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

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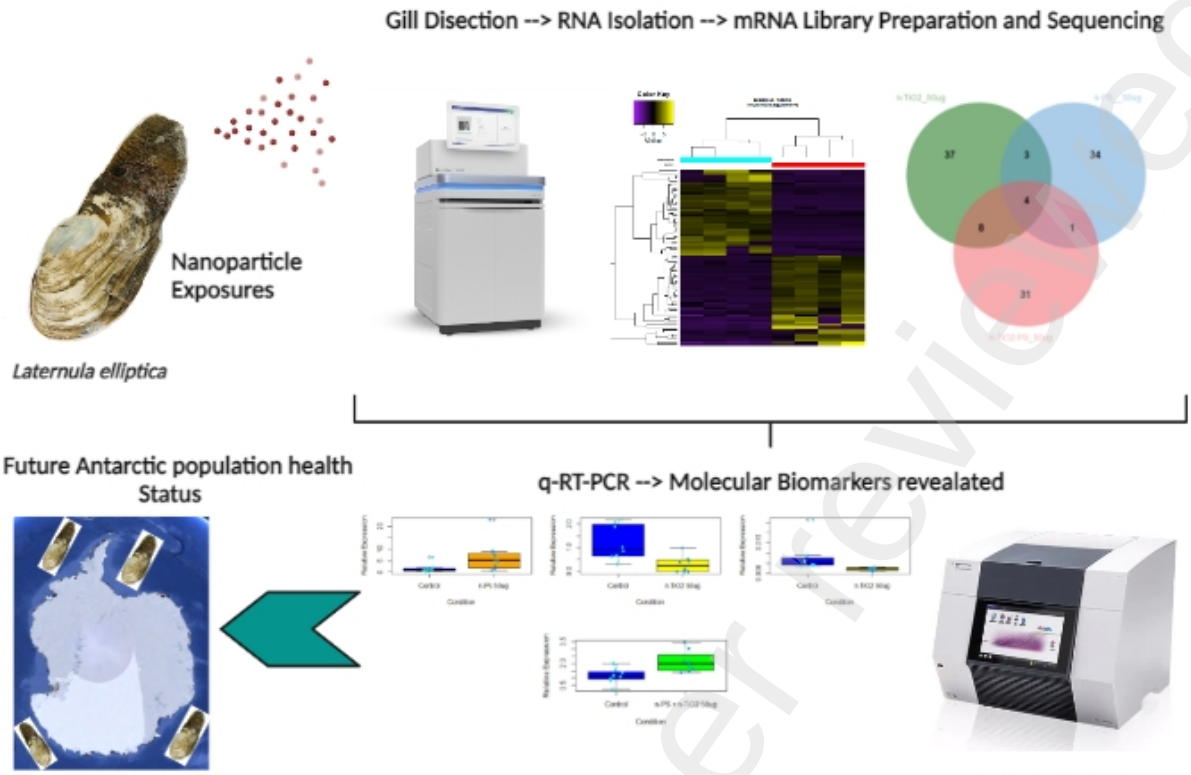
## Highlights

- Nano-PS-COOH and nano-$TiO_2$ analyzed with genome-wide gene expression in *L. elliptica*.
- Gill is a relevant tissue to evaluate toxic effects of nanoparticles in filter-feeders.
- Exposure to nanoparticles alters relevant molecular functions.
- Combined nanoparticle exposures produced a particular transcriptional impact.
- Transcript-target RT-qPCR reveals valuable biomarkers.


## Abstract

Contaminants of emerging concern (CEC) have been documented in surface waters, sediment and biota in Southern Ocean. Among CEC, in recent years micro- and nanoplastics and metal-oxide nanomaterials have been found in several environmental compartments. Ecotoxicological consequences to their exposure are almost unknown for Antarctic aquatic species and barely addressed so far. 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 CEC 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/ml). Transcript-target q-RT-PCR was performed with the aim to identify suitable biomarkers of exposure and effects. The experimental exposures showed gene expression profiles with the control group always clustered together (at both concentrations), however, as expected at the highest concentration 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. Although few transcripts were shared as differentially expressed between the experimental treatments compared to control, At function level relevant ones 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 nanoplastics, n-$TiO_2$ and their combination at 50 µg/ml, that could be used for monitoring clams' health status in different Antarctic localities.

