways at year 2. Red indicates up-regulated pathways at year 1.

| HALLMARK categories - Year 1 vs year 2 (p-adjusted<0.2) | | | |
|---|---|---|---|
| Winter | | | |
| 5SCA | | 6CAM | |
| Pathway | process | Pathway | process |
| HALLMARK_INTERFERON_RESPONSE | IMMUNE | HALLMARK_INTERFERON_RESPONSE | IMMUNE |
| HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY | STRESS RESPONSE | HALLMARK_TNFA_SIGNALING_VIA_NFKB | SIGNALING |
| HALLMARK_COMPLEMENT | IMMUNE | HALLMARK_FATTY_ACID_METABOLISM | METABOLIC |
| HALLMARK_PEROXISOME | CELLULAR COMPONENT | HALLMARK_OXIDATIVE_PHOSPHORYLATION | METABOLIC |
| HALLMARK_APICAL_JUNCTION | CELLULAR COMPONENT | HALLMARK_COMPLEMENT | IMMUNE |
| HALLMARK_APICAL_SURFACE | CELLULAR COMPONENT | HALLMARK_MYC_TARGETS_V2 | PROLIFERATION |
| | | HALLMARK_BILE_ACID_METABOLISM | METABOLIC |
| | | HALLMARK_IL6_JAK_STAT3_SIGNALING | IMMUNE |
| | | HALLMARK_COAGULATION | IMMUNE |
| | | HALLMARK_ANDROGEN_RESPONSE | SIGNALING |
| | | HALLMARK_IL2_STAT5_SIGNALING | SIGNALING |
| | | HALLMARK_PI3K_AKT_MTOR_SIGNALING | SIGNALING |
| | | HALLMARK_CHOLESTEROL_HOMEOSTASIS | METABOLIC |
| | | HALLMARK_UV_RESPONSE_DN | DNA DAMAGE |
| | | HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY | STRESS RESPONSE |
| | | HALLMARK_INFLAMMATORY_RESPONSE | IMMUNE |
| Spring | | | |
| 5SCA | | 6CAM | |
| Pathway | process | Pathway | process |

| | | | |
|---|---|---|---|
| HALLMARK_FATTY_ACID_METABOLISM | METABOLIC | HALLMARK_UV_RESPONSE_UP | DNA DAMAGE STRESS |
| HALLMARK_OXIDATIVE_PHOSPHORYLATION | METABOLIC | HALLMARK_HYPOXIA | RESPONSE |
| HALLMARK_GLYCOLYSIS | METABOLIC | | |
| HALLMARK_UNFOLDED_PROTEIN_RESPONSE | STRESS RESPONSE | | |
| HALLMARK_ADIPOGENESIS | DEVELOPMENT | | |
| HALLMARK_MYOGENESIS | DEVELOPMENT | | |
| HALLMARK_MTORC1_SIGNALING | SIGNALING STRESS | | |
| HALLMARK_PROTEIN_SECRETION | RESPONSE PROLIFERATION | | |
| HALLMARK_G2M_CHECKPOINT | | | |
| HALLMARK_PEROXISOME | CELLULAR COMPONENT | | |
| HALLMARK_BILE_ACID_METABOLISM | METABOLIC | | |
| HALLMARK_XENOBIOTIC_METABOLISM | METABOLIC | | |
| HALLMARK_PANCREAS_BETA_CELLS | DEVELOPMENT | | |
| HALLMARK_DNA_REPAIR | DNA DAMAGE | | |
| HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION | DEVELOPMENT STRESS | | |
| HALLMARK_HYPOXIA | RESPONSE | | |
| HALLMARK_SPERMATOGENESIS | DEVELOPMENT PROLIFERATION | | |
| HALLMARK_MYC_TARGETS_V1 | PROLIFERATION | | |
| HALLMARK_E2F_TARGETS | PROLIFERATION | | |
| HALLMARK_MITOTIC_SPINDLE | PROLIFERATION | | |
| HALLMARK_P53_PATHWAY | | | |

## 3.2 Microbiota analyses

Microbiota analyses aimed to evaluate changes in digestive gland microbial communities between the two investigated years. To reach such a goal, and to establish host-microbiota interactions, we applied the same approach described above for gene expression analyses.

