# Transcriptomic responses of Antarctic clam *Laternula elliptica* to nanoparticles, at single and combined exposures reveal ecologically relevant biomarkers

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## ABSTRACT

In recent years micro- and nanoplastics and metal-oxide nanomaterials have been found in several environmental compartments. The Antarctic soft clam *Laternula elliptica* is an endemic Antarctic species having a wide distribution in the Southern Ocean. Being a filter-feeder, it could act as suitable bioindicator of pollution from nanoparticles also considering its sensitivity to various sources of stress. The present study aims to assess the impact of polystyrene nanoparticles (PS-NP) and the nanometal titanium-dioxide (n-TiO$_2$) on genome-wide transcript expression of *L. elliptica* either alone and in combination and at two toxicological relevant concentrations (5 and 50 μg/L) during 96 h exposure. Transcript-target qRT-PCR was performed with the aim to identify suitable biomarkers of exposure and effects. As expected, at the highest concentration tested, the clustering was clearer between control and exposed clams. A total of 221 genes resulted differentially expressed in exposed clams and control ones, and 21 of them had functional annotation such as ribosomal proteins, antioxidant, ion transport (osmoregulation), acid-base balance, immunity, lipid metabolism, cell adhesion, cytoskeleton, apoptosis, chromatin condensation and cell signaling. At functional level, relevant transcripts were shared among some treatments and could be considered as general stress due to nanoparticle exposure. After applying transcript-target approach duplicating the number of clam samples, four ecologically relevant transcripts were revealed as biomarkers for PS-NP, n-TiO$_2$ and their combination at 50 μg/L, that could be used for monitoring clams' health status in different Antarctic localities.

## 1. Introduction

The Antarctic continent and surrounding waters are currently protected by internationals laws of the Antarctic Treaty and since the late 90 s, the Committee for Environmental Protection (CEP) has increased the efforts to develop management tools for environmental impact assessment, monitoring and marine pollution. Despite such efforts, Antarctica is currently under threat from increasing anthropogenic pressures. A number of studies have shown that plastic pollutants as macro- (>10 mm) and meso-sized (1–10 mm) plastics are present in the

Southern Ocean waters (Lacerda et al., 2019; Suaria et al., 2020), as well as microplastic (Isobe et al., 2017; Lacerda et al., 2019; Waller et al., 2017a). Further evidence of microplastics in Antarctica is represented by recent records in sediments of the Ross Sea (Munari et al., 2017) and Antarctic sea ice (Kelly et al., 2020), revealing the availability of these CEC (Contaminants of Emerging Concern) for the trophic web, including organisms as the Antarctic krill. Dawson et al. (2018) demonstrated that Antarctic krill can digest microplastic particles into nanoplastics, enhancing the transfer of such pollutants along trophic webs. Nanoplastic debris has been recorded in Antarctic sea ice with 52.3 ng/mL average concentration, demonstrating that the occurrence of plastic at nanosized scale in Antarctic ice and water is now real (Materić et al., 2022). In terms of ecotoxicological risks for Antarctic marine life, invertebrates, such as filter-feeders and bottom grazers, are probably the most exposed to the fraction of plastic litter accumulating in sediments, as shown by microplastics found in specimens from the Ross Sea (Bergami et al., 2023; Sfriso et al., 2020).

Metal-oxides nanoparticles belonging to CEC have barely been investigated in Antarctic marine species. Their presence has yet to be demonstrated in Antarctic ice and waters, but they are likely to be present as a result of the increasing activities of scientific stations, tourism, and fishery. In other latitudes, as temperate areas, the most commercially common titanium dioxide in the nanoscale form ($n-TiO_2$) has been detected at concentrations ranging from 20 to 900 µg/L in Mediterranean surface waters (Labille et al., 2020). Sunscreen and cosmetic products, as well as post-consumer material are likely to deliver metal-oxide nanomaterials into marine coastal waters (Haynes et al., 2017). We anticipate that the presence of nanoscale material in the Southern Ocean is highly possible, reaching this region by transport from other oceans and by human activities in Antarctica. Their toxicity toward benthic filter-feeding occupying an important level in the trophic network deserves further exploration.

The effect of nanoplastics and metal-oxide nanomaterials should be considered in marine Antarctic biota as previously performed in other regions of the world (Trevisan et al., 2022). Furthermore, their combined potential synergistic effects deserve to be explored in marine Antarctic metazoans that play a significant ecological role in the marine trophic webs. It is suggested that Antarctic filter-feeding mollusks are among the most sensitive marine metazoans (Peck, 2018), hence species such as the Antarctic soft clam *Laternula elliptica* can be considered as target organisms in coastal areas.

South Shetland Islands, such as King George Island, are constantly impacted by human activities, mainly associated with scientific bases, tourism and fishery vessels, thus it is crucial to assess the impact of nanopollutants on this filter feeder species. As many other marine bivalves worldwide (Li et al., 2019), *L. elliptica* can be elected a good sentinel of anthropogenic pollution in Antarctic marine coastal areas thanks to: its wide distribution in the Southern Ocean, occurring at high densities; its filtering activity of large seawater volumes, leading to accumulation of (micro/nano)pollutants; its trophic level; its easy sampling and organs dissection (Clark et al., 2010; Vodopivez et al., 2015; Waller et al., 2017b; Passos et al., 2022). Within marine mollusks, gills and digestive glands are the most affected target organs by CEC exposure, with histopathological alterations and inflammatory response observed (Jeyavani et al., 2022; Teng et al., 2021). Gills in particular play an essential role in feeding and respiration as a first barrier with the external environment, thus more exposed to nanoplastics and metal-oxide nanomaterials present in seawater (Shao et al., 2021; Zhou et al., 2022).

The potential for using RNA sequencing (RNA-seq) to study complex responses of non-model organisms to environmental pressure is evident in a rapidly growing body of literature (Oomen and Hutchings, 2017). The high dimensionality of transcriptomic responses enables their use as highly specific fingerprints of exposure, and these can be used to diagnose environmental stress (Reid and Whitehead, 2016). In addition, molecular biomarkers can be revealed with this approach, such as

biomarkers found in gastropods exposed to cadmium (Gu et al., 2019). Transcriptomic analysis can not only screen the ecotoxicity of CEC but also infer the function and specific regulatory mechanism of the corresponding unknown genes. Interestingly, Gardon et al. (2020) demonstrated a set of dose-specific transcriptional biomarkers in oysters exposed to microplastics involved in detoxification process, oxidative stress damage and immunity.

Transcriptional response to different stressors has been previously addressed in the Antarctic soft clam *L. elliptica*, including responses to shell damage (Clark et al., 2010; Sleight et al., 2015), injury and starvation (Husmann et al., 2014), and thermal stress (Truebano et al., 2010). This clearly validated *L. elliptica* as a good indicator for stress exposure, making this mollusk an ideal candidate to be a sentinel species in the Southern Ocean. Nevertheless, responses to CEC exposure have not yet to be addressed in this species. We hypothesized that CEC such as PS-NP and $n-TiO_2$ short exposures may provoke gene expression alterations, affecting important eco-physiological functions, with special effects with the exposure of these both nanopollutants acting at same time. We also anticipate the identification of value biomarkers of these short exposures (96 h) belonging to different genes functions. Among possible transcriptomic biomarkers, we could suggest those frequently found affected by nanoparticles, such as antioxidant, cytoskeleton, vesicular traffic, ion transport, metabolism and immunity, among others. In contrast to other model organisms, This is the first evaluation of synergetic effects of two types of nanoparticles in a marine metazoan. The aim of this work was to study the transcriptomics response to CEC (PS-NP and $n-TiO_2$) at single and synergetic combined exposures in gill tissues of *L. elliptica*, expanding the analysis to identify valuable and early stage biomarkers of nano-pollutants to concentrations in line with previous studies (Al-sid-cheikh et al., 2018; Sun et al., 2016).

