Venezia2021

Programma di ricerca scientifica per una laguna “regolata”

Linea 2.1

Qualità del sedimento lagunare a supporto della sua gestione sostenibile

D2.1.2.5

Risultati delle analisi ecotossicologiche mediante genomica

Massimo Milan, Ilaria Bernardini, (UNIPD-BCA)

22/02/2022

Sommario

1. Riassunto

La laguna di Venezia è un ambiente vulnerabile sottoposto a cicliche attività di dragaggio dei sedimenti dei canali navigabili, operazioni che possono indurre la sospensione dei contaminanti presenti nei sedimenti con possibili conseguenze per le specie che vivono quest'area. È pertanto fondamentale mettere a punto metodiche e protocolli per la valutazione del rischio ambientale ed in particolare per definire la pericolosità derivata dalla movimentazione del sedimento.

Nell'ambito del progetto, si è prestata particolare attenzione nel valutare la possibile tossicità del sedimento su una specie con rilevante interesse ecologico ed economico per la laguna di Venezia, la vongola filippina *Ruditapes philippinarum.* Nel dettaglio, sono state effettuate analisi molecolari volte ad indagare alterazioni nei profili di espressione genica (RNA-seq) ed eventuali cambiamenti nelle comunità microbiche (16S) in vongole esposte ai sedimenti dragati nel Canale Vittorio Emanuele III e nel Canale San Felice.

I risultati hanno evidenziato modificazioni trascrizionali negli animali, in particolate in seguito all'esposizione ai sedimenti campionati in aree prossime a Porto Marghera. A loro volta, le analisi del microbiota hanno messo in evidenza importanti modificazioni a livello di alterazione delle comunità microbiche nella ghiandola digestiva delle vongole, inclusi anche possibili patogeni opportunisti. Va tuttavia fatto presente che l'interpretazione di tali risultati necessita del supporto delle indagini chimiche relative al livello di contaminazione del sedimento e al bioaccumulo nei tessuti delle vongole, che non risultano ancora disponibili al momento della stesura del presente documento.

Inoltre, i risultati ottenuti dalle analisi trascrittomiche verranno inclusi all'interno di un modello Weight of Evidence per la valutazione del rischio ambientale.

2. Summary

The Venice Lagoon is a vulnerable environment subjected to cyclical dredging activities but specific investigations on sediment are scarce so far. The movement of sediments by these anthropogenic activities poses serious environmental concerns depending on the quantity and quality of removed sediment due to the suspension of the present contaminants giving more bioavailability for organisms. However, a specific regulation agreement for the environmental risk assessment in the Venice Lagoon related to the hazard of dredged sediments has not been defined yet.

Thus, in the context of the present line of research, investigation on molecular alterations in clams (*Ruditapes philippinarum*) were performed in order to provide useful information for the environmental risk assessment based on the Weight of Evidence approach. Analyses were carried out in order to evaluate changing in gene expression profiles (RNA-seq) and in microbiota communities (16S) in clams exposed to sediments from the Canale San Felice (control) and the Canale Vittorio Emanuele III, dredge at different distances from the Porto Marghera, a well-known polluted area of the Lagoon.

Results highlighted the up and down regulation of several genes mostly in clams treated with dredged sediments closer to the Porto Marghera, especially after the short-term treatment. Moreover, several pathways related to different processes were found altered in all treatments.

Furthermore, over and under representation of several microbial taxa were detected in all groups, including taxa referred to microbial species considered opportunistic pathogens.

However, for a complete interpretation of these data, chemical analyses are necessary and they are still ongoing.

Overall, as aforementioned, data reported in the present deliverable will be used to implement and improve technical specific regulation used for the monitoring of sediments and related anthropogenic activities in this environment, constituting a new fundamental approach to be included in the risk assessment process based on the Weight of Evidence.

3. Introduction

Sediment constitutes a matrix continuously in contact with the water column whose interaction produces sedimentation/resuspension of particulates and exchange with interstitial water. Furthermore, sediment hosts microorganisms that contribute to the proper functioning of fundamental biogeochemical cycles thanks to their biological processes. All these processes may influence the organic and inorganic contaminants distribution between water and sediment determining the bioavailability of pollutants to biological communities.

For all these reasons, the quality assessment of the sediment is fundamental in the monitoring and management of ecosystems, including and mostly in the transitional waters and costal lagoons, which are vulnerable environments like the Venice Lagoon. Here, sediment plays an essential role in maintaining the equilibrium of the ecological system and influencing the health of living organism, from the benthos to all organisms that live in direct contact with the sediment, including species of economic importance for the typical aquaculture activities of the lagoon.

Lagoon areas are complex highly productive ecosystems characterized by dynamic interconnections between freshwater and marine environments. Physical modifications of the direct human interventions are the main threats to these valuable and vulnerable environments (Chapman et al., 2013; Stefani et al., 2018).

The inherent complexity of lagoon systems and the limited knowledge of chemical tolerance of lagoon species make investigations of potential impacts difficult and expensive (Stefani et al., 2018).

Over the last two decades, the Venice Lagoon has been subjected to numerous studies and experimental investigations by public authorities and research institutes to understand the processes that characterize and involve the sediment, the state of contamination and the consequent implications for the health of lagoon ecosystems. Despite the multitude of projects, specific investigations on the sediments in the navigation channels subjected to cyclical dredging activities are lacking. Dredging activities are physical modifications of the direct human interventions periodically required in harbour areas to maintain proper depths to ensure boats passage. Likewise, these anthropogenic activities pose serious environmental concerns depending on the quantity and quality of removed sediment (d'Errico et al., 2021) since the movement of this matrix may suspend and increase bioavailability of contaminants during the operations, requiring hence more appropriate management options for the destination of dredged material (DelValls et al., 2004; Eggleton and Thomas, 2004). In this regard and in the monitoring programs framework related to the Lagoon of Venice, the technical document "*Protocollo d'Intesa*" (1993) was issued to define specific limits to the reuse of sediments for recovery and morphological reconstruction interventions, through the setting of concentration thresholds of a set of pollutants (metals; PAHs, PCBs, hydrocarbons and organochlorine pesticide). Although it was enacted with a temporary value, it is still used for the quality of *in situ* sediment assessment today.