## Abstract figure





## Keywords

Nanoplastic, nanometal, toxicity, Gene expression, biomarkers

## 1 Introduction

The Antarctic continent and surrounding waters are currently protected by internationals laws of the Antarctic Treaty and since the late 90s, 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. Recent studies have revealed various contaminants of emerging concern (CEC) in Southern Ocean ecosystems. Among CECs observed in Antarctic waters, pharmaceuticals and personal care products have been detected in seawaters around King George Island (Perfetti-Bolaño, A. et al., 2022; Szopińska et al., 2022). Furthermore, 54 CECs have been linked to human presence in the Northern Antarctic Peninsula, and thus potentially increasing environmental risk by affecting marine life and ecosystems (Olalla et al. 2020). Concerning persistent halogenated organic CECs, hexachlorobenzene (HCB), BDE-209, heptachlor, oxychlordane and mirex have been the main pollutant recorded in tissues of marine benthic invertebrates at Rothera Point in the Antarctic Peninsula (Cunha da Silva et al., 2023; Krasnobaev et al., 2020). The ever-increasing amount of plastic litter found in Southern Ocean is the most probable source of CEC pollution, originating both from research stations and fishing operations, but also from transport by ocean currents, and wind-generated water movements (Caruso et al., 2022; Rota et al., 2022).

A number of studies have shown that plastic pollutants as macro- (>10mm) 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., 2017). The origin of micro- (1-1000 μm) and nanoplastics (<1μm) is not yet determined, however Leistenschneider et al. (2021) showed that 45% of microplastic collected in the Weddell Sea derived from vessel-induced contamination. On the other hand, the hypothesis of transport by Antarctic base effluents and currents from other oceans should not be excluded. 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 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. Besides, 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).

Differently, metal-oxides nanoparticles belonging to CEC have been barely 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 definitely 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, the combined potential synergistic effects deserve to be explored in marine Antarctic metazoans, playing a significant ecological role in the marine trophic webs (Das et al., 2022). It is suggested that Antarctic filter-feeding mollusks are among the most sensitive marine metazoans (Peck, 2018), hence species such as *Laternula elliptica* could be considered as target in some areas of the Antarctic coast (Lister et al., 2015). Besides, this species has already been proposed as a suitable sentinel of anthropogenic pollution in Antarctic marine coastal areas, as marine bivalves in others regions of the world (Li et al., 2019). 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 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).

Transcriptomic is an approach which has been previously applied to pollution monitoring and effect assessment (Blalock et al., 2018), particularly performing experimental designs with control and treatment conditions to be compared. 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 usage as highly specific fingerprints of exposure, and these fingerprints can be used to diagnose environmental stress (Reid and Whitehead, 2016). In addition, molecular biomarkers can be revealed with this approach, for example, Förlin et al. (2019) identified potential transcriptomic biomarkers of toxic compounds in Baltic perch, and similar analysis produced biomarkers in gastropods exposed to Cadmium (Gu et al., 2019). In contrast to other model organisms few studies

have examined the impact of CEC in marine bivalves at the transcriptome level. Transcriptomic analysis can not only screen the ecotoxicity of CEC but also infer the function and specific regulatory mechanism of the corresponding unknown genes. As far as bivalve species from temperate areas such as Manila clam, Milan et al. (2013) obtained 162 transcripts correlated with at least one kind of pollutant found in the studied area, of which seven were assigned as correct biomarkers in the most polluted location. 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 anticipate that model nanoplastics such as polystyrene nanoparticles (PS NP) and n-$TiO_2$ exposures may provoke sub-lethal gene expression alterations, affecting some eco-physiological functions. The aim of this work was to study the transcriptomics response to CEC (PS NP and n-$TiO_2$) exposure in gill tissue of *L. elliptica*, expanding the analysis to identify biomarkers of nano-pollutants. Accordingly, we applied a whole genome-wide transcriptomic analysis (RNAseq) to unravel deferentially expressed transcripts of relevant molecular functions for each contaminant and synergic effect of co-exposure at two concentrations (5 and 50 µg/mL). Then, a transcript-target RT-qPCR analysis was applied on double the number of organisms (n = 8) to validate biomarkers that could be used *in situ.*

## 2 Methods

Sampling Antarctic soft clams: a total of 120 *Laternula 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 (Figure 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 250 and 25 µg/mL),

sonicating the suspensions using the bath sonicator DENTSPLY Ultrasonik 57H for 2 min (Murano et al., 2021) and vortexed prior to use as described by (Bergami et al., 2020). Two working suspensions at 250 and 25 µg/mL were then prepared and 1 mL of each suspension was added to 5 L tanks of natural sea water (NSW) to achieve final concentrations of 50 and 5 µg/mL, respectively.