## 2. Methods

Sampling Antarctic soft clams: a total of 120 *L. elliptica* adult (average shell length of 71.66±11.75 cm, mean ± standard deviation, SD) was collected by SCUBA diving in Fildes Bay, King George Island (South Shetland Islands), in January 2020 (Fig. 1).

### 2.1. Preparation of nanoparticles suspension for experimental exposure

Carboxyl-modified PS-NP (PS-COOH NP), with a nominal size of 62 nm, were purchased from Bangs Laboratories Inc. (catalog code: PC02N, suspension at 10.1 %), while $n-TiO_2$ was kindly provided by Degussa Evonik as Aeroxide ® P25 (powder composed of 82–18 % anatase - rutile crystal structure) with a nominal size of 25 nm. From the PS-COOH NP stock suspension, an intermediate suspension was prepared in MilliQ water at the concentration of 10 mg/mL and then two working solutions of 250 and 25 µg/mL were obtained by dilution, sonicating the suspensions using the bath sonicator DENTSPLY Ultrasonik 57 H for 2 min and vortexing them prior to use as described by (Bergami et al., 2020). The two working suspensions at 250 and 25 µg/mL were used in laboratory testing by adding 1 mL of each suspension to 5 L tanks of natural sea water (NSW) to achieve final concentrations of 50 and 5 µg/L, respectively.

As for $n-TiO_2$, a stock suspension (10 mg/mL) was prepared in MilliQ water, vortexed and sonicated for 60 minutes, always keeping the suspension in cold water, in order to not produce damage of nanometals-oxides (changing water each ~ 10 min) (Della Torre et al., 2015). Two intermediate suspensions were then prepared at 250 and 25 µg/mL, and 1 mL of each was added to 5 L tanks containing NSW to reach concentrations of 50 and 5 µg/L, respectively. Tested suspensions were replaced every 24 h for a semi-static *in vivo* exposure. The NSW used for the experiments (temperature of 0±1 °C, pH of 8.42 and salinity of 32.8.) was taken from Fildes Bay where the specimens where also collected.

Secondary characterization of PS-COOH NP (at 50 µg/mL, following
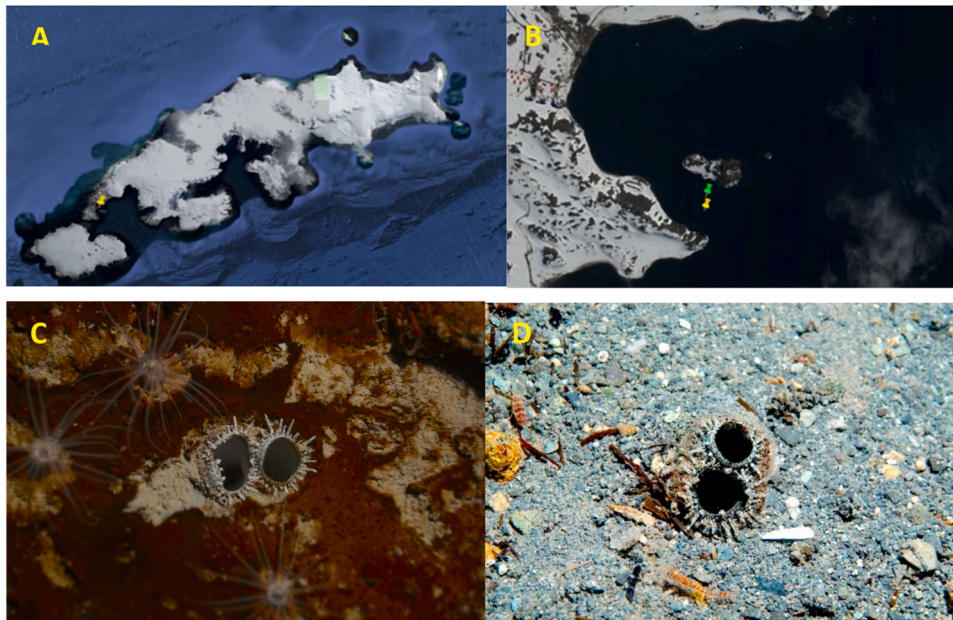
**Fig. 1.** Sampling points where *Laternula elliptica* clams were collected on King George Island (A) at Fildes Bay (B). The points correspond to geo-referential co-ordinates 62° 12' 17.32'' S, 58° 56' 56.48'' W and 62° 12' 19.34'' S, 58° 56' 57.0052'' W (C-D) Pictures of *L. elliptica* individuals in their natural environment (photos from Dr. Ignacio Garrido).

Bergami et al., 2019, 2020) and n-TiO$_2$ (at 5 μg/mL, based on Della Torre et al., 2015) in NSW was carried out by Dynamic Light Scattering and electrophoretic mobility. Such high concentrations were used only to run DLS analyses, in line with previous studies, due to limitation of the instrument to characterize particles below 1 μg/mL. For this purpose, NSW was first filtered at 0.20 μm to allow the removal of large suspended natural colloids that would interfere with the analysis. Nanoparticle size-related parameters (e.g., Z-average, expressed in nm) and surface charge (ζ-potential, expressed in mV) evaluations of nanoparticles resulted from 3 independent measurements and were obtained at 0 °C (Z-average) and 4 °C (ζ-potential).

### 2.2. Experimental design

After 2 days of acclimation of the specimens in well oxygenated NSW with constant temperature, pH and salinity, two independent experiments (96 h) were carried out. Individuals (n = 5) were placed in 5 L tanks with the following test solutions: NSW only (control), PS-COOH NP at 5 and 50 μg/L, n-TiO$_2$ at 5 and 50 μg/L and combined exposure (PS-COOH NP and n-TiO$_2$) at 5 and 50 μg/L. The 5 LTanks were permanently well oxygenated and specimens were mantained with a light/obscurity intervals photoperiod of 18/6 H. After 96 h, clams were dissected for tissue extractions (gills), frozen with liquid nitrogen, put in 1 mL of RNAlater and kept at −80 °C.

### 2.3. RNA isolation, library preparation and Illumina NovaSeq sequencing

Eight gill tissues for each treatment, between 15 and 30 mg, were weighed on an analytical balance, and RNA isolated using E.Z.N.A. ® Total RNA Kit I (Omega Bio-Tek), following the protocol instructions. The RNA concentrations of each sample were measured with Tecan infinite m200 pro spectrophotometer. Four sample replicates by conditions (n = 32) were used for library preparation (with TrueSeq Stranded mRNA Kit; Illumina) and Illumina NovaSeq sequencing in DNALink Company (San Diego, United States).