However, considering the scientific developments and the increased degree of knowledge of sediments of the Lagoon compared to the 1990s, the definition of a new specific regulation for the management and the evaluation of the quality of sediment of the Lagoon, in the context of the Decreto 173/2016, including also the integration of the results of the chemical and ecotoxicological lines of evidence, is ongoing.

Overall, the aim of WP2.1.2 of Venezia2021 research project is to evaluate the quality of dredged sediments for management purposes related to their movement through the implementation of appropriate experimental investigations referring to various aspects or "lines of evidence", from chemical to ecotoxicological, including analysis at molecular level.

The present deliverable is about results of ecotoxicological analyses obtained from molecular methodologies. Data will contribute to integrate information from other lines of evidence (i.e. chemistry, bioavailability, bioassays, biomarkers) to estimate the risk using a methodology based on the Weight of Evidence approach for assessing the quality of the sediment. The results will support the experimentation and implementation of the new legislation on sediment management.

In detail, in this deliverable the results obtained by molecular analyses that will be used to implement the "Transcriptomic Line of Evidence (LOE)" are reported. Since several previously published papers highlighted the importance of transcriptomic analyses in complementing other investigations in bivalves, *Ruditapes philippinarum* and *Mytilus galloprovincialis*, in relation to the presence of environmental contaminants (e.g. Milan et al., 2011; Milan et al., 2013; Matozzo et al., 2013; Milan et al., 2015; Milan et al., 2016; Milan et al., 2018; Iori et al., 2020; Bernardini et al., 2021), RNA-sequencing technique was adopted to analyse molecular responses of Manila clams to the exposure of sediments dredged in the Venice Lagoon. Furthermore, since the importance of microbiota in molluscs is well-known, 16S rRNA Amplicon Sequencing technique was used to understand potential changing in microbial communities of organisms following the exposure to sediment.

The obtained results are reported and preliminarily discussed in this deliverable. However, since a proper discussion will only be possible once the results of chemical analyses of sediments and animal tissues (bioaccumulation) will be available, it will be included in the scientific publications (in preparation) as soon as the data will be provided by the laboratory in charge of such analysis (i.e. Laboratorio di Voltabarozzo del Provveditorato).

4. Material and methods

4.1 Sediment sampling

Sediment cores were collected by SELC Company in 5 sampling points placed in the Vittorio Emanuele III fairway in the Venice Lagoon (Sediment I, II, III, IV and V) and one in Canale San Felice (Sediment VI), representing the control group (Fig.1). Two cores each site were collected and then homogenised through the quartering method to ensure the representativeness of the sediment bulk. Samples were maintained at +4°C until the laboratory experiments.

Figure 1. Sites of sediment cores collection.

4.2 Laboratory experiments

A laboratory experiment was carried out to test the toxicity of dredged sediments on clams (*R. philippinarum*), an edible bivalve species farmed in the Venice Lagoon, in January/February 2021. A second experiment was set in April/May 2021 to validate the previous data when necessary and to assess the possible effects in different period of Manila clam reproductive cycle.

In each experiment, after the acclimation period, 50 specimens per tank were placed. Each experimental group was performed in duplicate for a total of 100 clams per condition.

Digestive gland of clams was collected immediately before the exposure starting (time T0), after 3 and 14 days of exposure, stored in RNA later at -80 ◦C and addressed to molecular analyses. Furthermore, two samples of sediments from each tank were collected after 7 days of the experiment starting and stored at -80°C until the following analyses.

4.3 RNA extraction and sequencing

RNeasy Mini Kit Qiagen (Hilden, Germany) was used for the total RNA extraction from digestive gland of 10 specimens (5 in the second experiment) and from two replicates of sediment from each tank of each experimental group (4 samples per condition in total).

RNA purity, concentration, and integrity were checked using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Tape Station (Agilent, Waldbronn, Germany). RNA extracted was used for both gene expression (RNA sequencing) (only clams) and microbiota analysis (16S) (clams and sediments). Library preparation for gene expression analysis was performed using QuantSeq 3' mRNA-Seq Library Prep Kit. The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a single-end 75 bp setup.

For microbiota characterization, 1 µg of RNA was reverse-transcribed to cDNA using the Superscript IV kit

(Invitrogen, Life Technologies, Monza, Italy). Libraries and sequencing were performed by BMR Genomics (Padova, Italy) in a 50 µL reaction starting with diluted 0.2 ng/µL cDNA and both 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). The final libraries were then sequenced with MiSeq Illumina 300 PE.

4.4 Bioinformatics and statistical analyses for gene expression

Gene expression profiles were explored through the principal component analysis (PCA) as unsupervised method performed considering all samples within each sampling time, pairwise comparisons for each sampling time comparing each experimental group to the reference group (Sediment VI) and Enrichment analyses of differentially expressed genes.

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) and 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 20% of total libraries (out of 130 for the first and 65 for the second experiment), which would contribute to background noise (Peruzza et al. 2020, Pradhan et al. 2020, modified), were removed. Filtered reads were then normalized using the RUVs function (with parameter "k = 7") 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). Pairwise comparison was performed between the reference sediment VI and each experimental group within each sampling time. Genes with FDR < 0.05 and FC ≥ 2 were deemed differentially expressed. The Gene Set Enrichment Analysis (GSEA) on differentially expressed genes was performed using the Hallmark Gene Sets (Liberzon et al., 2015).