### *3.2.1 Microbiota analyses in Manila clam (R. philippinarum)*

PCoA analyses performed at OUT level considering samples collected in winter and spring are reported in Figure 7. Contrary to the results obtained by gene expression profiling, no evident separation between monitored years and sampling sites were found.

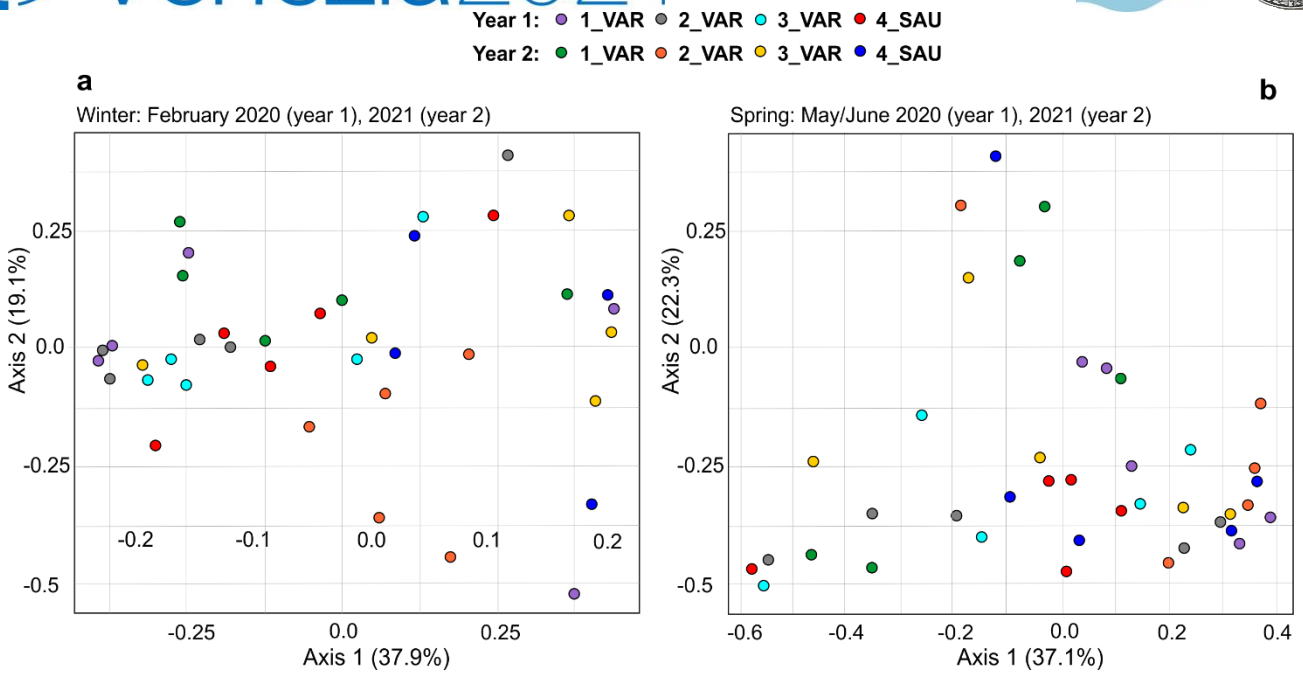


Figure 7. Principal Coordinates Analysis (PCoA) of clams' digestive gland microbiota. a) PCoA of clams collected in winter of the year 1 (2020) and year 2 (2021) in different farming sites (1VAR, 2VAR, 3VAR, 4SAU). b) PCoA of clams collected in spring of the year 1 (2020) and year 2 (2021) in different farming sites (1VAR, 2VAR, 3VAR, 4SAU).

Charts describing diversity among samples using different indices (Chao1, Shannon, Simpson index) are reported in Figure 8

Again, no significant differences in microbial diversity between the two investigated years were found, while 4SAU showed higher diversity than 1VAR independently from sampling season/year.