### 2.4. Bioinformatic analyses

32 paired-end libraries were obtained from Illumina NovaSeq 6000

sequencing (four biological samples by conditions). The raw sequences were analyzed under Galaxy Platform ABIMS from Station Biologique de Roscoff (https://galaxy.sb-roscoff.fr/) and from Institut Française de Bio-informatique (https://usegalaxy.fr), until finding the differentially expressed transcripts between controls and treatments. Firstly, the raw sequences were checked for quality, using a phred score > 30 as criteria. The 9 first nucleotides and last one did not pass this criterion. For these reasons, the first 9 nucleotides and the last one of all raw reads were trimmed with "Trim sequences" program Galaxy Version 1.0.2. Then, a reference transcriptome was assembled using all libraries with Trinity, followed by ortholog regrouping to reduce functional redundancy using with CDHit and the final transcripts were annotated with the Trinotate workflow. Completeness and redundancy degree were assessed with BUSCO software. Salmon was used to count reads using the assembled reference transcriptome as input, with the relative orientation of reads within a pair "Inward", strandedness "U" (not stranded) and type of index "quasi". The TPM normalized counts were used to generate expressions matrixes matrices of transcript expression among experimental conditions. Then, these matrixes were used to obtain the differentially expressed genes (DEG) at isoform level using DEseq2 software, considering DEG with a log2 (fold change) > 1.5 (fold-change>3) and false discovery rate < 0.05.

### 2.5. qRT-PCR amplification

cDNAs were synthesized from RNA samples, using M.MLV reverse transcriptase, reaction buffer RNAses-free, oligo(dt)$_{20}$, random hexamers, RNaseOUT™ Recombinant Ribonuclease Inhibitor and dNTP (all these reagents from Thermo-Fisher Invitrogen®), following protocol instructions and using conventional thermocycler SimpliAmp (Applied Biosystems). The amplification reaction cycles were performed according to manufacturer's instructions.The qPCRs primers were designed for all differentially expressed genes with functional annotation and the reference genes, using "Expasy translate" (https://www.expasy.org/) software to verify 5'→ 3' sense and "Amplifx" to find the best primers for each sequence. The primer design criteria were: 1) a length between 18 and 24 bp, 2) with a melting temperature difference no higher than 1 °C, 3) qPCR product length between 90 and 200 bp, 4) GC percent superior to 45 %. The "oligo Calc" software (http://biotools.nubic.northwestern.

edu/OligoCalc.html) was used to assess the melting temperature and confirm that no fork nor dimer formation occurs. Primer sequences are given in Table 1(Supplementary File 1). A conventional gradient PCR was performed for each pair of primers designed with 200 nM concentration, from 54.8 to 64.6, to determine the optimal temperature. The PCR program was: 1) hot start at 95 °C for 10 min; 2) 40 cycles with 95 °C for 30 s, the temperature gradients for 1 min, and finalize with 72 °C for 30 s. Then, the melting curve was produced with real-time thermocycler AriaMx (Agilent) to choose the best unique peak (taking the highest). The melting curve program was (0.5 °C of resolution): 1) 95 °C for 1 min; 2) 30 seconds at 55 °C and 3) 30 s at 95 °C. After this, an electrophoresis in agarose gel (1.7 %) was performed to ensure that only one transcript is amplified. Once the primers were validated at the best temperature, a qRT-PCR was performed with eight samples by conditions, With the Brilliant II SYBR® Green QPCR Master Mix, following product protocol instructions. Finally, relative expressions for eight samples per condition of each transcript were calculated following the $2^{-\Delta\Delta Ct}$ of Livak and schmittgen (2001), normalized with the reference transcripts "NADH deshydrogenase (Ubiquinone) 1 subunit beta subcomplex 8", recorded as stably expressed with RNAseq data. This taking into account a ratio between TPM average for all 32 samples and Standard Deviation, in order to choose the least variable with a high gene expression level in our RNAs samples. The gene expression log 2 (fold change) results were correlated with qRT-PCR log2 (fold change) ones for the same transcripts, and using the same individuals, in order to validate the RNAseq approach. Shapiro test was performed to evaluate the normal distribution, and this failed using afterward the Spearman non-parametric test to validate correlation, being < 0.05 considered a significant correlation and the Rho coefficient as degree of correlation.

### 2.6. Statistical analyses

Shapiro test and Bartlett test were performed to assess the normal distribution and homoscedasticity respectively using Rstudio scripts. According to these results t-test (parametric) or Mann-Whitney (non-parametric) were performed between the relative expression of control and treatment condition using GraphPad Prism 5, considering a 95 % of confidence (p-value < 0.05).

### 2.7. Data availability

Raw Illumina NovaSeq 6000 paired-end reads are available in the NCBI BioProject PRJNA962035 (http://www.ncbi.nlm.nih.gov/bioproject/962035).

### 3. Results and discussion

### 3.1. Nanoparticles behaviour

The characterization analysis confirmed the agglomeration behaviour of n-TiO$_2$ in high ionic strength media, with an average hydrodynamic size of 872.9 ± 73.8 nm in NSW and a weak negatively surface

**Table 1**
Sequencing, filters, alignments and assembly statistics of the present study using 32 paired-ends samples.

| Reads | |
|---|---|
| Raw Reads | 975 727 394 |
| Aligned and counted reads (%) | 91.48 |
| **Assembly** | |
| Total trinity transcripts | 603954 |
| Total trinity transcripts After CD-Hit | 251294 |
| **Assembly statistics After CD-Hit** | |
| N50 | 955 |
| **BUSCO statistics After CD-Hit** | |
| Total completeness (%) | **99.4** |

charge (–11.7 ± 0.7 mV). We previously reported instability and large agglomeration of n-TiO$_2$ (25 nm, anatase, from Sigma-Aldrich) in Antarctic rock pool waters (González-aravena et al., 2022) and our results are in line with previous studies in seawaters for this NP (reviewed in Corsi et al., 2020, 2021). For example, Della Torre et al. (2015) reported the formation of micrometric agglomerates (~970 nm) in artificial sea water for the same n-TiO$_2$ (Aeroxide© P25) at 10 μg/mL. Similarly, as far as PS-COOH NP, a large agglomeration (average size of 752.7 ± 137 nm) and a negative surface charge (-25.2 ± 1.8 mV) were observed in Antarctic NSW. Size-related parameters largely differ from our previous findings for this NP in seawater collected from the same coastal area (Bergami et al., 2019), in which we showed only slight initial agglomeration (173 ± 21 nm) compared to NP nominal size (50 nm). Such difference could be attributed to the different batch of NP used (Bangs Laboratories Inc. in the present study *vs* Invitrogen in Bergami et al., 2019) or to the different composition/abundance of natural colloids present in the NSW. Our results highlight the need to apply characterization analysis on a case-by-case study in nano-ecotoxicological studies (Corsi et al., 2021, 2020).