As for n-$TiO_2$, a stock suspension (10 mg/mL) was prepared in MilliQ water, vortexed and sonicated for 1 hour, always keeping the suspension in cold water (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/mL, respectively. Tested suspensions were replaced every 24h 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 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. 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) 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 NSW, two independent experiments (96h) were carried out. Individuals (n = 5) were placed in 5L tanks with the following test solutions: NSW only (control), PS-COOH NP at 5 and 50 µg/mL, n-$TiO_2$ at 5 and 50 µg/mL and combined exposure (PS-COOH NP and n-$TiO_2$) at 5 and 50 µg/mL. Tanks were permanently well oxygenated and specimens exposed with a photoperiod of 18/6 light/obscurity intervals. After 96h, clams were dissected for tissue extractions (gills), frozen with liquid nitrogen, put in 1ml of RNAlater and kept at -80 °C.

2.3 RNA isolation, library preparation and Illumina NovaSeq sequencing: Eight gill tissues for each treatment, between 15-30 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 (nt = 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 (Gordon 2010). Then, a reference transcriptome was assembled using all libraries with Trinity (Grabherr et al., 2011), followed by ortholog regrouping to reduce functional redundancy using with CDHit (Fu et al., 2012) and the final transcripts were annotated with the Trinotate workflow. Completeness and redundancy degree were assessed with BUSCO software (Simão et al., 2015). 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" (Patro et al., 2017). 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 (Love et al., 2014), considering DEG with a log2(foldchange) > 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 transcripts with functional annotation and the reference transcripts, 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 18-24 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 minutes; 2) 40 cycles with 95°C for 30 seconds, the temperature gradients for 1 minute, and finalize with 72°C for 30 seconds. 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 minute; 2) 30 seconds at 55°C and 3) 30 seconds 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 Q-RT-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.

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

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 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).

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%.

Results from experimental exposure showed the gene expression profiles at the lowest concentration tested of 5 µg/ml are shown in (Figure 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/ml, control group clustered separately, but without treatment samples in this case (Figure 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 global transcriptomic analysis recorded 25, 56 and 43 DEG for PS-COOH NP, n-$TiO_2$ alone and in combination for the 5 µg/ml in comparison with control condition (supplementary file 2). Regarding the highest concentration tested of 50 µg/ml, 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/ml, with

only 1 DEG shared among single and combined exposure conditions at 5 µg/ml (Figure 4A) and 4 DEG at 50 µg/ml, (Figures 4B). Furthermore, these diagrams also reveal few shared DEG between pairs of treatments, with from 2 to 5 shared DEG at 5 µg/ml, and from 1 to 8 shared DEG at 50 µg/ml. 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-$TiO_2$ and one shared DEG when combined (Figure 4C-E). A more pronounced effect of nanoparticles exposure at higher concentration was found (Figure 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.

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 different genes 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. The expressions of genes coding for ribosomal proteins were altered in response to both nanoparticles. A transcript (TRINITY_DN101707_c0_g1_i13) encoding for a "60S 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/ml respectively (table 2 and sup file 2). This is in line with a previous study on the marine rotifer *Brachionus koreanus*, 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). Furthermore, the expression of the gene (for transcript: TRINITY_DN127464_c0_g2_i14) encoding for a 60S ribosomal protein L23 was repressed by -2.74 and -3.49 log2Fold-Change respectively, in response to n-$TiO_2$ exposure and to both nanoparticles at 50 µg/ml (table 2 and sup file 2). The effects of silver NP on the expression of genes encoding for ribosomal proteins has previously been reported in *Danio rerio* embryos (Van Aerle et al., 2013), and 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. 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 (for transcripts TRINITY_DN246_c2_g2_i1 and