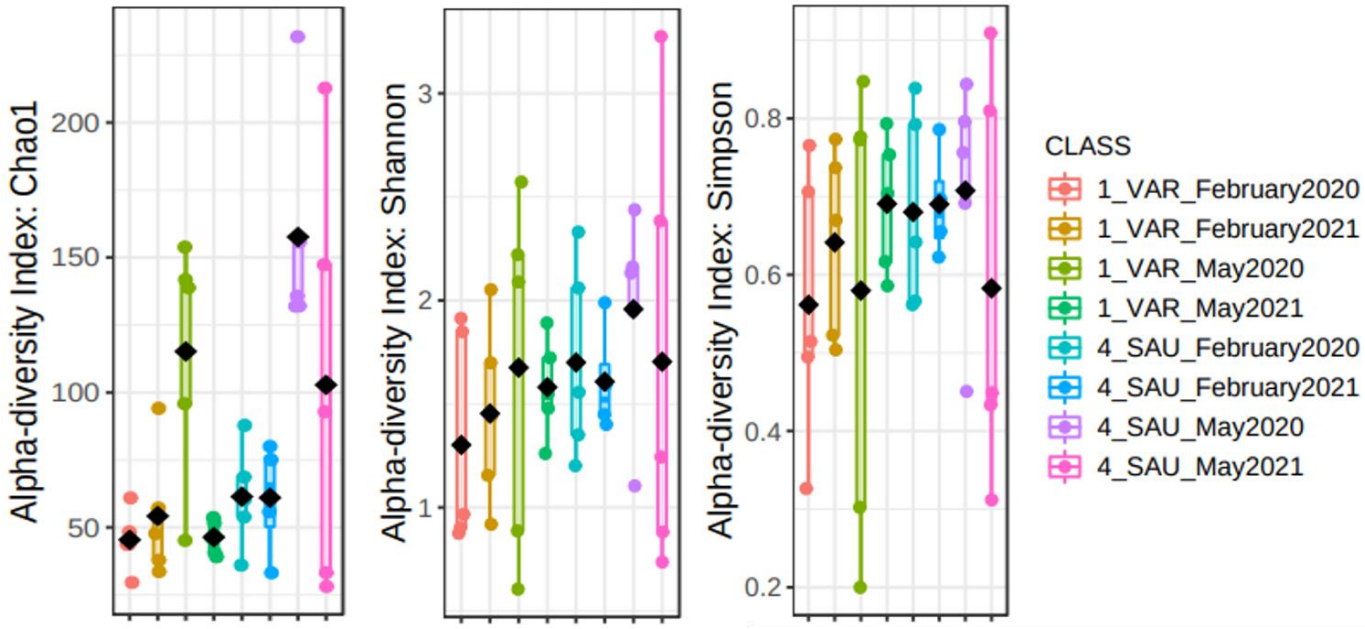


Figure 8. Chao1, Shannon's and Simpson's Index of clams collected in different seasons of year 1 and year 2.

The number of differentially represented species and genera detected comparing farming sites and sampling years (pre- vs post-MoSE) are summarized in Table 3.

Table 3. Number of significant taxa at species and genus level. On the left, the number of differently represented taxa in clams from the same farming site within the same season collected in year 1 compared to the year 2. On the right, the number of differently represented taxa between clams collected in 1VAR compared to 4SAU within the same season in year 1 and year 2.

| year 1 vs year 2 | | | | | 1VAR vs 4SAU | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species level | | | | | | | | | |
| | | ↓ | ↑ | TOT | | | ↓ | ↑ | TOT |
| 1VAR | Winter | 0 | 2 | 2 | Winter | Year 1 | 0 | 2 | 2 |
| | Spring | 1 | 6 | 7 | | Year 2 | 2 | 0 | 2 |
| 4SAU | Winter | 0 | 0 | 0 | Spring | Year 1 | 12 | 7 | 19 |
| | Spring | 3 | 6 | 9 | | Year 2 | 1 | 0 | 1 |
| Genus level | | | | | | | | | |
| | | ↓ | ↑ | TOT | | | ↓ | ↑ | TOT |
| 1VAR | Winter | 2 | 4 | 6 | Winter | Year 1 | 2 | 3 | 5 |
| | Spring | 1 | 8 | 9 | | Year 2 | 0 | 0 | 0 |
| 4SAU | Winter | 2 | 2 | 4 | Spring | Year 1 | 10 | 12 | 22 |
| | Spring | 3 | 6 | 9 | | Year 2 | 1 | 1 | 2 |