### 3.2. Sequencing and analyses results

The present genome-wide transcriptomic (RNAseq) analysis yielded 975,727,394 paired-end reads from 32 *L. elliptica* gills (four samples by experimental condition), having a high-quality data with an average nucleotide phred score of 36.11 and average of percent bases over 30 phred score of 94.75 %. After trinity assembly and CD-Hit orthologs grouping an exhaustive transcriptome was constructed with 251,294 transcripts for the 32 clam individuals with a N50 of 955 bp. A functional transcriptome assessment was performed with BUSCO reporting a high completeness for metazoan transcriptomes (99.4 %) and low duplication rate (7.0 %), having 92.4 single transcripts, only 0.6 % is fragmented and no transcripts missing (0 %) (Table 1). Among the 251,294 transcripts, a potential annotation could be attributed to 26,324 (10.48 %) using the trinotate workflow. After filtering transcripts with an expression level below 10.00 and isoforms below 10 % of representation, the annotation rate increased to 19.72 %.
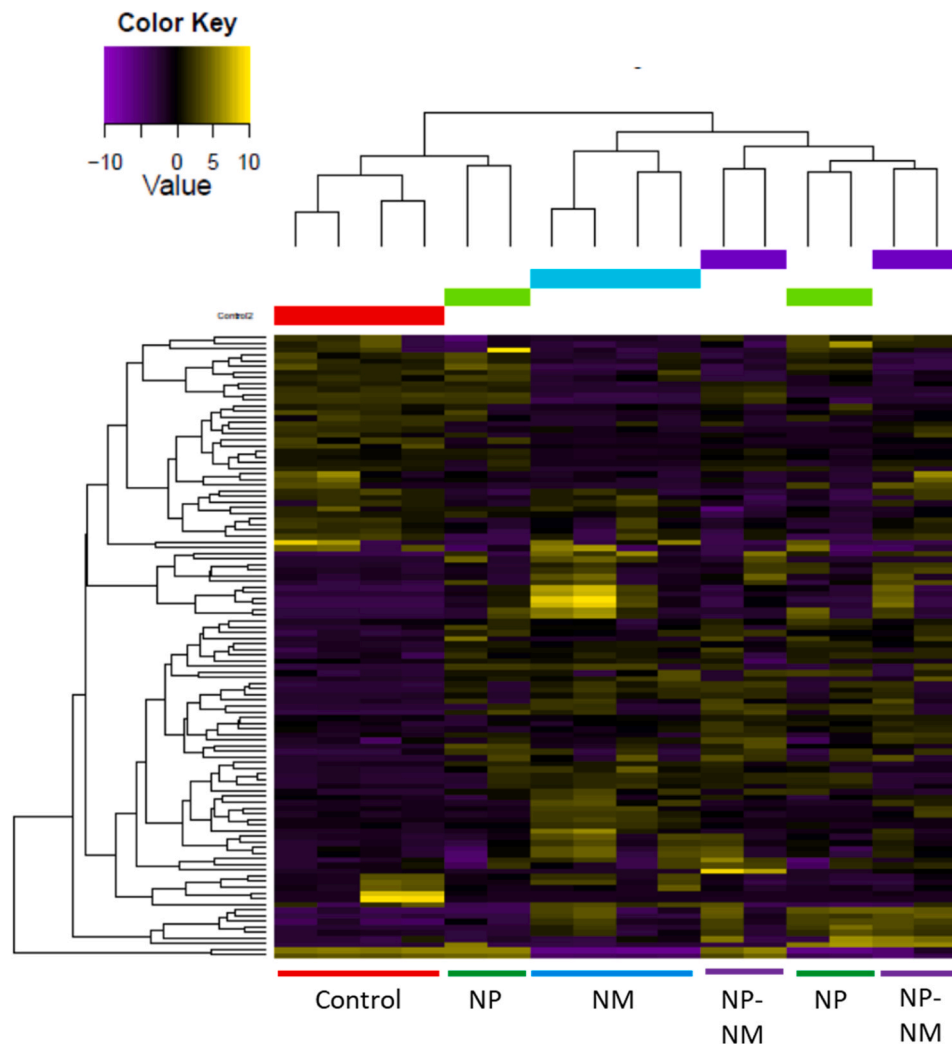
### 3.3. Global RNAseq outcomes

Results from experimental exposure showed the gene expression profiles at the lowest concentration tested of 5 μg/L are shown in (Fig. 2), with control samples clustered apart and only two individuals exposed to PS-COOH NP clustered closer to the control group than with the other 2 individuals of the same condition. On the contrary, specimens exposed to n-TiO$_2$ clustered together while the co-exposed ones were not clearly separated even though they still belonged to the clusters of exposed ones. Similarly, at the highest tested concentration of 50 μg/L, control group clustered separately, but without treatment samples in this case (Fig. 3). The findings of the clustering analysis suggest that a concentration-dependent gene expression profile could be hypothesized for both nanoparticles tested irrespective of the size and core composition (62 *vs* 25 nm, PS-COOH NP *vs* n-TiO$_2$). This dose depending nanoparticles effects has been recorded in others study with bivalve (Gardon et al., 2020), in another hand the heatmaps show a clear effects of nano-contaminants and the RNAseq differentially expressed genes showed relevant transcripts functions modulated by treatments.

This global transcriptomic analysis recorded 25, 56 and 43 DEG for PS-COOH NP, n-TiO$_2$ alone and in combination for the 5 μg/L in comparison with control condition (supplementary file 2). Regarding the highest concentration tested of 50 μg/L, 43, 53 and 45 DEG were detected for PS-COOH NP, n-TiO$_2$ and their combination in comparison to controls (supplementary file 2). The Venn diagram analysis shows a low overlap considering all DEG of each treatment at 5 and 50 μg/L, with only 1 DEG shared among single and combined exposure conditions at 5 μg/L (Figs. 4A) and 4 DEG at 50 μg/L, (Fig. 4B). Furthermore,

**Fig. 2.** Heatmap of Differentially Expressed Genes revealing the clustering of the control group (Control, in red), and treatments exposed to 5 μg/L nanoparticles: PS-COOH NP (NP, in green), n-TiO₂ (NM, in blue) and combined exposure (NP-NM, in violet).

these diagrams also reveal few shared DEG between pairs of treatments, with from 2 to 5 shared DEG at 5 μg/L, and from 1 to 8 shared DEG at 50 μg/L. Within a same treatment, no shared DEG between the two concentrations of PS-COOH NP were found, while five DEG were shared between the two concentrations of n-TiO₂ and one shared DEG when combined (Fig. 4C–E). A more pronounced effect of nanoparticles exposure at higher concentration was found (Fig. 4 A and B), with more shared DEG, also suggesting the concentration-dependent effect on gill transcriptome of *L. elliptica*. Gardon et al. (2020) reported a similar concentration-dependent transcriptomic effect of micro-PS exposure at three concentrations (0.25, 2.5, and 25 μg/L), with different responses recorded mainly for antioxidant and immune genes.

### 3.4. Eco-physiological alterations

Our differential analysis allowed us to identify a total of 221 genes which were differentially expressed when comparing nanoparticles with control conditions. Among them, 21 have been attributed to a potential function after the annotation with the trinotate workflow (supplementary file 3, Table 1). Interestingly, the annotation of these differentially expressed genes (DEGs) converges toward some similar functions depending on the treatment and the concentration tested. Notably, functions related to structural constituent of ribosome, ions and particles transport, cell signaling, oxidative stress and immunity are affected in

response to nanoparticles exposure regardless of size and core composition.