4.5 Bioinformatics and statistical analyses for microbiota analyses

Raw reads of microbiome sequencing 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 obtaining high-quality representative sequences. After the quality-filter steps, read merging and removal of chimaeric fragments were carried out. Representative sequence alignment was performed using MAFFT software (Katoh and

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. The statistical analysis was performed using CALYPSO software (Zakrzewski et al.,2017), using the features table and the taxonomy produced in QIIME2. All samples and samples within each sampling time, were studied through the principal coordinate analysis (PCoA; Bray-Curtis distance) at OTU level. ANOVA was carried out to perform the pairwise comparison through the identification of different taxa between experimental groups at species and genus level (FDR<0.05). In detail, PCoA and pairwise comparisons were performed within each sampling time comparing each experimental condition (Sediment I, II, III, IV and V) to the control group represented by the Sediment VI. Furthermore, the Evenness, Richness and Shannon's indices were investigated at OTU level.

5. Results

5.1 Gene expression analysis

Transcriptomic data has been firstly investigated through the Principal Component Analysis (PCA) reported in Fig. 2. Considering that experiments have been performed at different moment of Manila clam reproductive cycle, PCA has been performed separating the Experiment 1 (January-February) and Experiment 2 (April/May). In addition, for each experiment, PCA has been studied at the 3th and 14th day to evaluate potential samples separation within the sampling time.

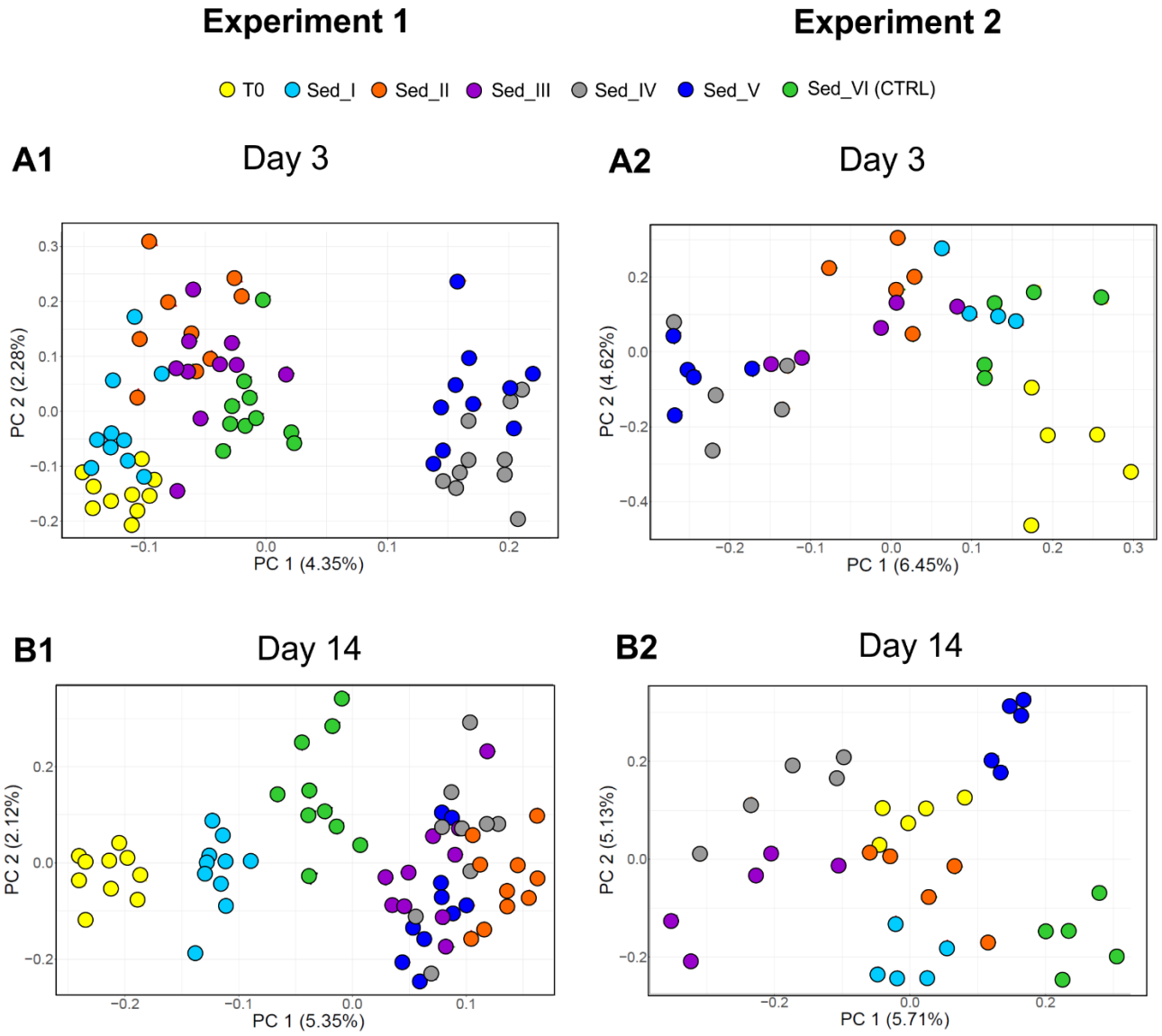

Figure 2. PCA of gene expression in clams of all treatments. On the left, the PCA related to the Experiment 1 after 3 (A1) and 14 (B1) days. On the right, the PCA related to the Experiment 2 considering samples collected after 3 (A2) and 14 (B2) days.

In the first experiment, after 3 days of exposure, samples of Sediment IV and V are separated along the x-axis from the other groups. PCA of the day 14 highlights a clear separation of clams exposed to Sediment I and Sediment VI (control) from all other treatments along the x-axis. The second experiment showed similar results, as the separation of clams treated with the Sediments IV and V at day 3 and the Sediment I and VI at day 14. Second, pairwise comparisons between control (Sediment VI) and the other treatments have been

performed for each experiment and sampling time. The number of different expressed genes (DEGs) obtained from each pairwise comparison is reported in Table 1.