TRINITY_DN3151_c0_g1_i1) encoding for Solute carrier family 23 members were up regulated in response to n-$TiO_2$ alone and in combination at 5 µg/ml (7.044 log2Fold-Change) at both concentrations tested (5 µg/ml and 50 µg/ml) (6.79 log2Fold-Change and 1.91 log2Fold-Change respectively) (Table 2 and sup file 2). 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*. Furthermore, this situation is accompanied by down-regulation of the expression of vesicular traffic gene (Ap-4 Complex accessory subunit tepsin, transcript id: TRINITY_DN24516_c0_g1_i10), upon n-$TiO_2$ exposure to 5 µg/ml with -2.29 log2Fold-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-$TiO_2$ (50 µg/ml), we observed a down-regulation of cytoskeleton function transcript Tubulin alpha-3 chain (TRINITY_DN82866_c4_g1_i1) with -5.75 log2FoldChange. Similar results were recorded in mollusks such as the bivalve *Mytilus 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). Also in gilthead sea bream *Sparus aurata* down-regulation of the expression of gene encoding for Alpha-actinin 1 was observed in liver in response to citrate coated gold NP of ~40 nm diameter at 50 µg/ml (Teles et al., 2019). 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.

Other molecular functions affected in response to nanoparticles and shared between the two exposures were related to oxidative stress and immunity. The expression of genes encoding for antioxidant functions were up-regulated: "TRINITY_DN4299_c0_g1_i16" encodes for a putative ferric-chelate reductase 1 and "TRINITY_DN6514_c1_g1_i6" 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/ml (table 2 and sup file 2). "TRINITY_DN3207_c5_g2_i2" transcript encodes for DBH-like monooxygenase protein 1 and "TRINITY_DN5798_c1_g1_i1" 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 log2FoldChange respectively in response to combined exposure to PS-COOH NP and n-$TiO_2$ at 50 µg/ml (table 2 and sup file 2). 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/ml respectively, similar to what was reported for the swamp crayfish, *Procambarus clarki*, upon PS NP orally administered (Capanni et al., 2021).

Regarding immune transcripts, we also recorded the downregulation of expression of a gene (TRINITY_DN7242_c3_g1_i1) annotated as Deleted in malignant brain tumors 1 protein (DMBT1), differentially expressed in response to n-$TiO_2$ at 50 µg/ml with -2.63 lof2Fold-Change (table 2 and sup file 2). This gene encodes for a member of scavenger receptor cysteine rich (SRCR) protein family and has possible functions in innate immunity, inflammation and epithelial cell differentiation (Madsen et al., 2010). Effect on immune response genes has been reported in zebrafish gill tissues in response to $Fe_3O_4$ NP exposure (Zheng et al., 2018). "TRINITY_DN7095_c0_g1_i22" is annotated as a transcript encoding for Tachylectin-5A and its expression is repressed by -1.95 log2Fold-Change in response to both nanoparticle exposure at 50 µg/ml. Tachylectins are pattern recognition molecules with a key role in the innate immune system, known to be involved in specific recognition of invading microbes through acetyl group-containing molecules (Angthong et al., 2017).

Another transcript potentially involved a lipase gene (TRINITY_DN4893_c0_g1_i22), which was significatively up-regulated in n-$TiO_2$-exposed clams and in those from the combined exposure group at 50 µg/ml with 3.67 and 4.01 log2Fold-Change respectively. This was the HRSL1 transcript, potentially involved in phospholipase metabolization as glycerophospholipids (Mardian et al., 2015). Membrane glycerophospholipids are known to affect not only the production of lipid mediators but also membrane properties, which could be affected during these exposures and compromise cell membrane permeability in gill tissues.

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-$TiO_2$ are source of concern. The down-regulation of immune genes and the up-regulation of antioxidant responses reveal potential detrimental consequences of 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.

Functions related to cell signaling were also affected in response to NP exposure. The expression of both genes "TRINITY_DN5301_c0_g1_i14" and "TRINITY_DN13854_c0_g1_i4" annotated as "alpha-protein kinase vwkA" and "CUB and sushi domain-containing protein 3", respectively, were induced in response to n-$TiO_2$, at 5 µg/ml (6.84 and 4.84 log2Fold-Change up-regulation, respectively). The first one is a cell signaling transcript potentially implicated in a large variety of cellular processes such as protein translation, $Mg^{2+}$ homeostasis, intracellular transport, cell migration, adhesion, and proliferation (Middelbeek et al., 2010). The second one is putatively involved in protein-protein interaction between extracellular and transmembrane proteins. At 50 µg/ml n-$TiO_2$ exposure, the expression of the gene "TRINITY_DN1668_c0_g1_i4" potentially encoding for a serine/threonine-protein kinase roco5 was down-regulated by 4.93 log2Fold-Change. This transcript belongs to Roco protein family that is characterized by having, among others, diverse regulatory and protein–protein interaction domains, potentially involved in cell division, chemotaxis, and development (Marín et al., 2008).