Considering negative effects with down-regulations, the expression of the gene encoding for a 60 S ribosomal protein L23 was repressed by $-2.74$ and $-3.49$ log2 (fold Change) respectively, in response to n-TiO₂ exposure and to both nanoparticles at 50 μg/L (sup file 2 and Table 2 of sup file 3). The effects of silver NP on the expression of genes encoding for ribosomal proteins has previously been reported in different developmental stages of *Chironomus riparius* (Nair et al., 2011). Altogether, our analyses are in line with previous works and they indicate that the expression of genes encoding for structural constituent of ribosome is sensitive to nanoparticles exposure, even at low concentration; this may result in dysregulation in ribosome protein configuration. Other function affected was the down-regulation of the expression of vesicular traffic gene (Ap-4 Complex accessory subunit tepsin, upon n-TiO₂ exposure to 5 μg/L with $-2.29$ log2 (fold Change), as described in zebrafish embryos (Jovanovic et al., 2011). This study showed that cell membrane transporter and vesicle transport transcripts were down-regulated in response to carbon-based nanoparticles (hydroxylated fullerenes at 40 μg/mL), suggesting NP interference in recycling process of vesicular organelles. Also, at the higher dose of n-TiO₂ (50 μg/L), we observed a down-regulation of cytoskeleton function transcript Tubulin alpha-3 chain with $-5.75$ log2 (fold change. Similar results were recorded in mollusks such as the bivalve *Mytilus*
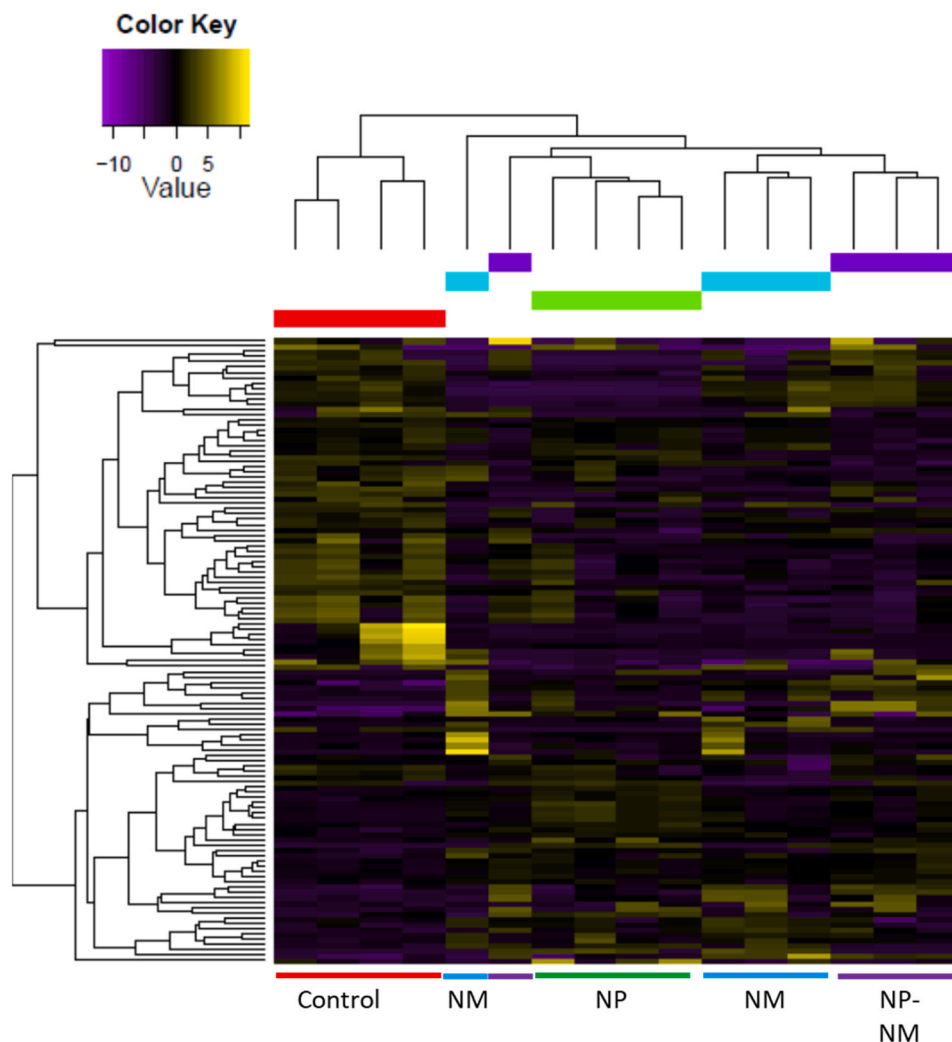
**Fig. 3.** Heatmap of Differentially Expressed Genes revealing the clustering of control group (Control, in red), and treatments exposed to 50 µg/L nanoparticles: PS-COOH NP (NP, in green), n-TiO$_2$ (NM, in blue) and combined exposure (NP-NM, in violet).

*galloprovincialis* with up-regulation of the expression of gene encoding for cytoskeletal transcript in digestive gland after n-TiO$_2$ exposure (Banni et al., 2016).

Regarding immune transcripts, we also recorded the downregulation of expression of a gene annotated as Deleted in malignant brain tumors 1 protein (DMBT1), differentially expressed in response to n-TiO$_2$ at 50 µg/L with −2.63 lof2 (fold change) (sup file 2 and Table 2 of sup file 3). A gene encoding for Tachylectin-5A and its expression is repressed by −1.95 log2 (fold Change) in response to both nanoparticle exposure at 50 µg/L. Effect on immune response genes has been reported in zebrafish gill tissues in response to Fe$_3$O$_4$ NP exposure (Zheng et al., 2018). In accordance to previously published studies, our analysis also highlighted that the expression of the gene which potentially encodes for caspase 8 is down-regulated by 5.17 log2 (fold change) in response to n-TiO$_2$ at the highest exposure concentration of 50 µg/L (shown in Table 2 of sup file 2 and sup file 3). A differential expression of genes involved in the apoptotic pathway in response to NP has been previously reported in the digestive glands of the freshwater clam *Corbicula fluminea* potentially associated with the oxidative stress induced by PS-NP (Li et al., 2021).

Regarding the gene expression in response to nanoparticle exposures, the expression of genes encoding for antioxidant functions were up-regulated: One encodes for a putative ferric-chelate reductase 1 and other encodes for glutathione S-transferase omega-like 2; the expression of both these genes is upregulated by2.17 and 3.35 log2FoldChange

respectively in response to PS-COOH NP at 50 µg/L (sup file 2 and Table 2 of sup file 3). Another transcript encodes for DBH-like monooxygenase protein 1 and other one encodes for a putative Thioredoxin, which plays a key role in cell protection from the detrimental effects of reactive oxygen species (Lee et al., 2013). The expression of these two genes is up-regulated by 1.98 and 22.78 log2 (fold change respectively in response to combined exposure to PS-COOH NP and n-TiO$_2$ at 50 µg/L (sup file 2 and Table 2 of sup file 3). This up-regulation of the expression of oxidative stress responsive genes is concordant with previous studies that observed antioxidant transcripts dysregulation in marine invertebrates, such as in the mussel *Mytilus spp.* in response to PS-NP (Paul-Pont et al., 2016). Antioxidant and xenobiotic responses have been described upon exposure to PS-NP, such as the up-regulation of glutathione S-transferase, in aquatic invertebrates like *Daphnia pulex* (Liu et al., 2021), and the crayfish *Cherax quadricarinatus* (Cheng et al., 2022). Interestingly, translation and antioxidant transcript expression has been altered at 5 and 50 µg/L respectively, similar to what was reported for the swamp crayfish, *Procambarus clarkii*, upon PS-NP orally administered (Capanni et al., 2021).