In both experiments, clams exposed to Sediment IV and V showed the highest number of DEGs after 3 days of exposure, while an opposite result was evident in clams exposed to Sediment III in the second experiment where a total of 137 DEGs was found at the last sampling time. Furthermore, in both experiments, sediments leading to most important transcriptional changes in clams were represented by Sediment IV (282, 159) and Sediment V (302, 148). Overall, in the longest-lasting treatments (day 14), the highest number of DEGs was found in the Experiment 2 that was performed in April/May when Manila clam is approaching the reproductive season. The full lists of DEGs and the corresponding annotation are reported in the Supplementary File 1 and Supplementary File 2 for Experiment 1 and Experiment 2, respectively. To date, the lack of information about the chemical characterization of sediments and bioaccumulation in clams' tissues make extremely difficult a proper discussion of molecular results obtained. However, GSEA applied to each dataset allowed to obtain an overview of disrupted molecular pathways following each treatment. Results regarding the first and the second experiment are reported in Table 2 and Table 3 respectively. First, GSEA confirmed the results obtained by pairwise comparisons and PCA, underlying that Sediment IV and V led to most important changes in Manila clam gene expression profiling. Indeed, these treatments showed the disruption of many pathways involved in cell proliferation (myc targets; cell cycle related targets of E2F transcription factors), signalling (mTORC1 signaling; Wnt pathway; PI3K-AKT-mTOR signaling pathway) and metabolism (cholesterol homeostasis; glycolysis; xenobiotic metabolism) in clams exposed for 3 days. These findings are confirmed at day 14, when the disruption of pathways involved in DNA repair was detected in clams in Experiment 1 and immune and inflammatory response in the Experiment 2. Overall, responses of clams to the exposure to the Sediment IV and V appear very similar in both experiments and sampling times. Thus, to our opinion one of the most interesting result of this study is represented by the repeatability of results obtained at transcriptional level. In fact, although there is a difference in the gonadal maturation stage of Manila clam in the two experiments, due to the different period in which the experiments were performed, clams of almost all treatments, except for the treatments with Sediment III and IV, showed similar or identical responses in both experiments.

To conclude, the exposure to Sediment I led also to important changes in Manila clam gene expression profiles, including pathways involved in development (epithelial mesenchymal transition, pancreas beta cells, spermatogenesis, myogenesis), immune response (interferon response, coagulation, complement) and stress response (hypoxia, protein secretion).

As anticipated, the discussion of the obtained results will be completed once chemical characterization of sediments and bioaccumulation in clams' tissues will be available. Moreover, they will also be included within a Weight of Evidence model aiming to assess sediments environmental risk.

Table 1. Number of differentially expressed genes (DEG) in the experiment 1 and experiment 2. The up-regulated genes compared to the control are shown in red, the down-regulated genes in green.

Sediments Vittorio Emanuele III (I-V) vs CTRL (Sediment VI) (FDR<0.05)												
	Experiment 1						Experiment 2					
	Day 3			Day 14			Day 3			Day 14		
	N° DEGs tot	down-reg.	up-reg.	N° DEGs tot	down-reg.	up-reg.	N° DEGs tot	down-reg.	up-reg.	N° DEGs tot	down-reg.	up-reg.
Sediment I	1	0	1	5	0	5	87	51	36	68	50	18
Sediment II	21	6	15	36	28	8	61	31	30	57	28	29
Sediment III	0	0	0	6	5	1	57	23	34	137	104	33
Sediment IV	282	100	182	22	6	16	159	39	120	72	38	34
Sediment V	302	144	158	19	8	11	148	40	108	72	39	33

Table 2. Significant HALLMARK gene set in clams treated with each sediment at 3 (2A) and 14 days (2B) in both experiments.

Table 2A. Day 3.

	I	II	III	IV	V
Exp. 1	EPITHELIAL_MESENCHYMAL_TRANSITION PI3K_AKT_MTOR_SIGNALING INTERFERON_RESPONSE ANGIOGENESIS APICAL_JUNCTION PANCREAS_BETA_CELLS MTORC1_SIGNALING APICAL_SURFACE HYPOXIA	CHOLESTEROL_HOMEOSTASIS PROTEIN_SECRETION UNFOLDED_PROTEIN_RESPONSE ANGIOGENESIS APICAL_JUNCTION	UNFOLDED_PROTEIN_RESPONSE ANGIOGENESIS COAGULATION APICAL_SURFACE	MTORC1_SIGNALING UNFOLDED_PROTEIN_RESPONSE MYC_TARGETS_V1 PROTEIN_SECRETION HYPOXIA WNT_BETA_CATENIN_SIGNALING ANGIOGENESIS E2F_TARGETS CHOLESTEROL_HOMEOSTASIS PI3K_AKT_MTOR_SIGNALING	MTORC1_SIGNALING INTERFERON_RESPONSE MYC_TARGETS_V2 UNFOLDED_PROTEIN_RESPONSE E2F_TARGETS MYC_TARGETS_V1 HYPOXIA GLYCOLYSIS PROTEIN_SECRETION CHOLESTEROL_HOMEOSTASIS XENOBIOTIC_METABOLISM
Exp. 2	EPITHELIAL_MESENCHYMAL_TRANSITION INTERFERON_RESPONSE PI3K_AKT_MTOR_SIGNALING ANGIOGENESIS PANCREAS_BETA_CELLS APICAL_JUNCTION HYPOXIA PROTEIN_SECRETION APICAL_SURFACE HEDGEHOG_SIGNALING MTORC1_SIGNALING	CHOLESTEROL_HOMEOSTASIS PROTEIN_SECRETION	UNFOLDED_PROTEIN_RESPONSE COAGULATION ANGIOGENESIS HYPOXIA PROTEIN_SECRETION	MTORC1_SIGNALING UNFOLDED_PROTEIN_RESPONSE MYC_TARGETS_V1 PROTEIN_SECRETION HYPOXIA ANGIOGENESIS CHOLESTEROL_HOMEOSTASIS WNT_BETA_CATENIN_SIGNALING	MTORC1_SIGNALING INTERFERON_RESPONSE MYC_TARGETS_V2 UNFOLDED_PROTEIN_RESPONSE E2F_TARGETS MYC_TARGETS_V1 HYPOXIA GLYCOLYSIS PROTEIN_SECRETION CHOLESTEROL_HOMEOSTASIS XENOBIOTIC_METABOLISM

Table 2B. Day 14.