In accordance to previously published studies, our analysis also highlighted that the expression of the gene "TRINITY_DN2086_c1_g1_i14" which potentially encodes for caspase 8 is down-regulated by 5.17 log2Fold-Changes in response to n-$TiO_2$ at the highest exposure concentration of 50 µg/ml (shown in Table 2 and sup file 3). A differential expression of genes involved in the apoptotic pathway in response to NP has been previously reported in zebrafish embryos exposed to Cu/$TiO_2$ and $TiO_2$ (Yeo and Park, 2012). Similarly, a set of apoptosis genes resulted differentially expressed in the digestive glands of the freshwater benthic clam *Corbicula fluminea* probably by oxidative stress induced by PS NP in this case (Li et al., 2021). Also, different concentrations of silicon dioxide and copper oxide NP affected Caspase3 gene expression in gill and liver of *Oreochromis niloticus* fish (Abdel-latif et al., 2021). The transcript "TRINITY_DN88636_c0_g1_i7" encodes 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 log2Fold-Change in response to 50 µg/ml n-$TiO_2$ exposure (Table 2 and sup file 2). Furthermore, the combined exposure to 5 µg/ml revealed the up-regulation of another gene, coding the Carbonic anhydrase 9 (TRINITY_DN494_c17_g1_i11), which catalyzes the reversible hydration of carbon dioxide in the reaction: $CO_2 + H_2O$ $H^+ + HCO^{3-}$. This transcript is potentially implicated in several physiological processes, such as acid-base balance, $CO_2$ and $HCO^{3-}$ transport and respiration (Kallio et al., 2010), being highly relevant in *L. elliptica* gill function and homeostasis. Finally, the significant expression increase in the gene "TRINITY_DN1607_c2_g1_i8" potentially encoding for Sushi von

Willebrand factor type A transcript was observed in response to combined exposure to NP at 50 µg/ml with 6.23 log2Fold-Change, being potentially implicated in cell adhesion (Glait-santar et al., 2012).

RNAseq analysis led us to identify DEG that may be relevant transcripts for biomarkers of early NP exposure (96 h) at toxicologically relevant concentrations (Al-sid-cheikh et al., 2018; Sun et al., 2016). We first 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/ml DEG detected) (supplementary file 3, Figure 1). We then performed a target transcript q-RT-PCR using eight *L. elliptica* samples in 19 relevant genes (Supplementary File 3, Figures 2-4). Interestingly, at the 5 µg/ml 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 (supplementary file 3, Table 2) 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/ml. Working with samples obtained after exposure at the highest concentration (50 µg/ml), a tendency toward the same results as for the RNA-seq analysis in 12 genes out of the 15 tested was observed (supplementary file 3, Table 2). 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, Figure 2). This suggests that antioxidant and metabolism of xenobiotic transcript could be suitable biomarker to indicate the presence of relevant concentrations of PS NP. Several studies have showed the use of these transcripts as a biomarker in response to NP exposures in bivalves, but most of them were assessed in response to metal-oxide NP only (Garaud et al., 2016). 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, Figure 3). Transcripts involved in inflammatory immune process have been previously observed as biomarkers of the exposure to diverse nano-metals in aquatic metazoans (Kulasza and Skuza, 2021). We also revelaed 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, Figure 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, Figure 4). We suggest that these

two 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 nano-metals (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).

## 4 Conclusions

The observed differential expression analysis in gills of *L. ellipica in vivo* exposed to nanoplastics and n-$TiO_2$ alone and in combination revealed a concentration-dependent effect, which stimulate further studies at concentrations closer to those environmentally relevant in Antarctic coastal waters (e.g., ~50 ng/ml for nanoplastics, as reported in Antarctic sea ice). Exposing *L. elliptica* to 5 µg/ml have clearly impacted clams ecophysiology and related functions but to a lesser extent than at 50 µg/ml (Figure 5). The functional annotation of the transcripts clearly revealed that structural constituent of ribosome, ions and particle transport, cell signaling, oxidative stress and immunity, or apoptosis pathway are affected by NP exposure alone and in combination. The observed gene modulation responses were already evident after 96h 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. These also allowed identifying biomarkers that could be used to monitor clam 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.