The expressions of genes coding for ribosomal proteins were also upregulated in response to both nanoparticles. A transcript encoding for a "60 S ribosomal protein L7", is up-regulated by 6.96 and 7.07 log2 (fold change) in response to PS-COOH NP and both nanoparticles exposure at 5 µg/L respectively sup file 2 and Table 2 of sup file 3). This is in line with a previous study on the marine rotifer *Brachionus koreanus*,
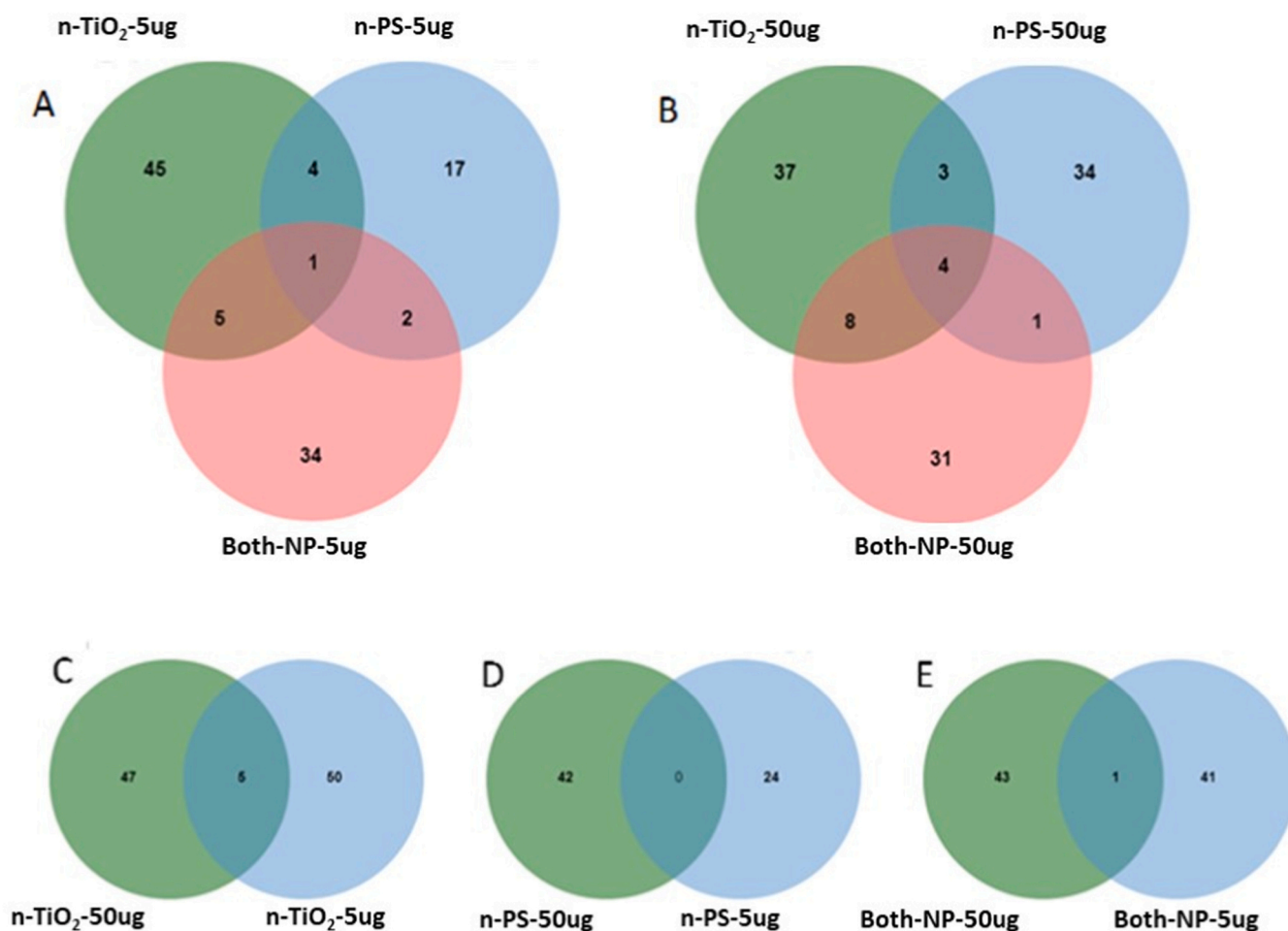
**Fig. 4.** Venn diagrams showing shared Differentially Expressed Genes (DEG) when comparing individuals exposed to control end experimental conditions at 5 µg/L (A) and 50 µg/L (B). The green circles represent the n-TiO2 conditions, the blue ones the PS-COOH NP conditions and the pink ones the co-exposure to both nanoparticles. The overlaps of circles represent the shared DEG. Barplots show DEG numbers by conditions with the same colors of circles. Venn diagrams showing shared transcripts between pair of conditions for n-TiO2 (A), PS-COOH NP (B) and combined exposure (C). Green circles represent the 50 µg/L treatments for each pollutant and the blue ones represent the 5 µg/L treatments. The overlaps of the circles represent the shared transcripts.

where Ribonucleoprotein Complex Biogenesis genes were differentially expressed in response to single exposure to non-functionalized PS-NP of 50 nm at 1 µg/mL (Jeong et al., 2021). Another shared molecular function altered in response to both nanoparticles exposure was related to "ion transport gene Solute Carrier family 23". The expression of two genes encoding for Solute carrier family 23 members were up regulated in response to n-TiO2 alone and in combination at 5 µg/L (7.044 log2 (fold Change)) at both concentrations tested (5 µg/L and 50 µg/L) (6.79 log2 (fold change) and 1.91 log2 (fold change) respectively) (sup file 2 and Table 2 of sup file 3). Considering that these genes are potentially involved in ion transport (Zhang et al., 2017), it could be expected that the osmoregulation process might be affected in response to nanoparticles exposure in gills of *L. elliptica*. Altogether, our work indicates convergent molecular patterns in response to nanoparticles exposure, revealing how which gill cell uptake and transport of ions and particles are affected by nanoparticles exposure. Another transcript potentially involved a lipase function, which was significatively up-regulated in n-TiO2-exposed clams and in those from the combined exposure group at 50 µg/L with 3.67 and 4.01 log2 (fold change) respectively. This could not only affect the production of lipid mediators but also membrane properties, which could be affected during these exposures and compromise cell membrane permeability in gill tissues.

Functions related to cell signaling were also affected in response to NP exposure. The expression of both genes annotated as "alpha-protein kinase vwkA" and "CUB and sushi domain-containing protein 3", were

induced in response to n-TiO2, at 5 µg/L (6.84 and 4.84 log2 (fold change) up-regulation, respectively). At 50 µg/L n-TiO2 exposure, the expression of other signaling gene "TRINITY_DN1668_c0_g1_i4" potentially encoding for a serine/threonine-protein kinase roco5 was downregulated by 4.93 log2 (fold change).