	I	II	III	IV	V
Exp. 1	ANGIOGENESIS COAGULATION MYOGENESIS NOTCH_SIGNALING APICAL_SURFACE SPERMATOGENESIS	UV_RESPONSE_DN INTERFERON_RESPONSE PEROXISOME INFLAMMATORY_RESPONSE	ANGIOGENESIS TGF_BETA_SIGNALING UV_RESPONSE_DN EPITHELIAL_MESENCHYMAL_TRANSITION PANCREAS_BETA_CELLS E2F_TARGETS P53_PATHWAY	SPERMATOGENESIS MYOGENESIS UV_RESPONSE_DN MTORC1_SIGNALING NOTCH_SIGNALING TGF_BETA_SIGNALING	E2F_TARGETS MYC_TARGETS_V1 G2M_CHECKPOINT DNA_REPAIR UV_RESPONSE_DN MYC_TARGETS_V2 P53_PATHWAY TGF_BETA_SIGNALING NOTCH_SIGNALING MTORC1_SIGNALING
Exp. 2	COAGULATION COMPLEMENT	E2F_TARGETS INTERFERON_RESPONSE NOTCH_SIGNALING G2M_CHECKPOINT	INTERFERON_RESPONSE APICAL_SURFACE E2F_TARGETS G2M_CHECKPOINT MTORC1_SIGNALING NOTCH_SIGNALING PI3K_AKT_MTOR_SIGNALING INFLAMMATORY_RESPONSE P53_PATHWAY	PEROXISOME FATTY_ACID_METABOLISM	E2F_TARGETS EPITHELIAL_MESENCHYMAL_ TRANSITION COMPLEMENT INFLAMMATORY_RESPONSE PEROXISOME

5.2 Microbiota analyses

Similar to gene expression profiles, microbiota analyses have been firstly investigated by Principal Coordinates Analyses (PCoA). Despite all sequencing data are already available, statistical analyses of results of the second experiment are ongoing and they will be included in the scientific publications (in preparation). However, firstly, the PCoA has been performed considering all samples independently by sampling time (3 and 14 days; Figure 3). This analysis showed a clear separation along the Y-axis between sample collected at day 3 and day 14, suggesting that digestive gland microbial communities changed in all treatments during controlled exposures. This result, already observed in previous studies, can be explained by laboratory conditions having different features by natural environment (e.g. different temperature; artificial feeding).

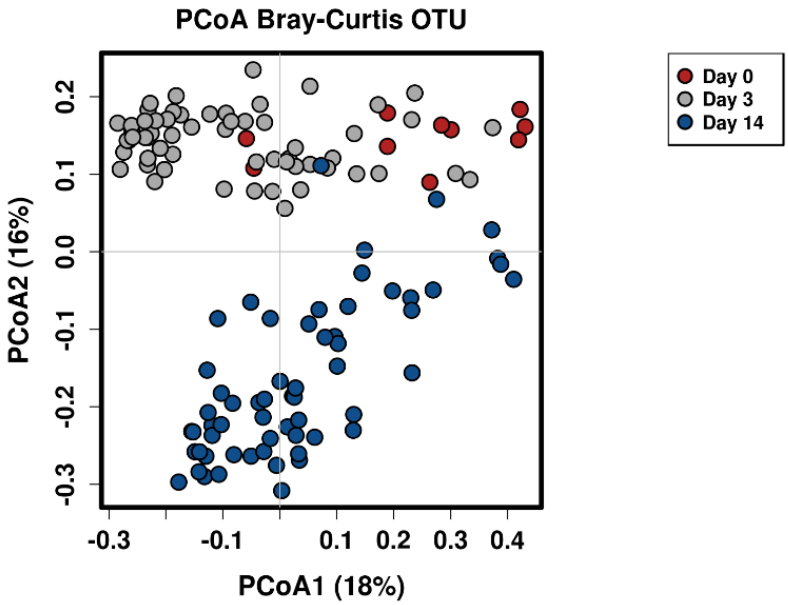

Figure 3. PCoA on microbial communities of digestive gland of clams exposed for 3 and 14 days to different sediments in the first experiment.

PCoA was also performed within each sampling time to investigate microbiota changes occurring in different treatments (Fig.4A, 4B). At the day 3, a weak separation among different treatments was detected. In particular, clams exposed to the Sediment VI (control) were separated along both axis from other treatments. Noteworthy, other treatments appear to be distributed along x-axis according to the sampling site and geographical coordinates. At the day 14, separation of control group (VI) from other treatments has been maintained, while clusters of samples belonging to other treatments have not been observed, excepted for a little more evident clusterization of clams exposed to Sediment I.

PCoA was also performed considering microbial communities of sediments collected after 7 days (Fig.4C). This analysis showed that microbial communities of Sediment I and Sediment VI are clearly separated from other treatments. This result partially reflects the results obtained for digestive gland microbiota at day 14.