First, comparisons between pre- and post-MoSE functioning showed few microbial changes in both farming sites, mainly occurring at the last sampling time (T4; spring). Second, following MoSE functioning, lower number of significant taxa was found in the second year comparing the two farming sites as observed in Manila clam gene expression profiles. Among the most interesting changes observed in clam's microbial communities between year 1 and year 2, down-representation of *Vibrio gigantis* was observed in both seasons of year 2 compared to year 1 in clams from 1VAR. The over-representation of *Vibrio* species in the farming site close to Chioggia inlet and the over-representation of *Arcobacter* spp. in the innermost site 4SAU observed during the first monitoring year (see previous RTS; Milan et al. in publication), were less evident during the second monitoring year (after MoSE functioning). In detail, considering the second monitoring year, just Vibrio genus was over-represented in 1VAR compared to 4SAU in spring 2021.

### *3.2.2 Microbiota analyses in Mediterranean mussel (M. galloprovincialis)*

PCoA analysis showed a weak separation along the axis 2 (14.1%) for mussels collected in the two seasons pointing out no separations in defined clusters based on monitored years and/or farming sites (Fig.9).

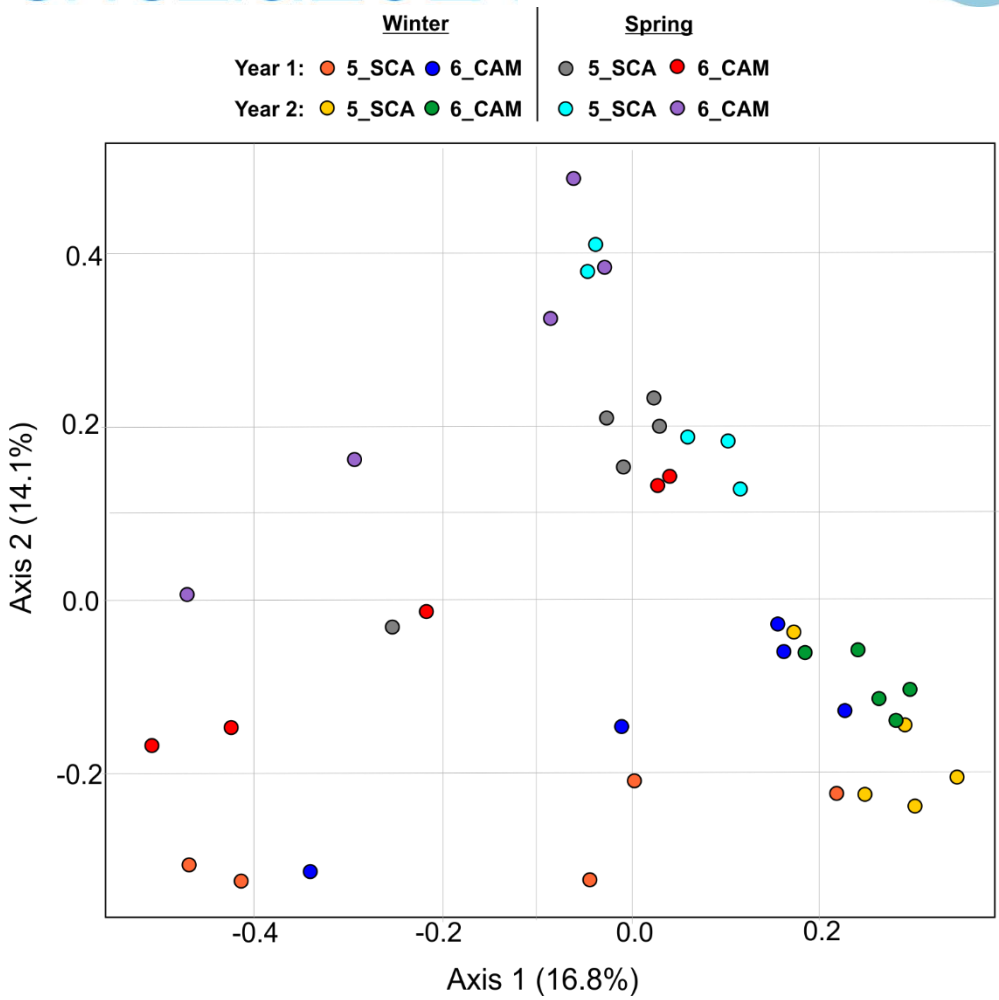


Figure 9. Principal Coordinates Analysis (PCoA) of mussels' digestive gland microbiota collected in winter of the year 1 (2020) and year 2 (2021) in different farming sites (5SCA, 6CAM).