A transcript that encodes for a putative "Barrier to Autointegration Factor" with function related to chromatin condensation for multiple pathways including mitosis, post-mitotic nuclear assembly, intrinsic immunity against foreign DNA, transcription regulation, and the DNA damage response (Sears and Roux, 2020). It is induced by 4.8 log2 (fold change) in response to 50 µg/L n-TiO2 exposure (sup file 2 and Table 2 of sup file 3), being this a novel result. Furthermore, the combined exposure to 5 µg/L revealed the up-regulation of another gene, coding the carbonic anhydrase 9, which catalyzes the reversible hydration of carbon dioxide in the reaction: $CO_2 + H_2O \ H^+ + HCO_3^-$. This transcript is being highly relevant in *L. elliptica* gill function and acid-base homeostasis and possibly implicated in shell biomineralization, being also a novel result. Finally, the significant expression increase in a gene potentially encoding for Sushi von Willebrand factor type A transcript was observed in response to combined exposure to NP at 50 µg/Lwith 6.23 log2 (fold change), being potentially implicated in cell adhesion (Glait-santar et al., 2012).

The alterations in eco-physiologically relevant gene functions as those observed in the present study upon single and combined exposure to PS-COOH NP and n-TiO2 are source of concern. The down-regulation

of immune genes and the up-regulation of antioxidant responses reveal potential detrimental consequences of the ability of clam's gill cells to cope with waterborne nanoparticles exposure. Indeed, such responses at gene level suggest that eco-physiological relevant functions of the Antarctic clams could be compromised from nanoplastics and metal-oxide NP exposure.

### 3.5. Global picture and biomarkers revealed

RNAseq analysis led us to identify DEG that may be relevant transcripts for biomarkers of early NP exposure (96 h) at toxicologically relevant concentrations used in previous studies (Al-sid-cheikh et al., 2018; Sun et al., 2016). The global picture observed in the set of exposures with genome-wide expression evaluation showed *L. elliptica* individuals deployed an antioxidant, osmoregulation and acid-base balance response in gill cells, with a compromised effects mainly in immunity and cytoskeletal and vesicular traffic configuration. We found a statistically significant correlation (p-value = 0.0416, Spearman rank correlation rho = 0.428) of log2 qRT-PCR Fold-Change against log2 RNAseq Fold-Change with all transcripts (of 5 and 50 µg/L DEG detected) (Figs. 5 and 6). We then performed a target transcript qRT-PCR using eight *L. elliptica* samples in 19 relevant genes (Supplementary File 3, Figs. 1–3). Interestingly, at the 5 µg/L exposure, a tendency was observed toward the same results in qPCR as for the RNA-seq analysis for some candidate genes (5 genes out of the 8 tested), AP-4 complex accessory subunit tepsin, Solute carrier family 23 member 2 (for two treatments), Alpha-protein kinase vwkA and Carbonic anhydrase 9 (Table 2 of supplementary file 3) but not to a statistically significant level. This clearly indicates that the consequences of NP exposure at this concentration is mitigated, confirming our previous observation on the clustering of the data at the 5 µg/L. Working with samples obtained after exposure at the highest concentration (50 µg/L), a tendency toward the same results as for the RNA-seq analysis in 12 genes out of the 15 tested was observed (Table 2 of supplementary file 3). The differential expression of four relevant genes at a statistically significant level in the 8 individuals tested was revealed by qPCR. The gene encoding for the transcript "Glutathione S-transferase omega-like 2" had significant relative expression difference (p-value = 0.0379) in response to PS-COOH NP exposure (Supplementary File 3, Fig. 1). This suggests that antioxidant and metabolism of xenobiotic transcript could be suitable

biomarker to indicate the presence of relevant concentrations of PS-NP. We suggest here to further use of this Glutathione transcript as a biomarker in response to PS-COOH NP challenge. Furthermore, we found by qPCR that the expression of the gene related to inflammatory process and protection ("Deleted in malignant brain tumors 1 protein") (p-value = 0.0070) was down-regulated in response to n-TiO$_2$ exposure as for the RNAseq analysis (Supplementary File 3, Fig. 2). We also revealed by qPCR on the 8 individuals the same expression results as for RNAseq for the transcripts annotated as "Tubulin alpha-3 chain" (Supplementary File 3, Fig. 3) in response to n-TiO2 exposure (p-value = 0.0007853) and the transcript annotated as "Solute Carrier Family Member 1" in response to both nano-pollutants (p-value = 0.0085) (Supplementary File 3, Fig. 3). We suggest that these three novel transcripts could be used as valuable novel biomarkers indicating the presence of NP in the environment, including remote regions such as the Southern Ocean.

Only few studies have performed gene expression analyses in aquatic Antarctic invertebrates following exposure to nanoplastics or nanometals (Bergami et al., 2022, 2019; González-aravena et al., 2022), however the combined effects more relevant in terms of realistic exposure scenarios have been overlooked (Corsi et al., 2021, 2020). However, no data of genome-wide transcriptomic analyses are yet available for most of polar organisms, including the endemic Antarctic clams *L. elliptica*. Meanwhile, a size-depending effect of nanoplastics have been proposed, with the smaller ones (< 100 nm, as those used in the present study) associated with higher toxicity (Hao et al., 2023). Similarly, nano-metals have been reported having a size-depending effect, with those having a low nominal size (e.g., 25 nm in the present study) an highest influx rate in micro-invertebrates such as *Daphnia magna*, and augmenting the possibility of toxic effects (Zhao and Wang, 2012). The outcomes of the present study revealed a molecular impact in *L. elliptica* with some gene expression attempt to cope with toxicity such as antioxidant, ion transport (osmoregulation), acid-base balance and lipid metabolism, meanwhile others are affected such as immunity, apoptosis, cytoskeleton and vesicular traffic (Fig. 5). This clearly evidenced that the gill tissue assessed respond and suffer by nano-contaminants of emerging concern, highlighting the importance to perform global transcriptional analysis in Antarctic metazoans. Furthermore, these contaminants are not isolated in nature, but combined the nanoparticles of plastic and metal-oxide, herein the importance and novelty of evaluate
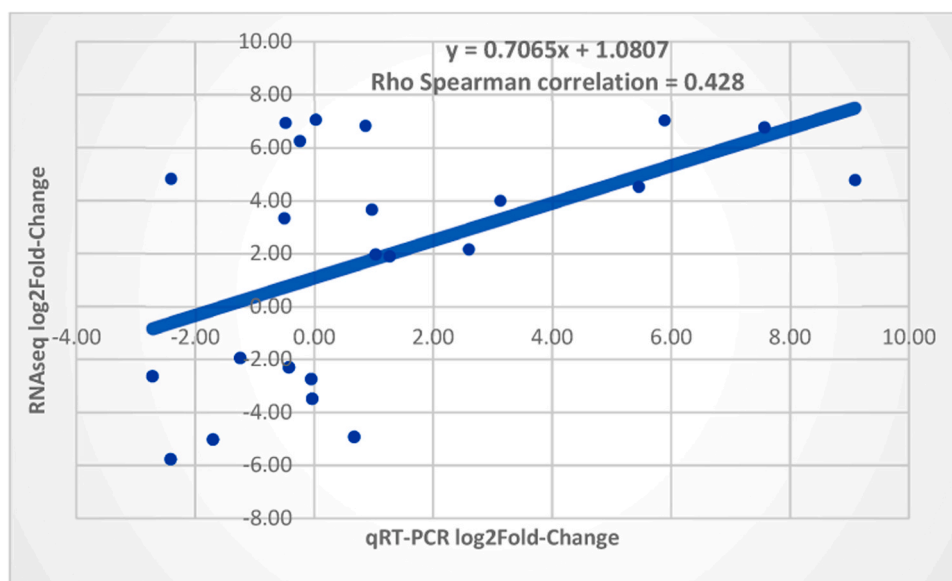