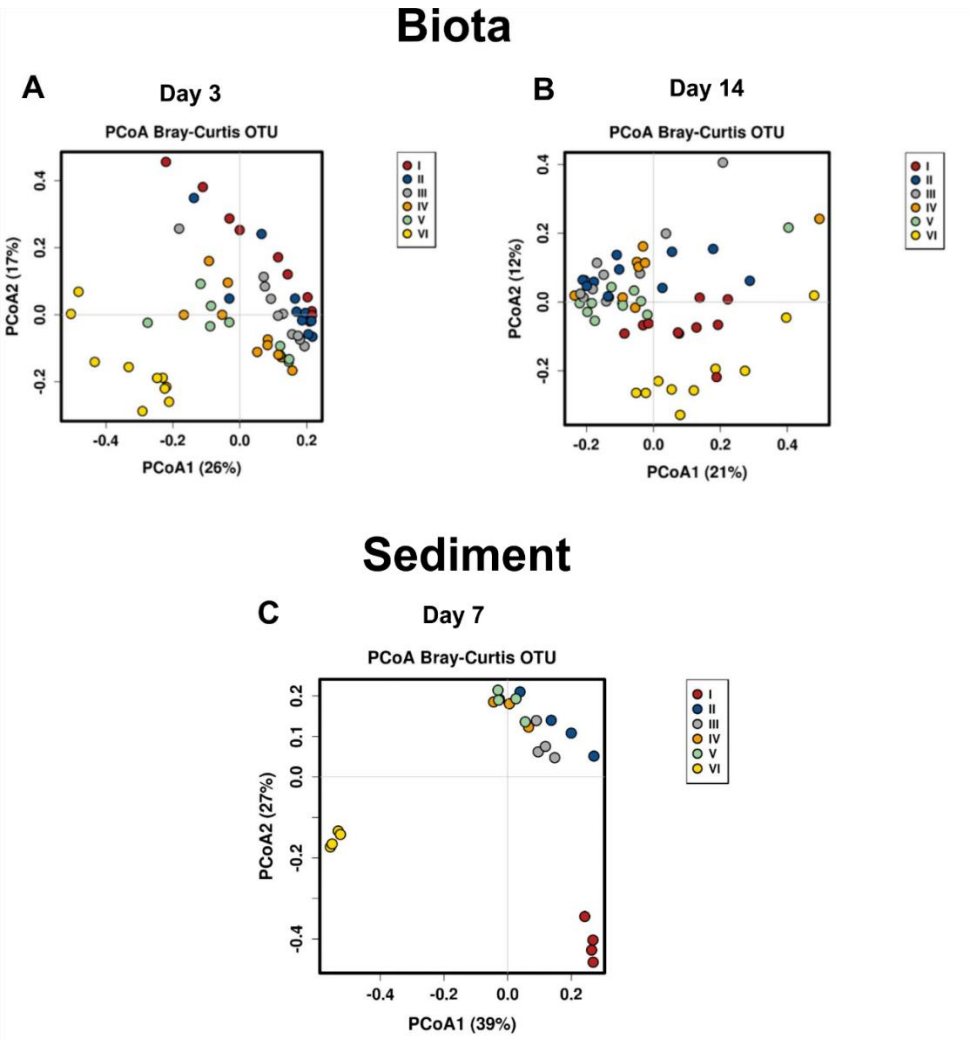

Figure 4. PCoA of microbial communities in digestive gland of clams sampled after 3 (A) and 14 days (AB) and in the sediment sampled after 7 days from the start of the experiment (C).

Microbial diversity (Richness and Shannon's Index) and equitability (Evenness Index) did not highlight any significant differences in clams neither at 3 nor at 14 days of exposure (data not shown), while, on the contrary, significant differences were found in sediments (Fig.5). In detail, the Richness and the Shannon's Index are clearly higher in the Sediment I compared to the other groups, while the Evenness Index showed lower value in the Sediment VI compared to the other groups (except for Sediment II).

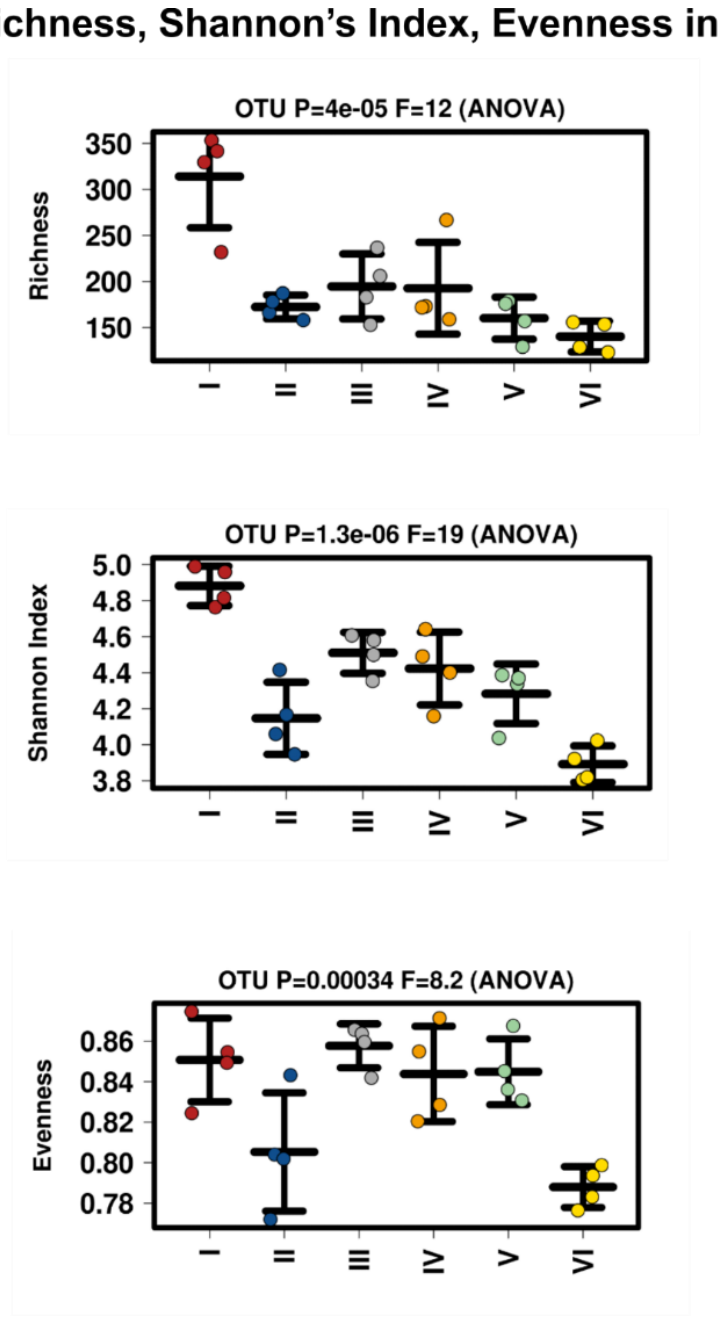

Figure 5. Richness, Shannon's Index and Evenness of microbial communities in sediments after 7 days of exposure.