## 5 Declaration of competing interest

The authors declare that they have not conflict of interests.

## 6 Authors contributions

**Rondon**: Designed experimentations, carried out experimental exposure experiments in Antarctica, performed bioinformatic analyses and wrote the manuscript. **Valdés**: Performed q-RT-PCR, statistical analyses and participated in manuscript drafting. **Cosseau**: Design experimentations, performed transcriptome orthologs clustering analyses (CD-Hit) and participated in manuscript drafting. **Bergami**: Designed experimentations, carried out characterization of nano-pollutant suspensions and participated in manuscript drafting. **Cárdenas**: participated in manuscript drafting. **Balbi:** participated in manuscript drafting**. Pérez-Toledo**: Carried out RNA-extractions, RT-PCR, and participated in q-RT-PCR. **Garrido**: Performed diving sampling in Antarctica and participated in experimental analyses. **Perrois**: carried out experimental exposure experiments in Antarctica. **Chaparro**: Participated in bioinformatics analyses in IHPE Galaxy Platform and participated in manuscript drafting. **Corre**: Participated in bioinformatics analyses in ABIM Galaxy platform. **Corsi**: Designed experimentations, provided nanopollutants and participated in manuscript drafting. **González-Aravena**: Participated in experimentation design and drafting manuscript.