**Fig. 5.** Correlation plot of log2Fold-Change obtained with RNAseq and q-RT-PCR approaches for each transcript differentially expressed in RNAseq analysis. At the top of figure the equation and Spearman Rho correlation factor. The lineal correlation was significative with correlation Spearman Test (the data have not a normal distribution with Shapiro test), with a p-value = 0.042.
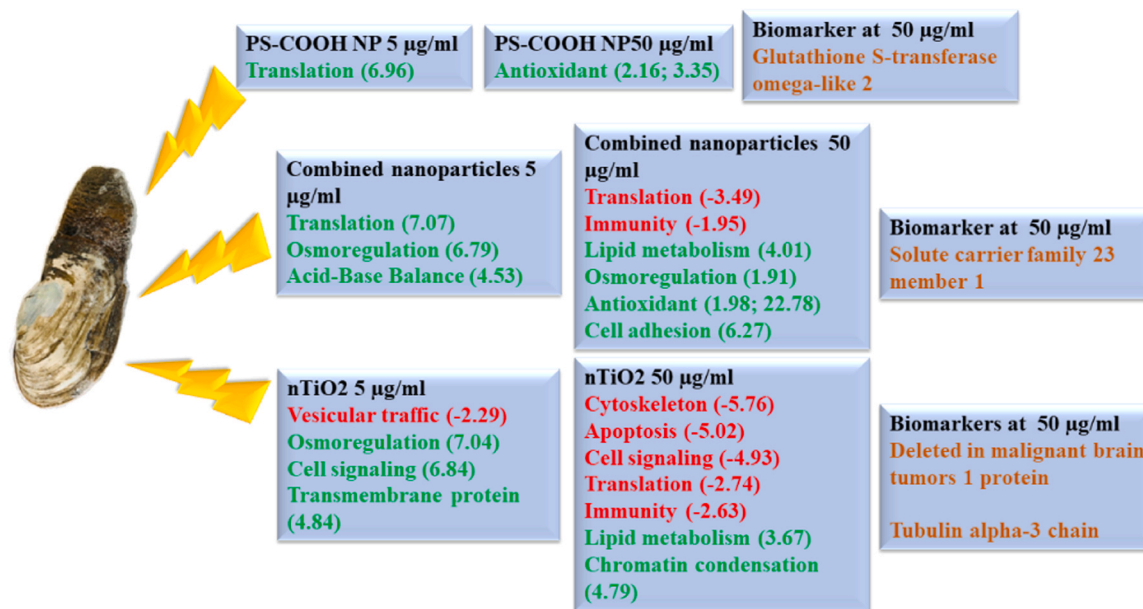
**Fig. 6.** Molecular effects of the exposure to PS-COOH NP and/or n-TiO$_2$ in the Antarctic soft clam *L. elliptica* at 5 and 50 µg/L. Molecular functions with up-regulated transcripts are shown in green and down-regulated ones in red (log2 of fold-change are in parentheses). The molecular biomarkers validated with eight clams are marked in brown.

the combination of the nanoplastic and the nanometal, producing different clam responses and effects in comparison of separate exposures. A strong point of the present study is the short time of exposure used to obtain the early biomarkers to a relevant concentration of nanoparticles (50 µg/L), but with the limitation of what could happen at longer times of exposures as weeks or months, which remains unexplored.

### 3.6. Comparison to other bivalve species

Micro- and nanoparticles exposures in bivalves have some known effects between species. For example, n-TiO$_2$ provoke up-regulation of cytoskeletal genes and ABC transporter in *Mytilus galloprovincialis* (Banni et al., 2016; De La Torre et al., 2015). Regarding cytoskeleton genes this was rather down-regulated by this nano-contaminant in the present study showing a different response in the Antarctic *L. elliptica*. In *Mytilus spp.* species Micro-Polystyrene exposures enhance antioxidant and glutathione-related enzymes in mussel tissues (Paul-Pont et al., 2016), suggesting a response to oxidative stress, similar to PS-COOH-NP exposures in the present study. Also, Micro-Polystyrene produce alteration in detoxification and immune genes in *Pinctada margaritifera* (Gardon et al., 2020), being detoxification gene found also upregulated in the present study by PS-COOH-NP, the GST but immune genes were instead found in n-TiO$_2$-NP exposure. In another hand, PE- and PET-MP inhibited lipid metabolism in the *Crassostrea gigas* (Teng et al., 2021), but this gene function was rather induced but by n-TiO$_2$-NP in the present study. Energy metabolism genes implicated in glycolysis and tricarboxylic acid cycle genes were disrupted after exposures to nZnO, nFe2O3, nCuO, and multi-walled carbon tube (MWCNT) in blood of *Tegillarca granosa* (Zha et al., 2022), but this result was not found in gills of *L. elliptica*. Interestingly, as expected, in synergy exposures to both concentrations studied novel gene function were found altered in *L. elliptica* such as acid-balance homeostasis and ion transport potentially implicated in osmoregulation.

### 4. Conclusions

The observed differential expression analysis in gills of *L. elliptica in vivo* exposed to nanoplastics and n-TiO$_2$ alone and in combination

revealed a concentration-dependent effect, which stimulate further studies with bivalves. Exposing *L. elliptica* to 5 µg/L has clearly impacted clams ecophysiology and related functions but to a lesser extent than at 50 µg/L. The functional annotation of the transcripts revealed that structural constituent of ribosome, ions and particle transport, cell signaling, oxidative stress and immunity, and apoptosis pathway are affected by NP exposure alone and in combination. The observed gene modulation responses were already evident after 96 h of exposure, nevertheless, such short exposure time only partially reflects lifelong exposure that Antarctic clams encounter in their natural environment. Applying genome-wide transcriptomic analyses combined with the transcript-target approach using a larger sampling number, valuable ecologically relevant biomarkers were obtained helping to understand how nanoparticles affect molecular functions. This approach also allowed identifying biomarkers that could be used to monitor clams' health status along Antarctic coasts, which are more prone to local anthropogenic pressures. In perspective of this work, it will be necessary to validate the biomarkers on individuals sampled at different Antarctic locations and exposed to different anthropogenic sources of pollution.

### CRediT authorship contribution statement

**Carolina Pérez-Toledo:** Data curation, Formal analysis, Methodology, Software. **Teresa Balbi:** Visualization, Writing – review & editing. **César Antonio Cárdenas:** Conceptualization, Writing – review & editing. **Elisa Bergami:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Céline Cosseau:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – review & editing. **Marcelo González-Aravena:** Investigation, Writing – review & editing. **Catalina Valdés:** Formal analysis, Investigation, Methodology, Software. **Ilaria Corsi:** Conceptualization, Formal analysis,

Investigation, Supervision, Validation, Writing – review & editing. **Rodolfo Rondon:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Writing – original draft. **Erwan Corre:** Methodology, Software. **Cristian Chaparro:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing, Software. **Garance Perrois:** Formal analysis, Methodology. **Ignacio Garrido:** Formal analysis, Methodology.

## Declaration of Competing Interest

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.

## Data Availability

the data will be available after publishing the article as NCBI Bioproject

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116523.

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