In addition, as for gene expression analyses, the pairwise comparison was performed between clams of the control group (Sediment VI) and clams exposed to the different sediments collected in the Vittorio Emanuele III canal in order to evaluate the number of significant over- and under- represented taxa in the different treatments at genus and species level. Results are summarized in Table 4, while full lists of significant taxa in digestive gland and sediments are reported in Supplementary File 3 and Supplementary File 4, respectively.

Table 4. Number of significant taxa (FDR ≤ 0.05) in clams and sediment at different sampling times. ↑ indicates the taxa over-represented in each treatment/sediment compared to the control. ↓ indicates the taxa under-represented in each treatment/sediment compared to the control.

	Biota (FDR ≤ 0.05)											
	Day 3						**Day 14**					
	Genus			***Species***			***Genus***			***Species***		
	TOT	↑	↓	**TOT**	↑	↓	**TOT**	↑	↓	**TOT**	↑	↓
Sediment I	**18**	10	8	**23**	11	12	**9**	5	4	**10**	5	5
Sediment II	**22**	10	12	**26**	19	7	**12**	4	8	**21**	16	5
Sediment III	**24**	13	11	**26**	19	7	**15**	10	5	**21**	16	5
Sediment IV	**18**	11	7	**27**	19	8	**2**	0	2	**10**	4	6
Sediment V	**12**	5	7	**22**	12	10	**11**	6	5	**17**	12	5

	Sediment (FDR ≤ 0.05)					
	Day 7					
	Genus			***Species***		
	TOT	↑	↓	**TOT**	↑	↓
Sediment I	**91**	57	34	**107**	70	37
Sediment II	**45**	22	23	**67**	36	31
Sediment III	**64**	37	27	**50**	28	22
Sediment IV	**43**	22	21	**43**	23	20
Sediment V	**40**	17	23	**36**	18	18

First, as showed in table 4, number of differentially represented taxa compared to the control group are similar among treatments. Second, clams of treatments lasted 3 days showed a number of differentially represented taxa higher than clams of treatments lasted 14 days. However, the most important differences have been observed in sediments microbiota, with much higher number of differentially represented taxa. In particular Sediments I and Sediments II showed a total of 107 and 67 differentially represented taxa respectively compared to control (Sediment VI). Thus, comparing microbial changes in the biota and sediment, the lowest number of significant taxa was observed in Manila clam confirming the potential ability of bivalves to control digestive gland microbial communities that appear to be just partially influenced by sediments microbiota.

Moreover, particular attention should be paid to the experimental groups of Sediments IV and Sediment V where the increase of *Vibrio vibrio aesturianus* and Arcobacter genus in digestive gland microbiota of clams, two opportunistic species often related to bivalve mortality events, was observed. Conversely, Manila clams exposed to Sediments I, II and III showed a down-representation of Vibrio genus compared to control at day 3. However, opposite trends have been observed at 14 days in clams treated with Sediment I and II. Noteworthy, regarding the microbiota in sediment, none over-representation of *Vibrio spp* was observed in sediments IV and V, while a down-representation of *Vibrio Vibrio gigantis* species have been observed in Sediment II, III and IV compared to control. Summarizing, these results suggest that increased of *Vibrio aesturianus* observed in clams exposed to Sediment IV could be related to the clams inability to control microbial communities following chemical stress. As aforementioned, data of chemical analyses are crucial for a proper interpretation of microbiota analyses.

6. Conclusions

Results obtained by the study confirmed that Manila clam is a good model to investigate the effects of biotic and abiotic stressors. In particular, gene expression and microbiota characterization analyses suggested a greater potential hazard from sediments dredged at site IV and site V than the others. However, GSEA highlighted the disruption of several molecular pathways in all treatments. The lack of chemical data (due to technical and administrative problems in the laboratory in charge of these analyses) make quite impossible a comprehensive interpretation of the obtained results. The discussion will therefore be completed once such data will be available and will support the subsequent integration of the transcriptomic results in the novel “Transcriptomic Line of Evidence” that will be included in the Weight of Evidence approach for sediment environmental risk assessment.

7. References

Bernardini, I., Matozzo, V., Valsecchi, S., Peruzza, L., Rovere, G.D., Polesello, S., Iori, S., Marin, M.G., Fabrello, J., Ciscato, M., Masiero, L., Bonato, M., Santovito, G., Boffo, L., Bargelloni, L., Milan, M., Patarnello, T., 2021. The new PFAS C6O4 and its effects on marine invertebrates: First evidence of transcriptional and microbiota changes in the Manila clam Ruditapes philippinarum. Environ. Int. 152, 106484. https://doi.org/10.1016/j.envint.2021.106484

Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13 (7), 581–583. https://doi.org/10.1093/molbev/mst010