Microbial diversity analysis (Chao1, Shannon's and Simpson's indices) performed in mussels farmed in 5SCA and 6CAM is reported in Figure 10.

Diversity index did not highlight significant changes between year 1 and year 2 in both sampling sites, while higher microbial diversity was found in 6CAM compared to 5SCA.

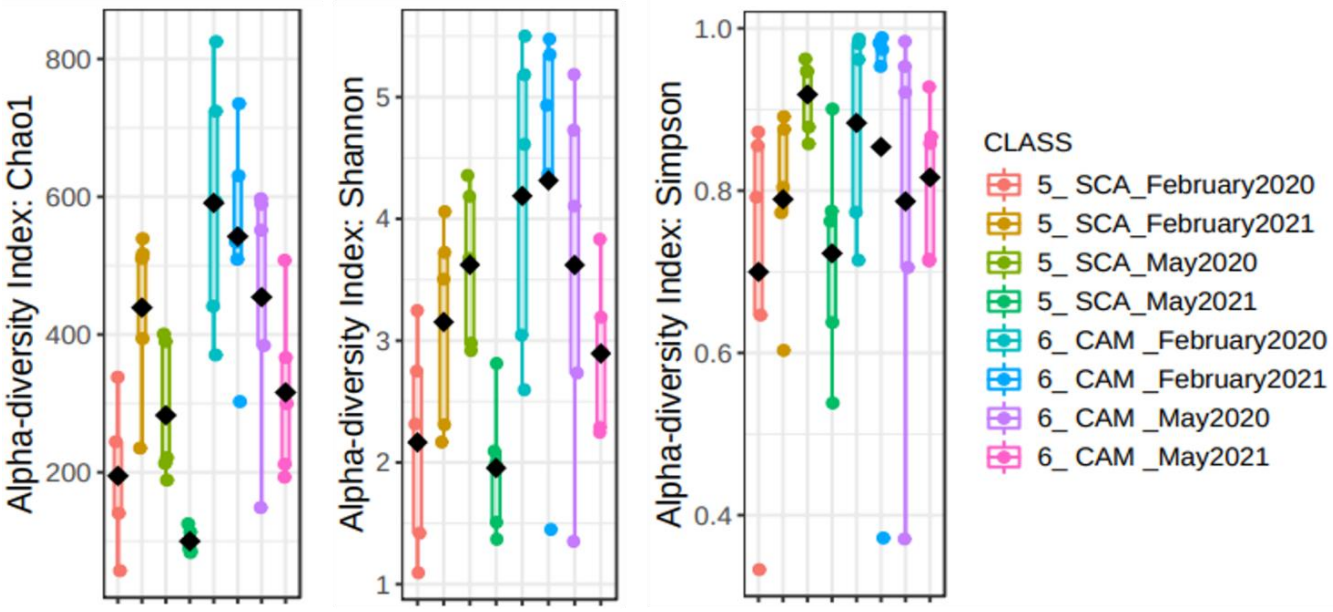


Figure 10. Chao1, Shannon's and Simpson's Index of mussels collected in different seasons of year 1 and year 2.

The number of significant taxa (FDR < 0.05) between sampling years and farming sites are reported in Table 4.

Table 4. Number of significant taxa at species and genus level. On the left, the number of differently represented taxa in mussels from the same farming site within the same season collected in year 1 compared to the year 2. On the right, the number of differently represented taxa between mussels collected in 5SCA compared to 6CAM within the same season in year 1 and year 2. In ↑ red the over-represented taxa. In ↓ green the down-represented taxa.

| year 1 vs year 2 | | | | | 5SCA vs 6CAM | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species level | | | | | | | | | |
| | | ↓ | ↑ | TOT | | | ↓ | ↑ | TOT |
| 5SCA | Winter | 6 | 6 | 12 | Winter | Year 1 | 5 | 5 | 10 |
| | Spring | 5 | 14 | 19 | | Year 2 | 3 | 3 | 6 |
| 6CAM | Winter | 12 | 11 | 23 | Spring | Year 1 | 5 | 6 | 11 |
| | Spring | 8 | 15 | 23 | | Year 2 | 16 | 0 | 16 |
| Genus level | | | | | | | | | |
| | | ↓ | ↑ | TOT | | | ↓ | ↑ | TOT |
| 5SCA | Winter | 9 | 10 | 19 | Winter | Year 1 | 26 | 10 | 36 |
| | Spring | 2 | 32 | 34 | | Year 2 | 5 | 5 | 10 |
| 6CAM | Winter | 14 | 23 | 37 | Spring | Year 1 | 10 | 10 | 20 |
| | Spring | 14 | 22 | 36 | | Year 2 | 29 | 0 | 29 |

Differently from results obtained in Manila clam, microbiota of mussel's digestive gland showed significant changes between year 1 and year 2, with several differentially represented taxa identified in both farming site at species and genus levels. Additionally, comparisons between investigated sites showed similar results at year 1 and year 2 for both spring and winter sampling time. Lists of differentially represented taxa at species and genus level are reported in Supplementary File 4. Noteworthy, at species level, none of the significant taxa was in common in winter of year 1 and year 2 in mussels from 5SCA and 6CAM, while just 2 genera (7% of significant taxa; Mycoplasma and Endozoicomonas) were commonly found over-represented in 5SCA at both monitored years. Similarly, considering spring, at species level just one taxa (*uncultured*

*gamma proteobacterium*) was confirmed differentially represented between sampling sites at both monitored years. These results can be summarized in two key points. First, despite the two investigated farming sites are both located close to Chioggia inlet, digestive gland microbiota differs between farming sites independently from sampling season/year. Second, differences observed in microbial communities of the two investigated sites differ at two monitored year (Supplementary File 4). However, considering that Mediterranean mussels are less susceptible to pathogens than Manila clam, our preliminary data did not show criticalities about presence/spread of pathogens in both pre- e post-monitoring periods.

# 4 Conclusions

In this deliverable, molecular analyses have been applied in Manila clam and Mediterranean mussels farmed in different sites of south Venice lagoon to characterize spatio-temporal changes in gene expression profiles and digestive gland microbiota. In detail, we monitored four clam and two mussels farming sites for 2 years reflecting periods pre- and post- the MoSE functioning. With regard to Manila clam gene expression profiling, results obtained suggest variations between the two monitored years mainly occurring in farming site close to Chioggia inlet (1VAR and 2VAR). In addition, our data suggest less important differences between clams grown at the two geographic extremes (1VAR and 4SAU) after the entry into operation of MoSE system. Mussels gene expression profiles showed most important variations between monitored years in terms of number of DEGs. However, both species showed the up-regulation of several molecular pathways related to immune response, stress response, energy metabolism, DNA damage and peroxisome in the second year (post-MoSE functioning). From clams microbiota analyses, no significant variations were observed after the MoSE functioning. In addition, microbial composition of clams inhabiting the two investigated areas appears more similar after MoSE functioning, mirroring the results obtained by gene expression analysis. It should be also highlighted the over-representation of the genus Vibrio in farming sites located close to Chioggia inlet during the second year. Conversely, mussels microbiota showed several changes between the two monitored years and between sites. However, no pathogens or opportunistic pathogens were observed, suggesting none microbiological criticalities. While to establish direct relationships between results obtained and MoSE functioning appears absolutely speculative, data collected open the way to long term monitoring of bivalve farming sites, related to possible effects of anthropogenic activities (e.g. MoSE) and climate changes. Correlations between molecular data and collected biometric parameters, chemical analyses and chemical-physical parameters (in validation) will allow to shed light on the ongoing environmental changes occurring in the Venice lagoon and their possible effects on bivalve farming.

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