Chapman, P.M., Wang, F., Caeiro, S.S., 2013. Assessing and managing sediment contamination in transitional waters. Environ. Int. 55, 71–91. https://doi.org/10.1016/j.envint.2013.02.009

d'Errico, G., Nardi, A., Benedetti, M., Mezzelani, M., Fattorini, D., Di Carlo, M., Pittura, L., Giuliani, M.E., Macchia, S., Vitiello, V., Sartori, D., Scuderi, A., Morroni, L., Chiaretti, G., Gorbi, S., Pellegrini, D., Regoli, F., 2021. Application of a Multidisciplinary Weight of Evidence Approach as a Tool for Monitoring the Ecological Risk of Dredging Activities. Front. Mar. Sci. 8, 765256. https://doi.org/10.3389/fmars.2021.765256

DelValls, T.A., Andres, A., Belzunce, M.J., Buceta, J.L., Casado-Martinez, M.C., Castro, R., Riba, I., Viguri, J.R., Blasco, J., 2004. Chemical and ecotoxicological guidelines for managing disposal of dredged material. TrAC Trends Anal. Chem. 23, 819–828. https://doi.org/10.1016/j.trac.2004.07.014

Eggleton, J., Thomas, K.V., 2004. A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. Environ. Int. 30, 973–980. https://doi.org/10.1016/j.envint.2004.03.001

Gerstner, J.R., et al., 2016. Removal of unwanted variation reveals novel patterns of gene expression linked to sleep homeostasis in murine cortex. BMC Genomics 17, 727. https://doi.org/10.1186/s12864-016-3065-8.

Katoh, K., Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780. https://doi.org/10.1093/molbev/mst010.

Iannello M., Mezzelani M., Dalla Rovere G., Patarnello T., Ciofi C., Carraro L., Boffo L., Ferraresso S., Babbucci M., Mazzariol S., Centelleghe C., Cardazzo B., Carrer C., Varagnolo M., Nardi A., Pittura L., Benedetti M., Fattorini D., Regoli F., Ghiselli F., Gorbi S., Bargelloni, L., Milan, M., 2021. Long-lasting effects of chronic exposure to chemical pollution on the hologenome of the Manila clam. Mol. Ecol. (submitted for publication).

Iori, S., Rovere, G.D., Ezzat, L., Smits, M., Ferraresso, S.S., Babbucci, M., Marin, M.G., Masiero, L., Fabrello, J., Garro, E., Carraro, L., Cardazzo, B., Patarnello, T., Matozzo, V., Bargelloni, L., Milan, M., 2020. The effects of glyphosate and AMPA on the mediterranean mussel Mytilus galloprovincialis and its microbiota. Environ. Res. 182, 108984. https://doi.org/10.1016/j.envres.2019.108984

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J.P., Tamayo, P., 2015. The Molecular Signatures Database Hallmark Gene Set Collection. Cell Syst. 1, 417–425. https://doi.org/10.1016/j.cels.2015.12.004

Milan M, Carraro L, Fariselli P, Martino ME, Cavalieri D, Vitali F, Boffo L, Patarnello T, Bargelloni L, Cardazzo B. (2018). Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. Aquatic Toxicology 194:195-207.

Milan M, Matozzo V, Pauletto M, Di Camillo B, Giacomazzo M, Boffo L, Binato G, Marin MG, Patarnello T, Bargelloni L. (2016). Can ecological history influence response to pollutants? Transcriptomic analysis of

Manila clam collected in different Venice lagoon areas and exposed to heavy metal. Aquatic Toxicology 174:123-33.

Milan M, Pauletto M, Boffo L, Carrer C, Sorrentino F, Ferrari G, Pavan L, Patarnello T, Bargelloni L. (2015). Transcriptomic resources for environmental risk assessment: a case study in the Venice lagoon. Environmental Pollution 197:90-98.

Milan M, Ferraresso S, Ciofi C, Chelazzi G, Carrer C, Ferrari G, Pavan L, Patarnello T, Bargelloni L. (2013). Exploring the effects of seasonality and chemical pollution on the hepatopancreas transcriptome of the Manila clam. Molecular Ecology 22(8):2157-72.

Milan M, Coppe A, Reinhardt R, Cancela LM, Leite RB, Saavedra C, Ciofi C, Chelazzi G, Patarnello T, Bortoluzzi S, Bargelloni L. (2011). Transcriptome sequencing and microarray development for the Manila clam, Ruditapes philippinarum: genomic tools for environmental monitoring. BMC Genomics 12:234.

Peruzza, L., et al., 2020. A comparative synthesis of transcriptomic analyses reveals major differences between WSSV-susceptible Litopenaeus vannamei and WSSVrefractory Macrobrachium rosenbergii. Develop. Comp. Immunol. 104, 103564. https://doi.org/10.1016/j.dci.2019.103564.

Pradhan, P.K., et al., 2020. Molecular insights into the mechanisms of susceptibility of Labeo rohita against oomycete Aphanomyces invadans. Scientific Rep. 10, 19531.

Robinson, M.D., et al., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi. org/10.1093/bioinformatics/btp616.

Stefani, F., Casatta, N., Ferrarin, C., Izzotti, A., Maicu, F., Viganò, L., 2018. Gene expression and genotoxicity in Manila clam (Ruditapes philippinarum) modulated by sediment contamination and lagoon dynamics in the Po river delta. Mar. Environ. Res. 142, 257–274. https://doi.org/10.1016/j.marenvres.2018.10.010

Verma, D.K, Peruzza, L., Trusch, F., Yadav, M.K., Ravindra, Shubin, S.V., Morgan, K.L., Mohindra, V., Hauton, C., Van West, P., Pradhan, P.K., Sood, N., 2020. Transcriptome analysis reveals immune pathways underlying resistance in the common carp Cyprinus carpio against the oomycete Aphanomyces invadans.

Genomics 28;S0888-7543(20)31987-X. doi: 10.1016/j.ygeno.2020.10.028.

Zakrzewski M, Proietti C, Ellis JJ, Hasan S, Brion M-J, Berger B, Krause L. Calypso: A user-friendly web-server for mining and visualizing microbiome-environment interactions (2017). Bioinformatics 33:782–3. PMID:28025202.