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## 10 Figures and Tables

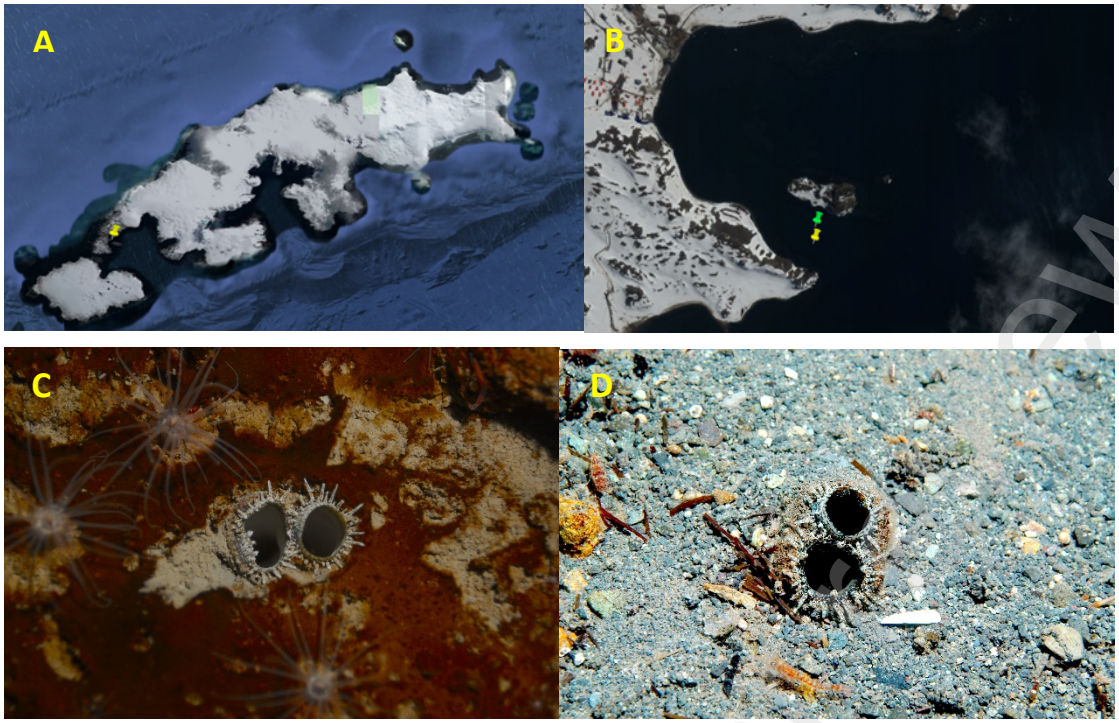
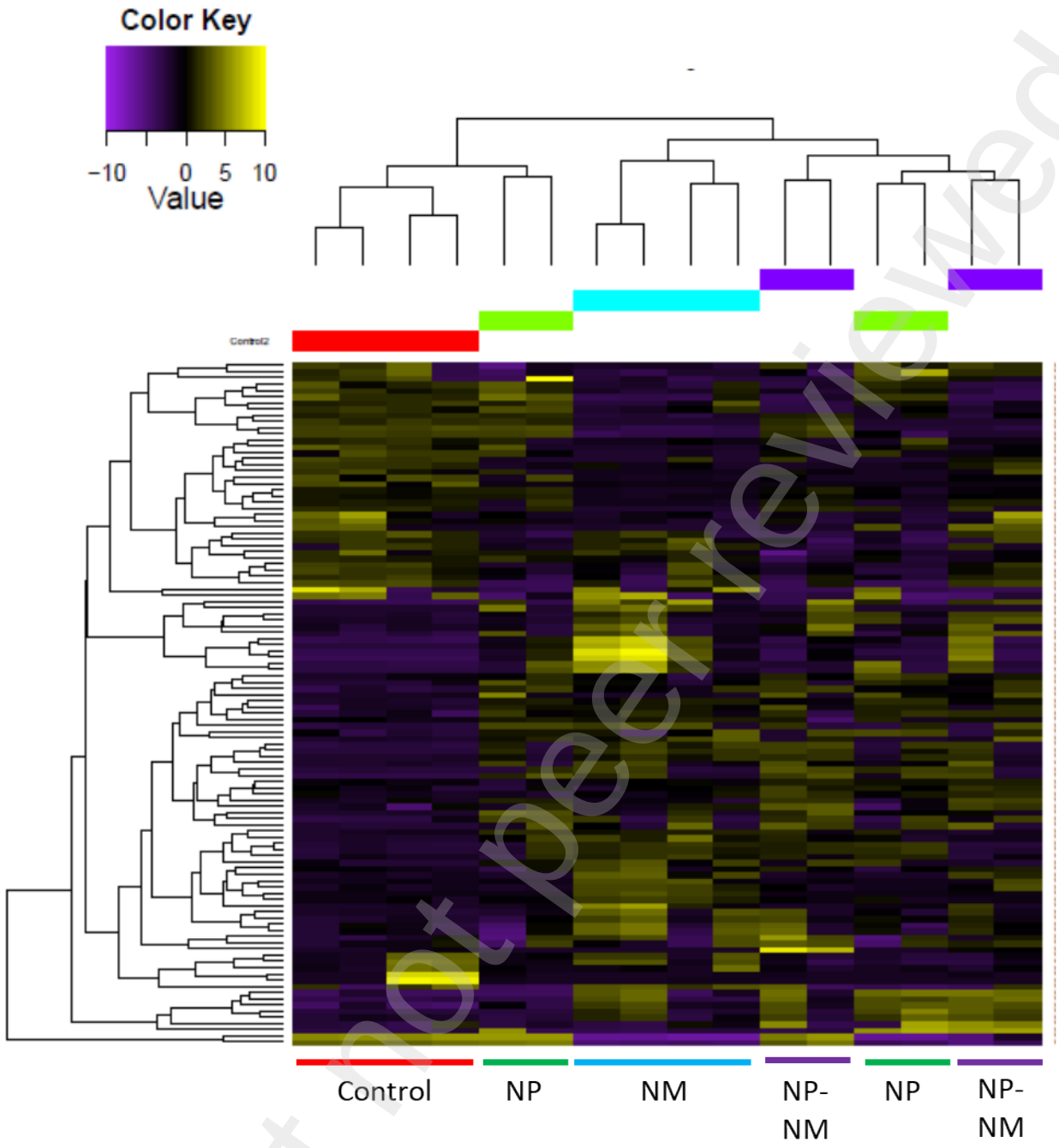



**Figure 1**. Sampling points where *Laternula elliptica* clams were collected on King George Island (A) at Fildes Bay (B). The points correspond to geo-referential coordinates 62° 12' 17.32" S, 58° 56' 56.48" W and 62° 12' 19.34" S, 58° 56' 57.0052" W (C-D) Pictures of *L. elliptica* individuals in their natural environment (photos from Dr. Ignacio Garrido).

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

| | |
|---|---|
| **Reads** | |
| Raw Reads | 975 727 394 |
| Filtered Reads | 975 727 394 |
| Total Aligned and counted reads | 892 620 127 |
| Aligned and counted reads (%) | 91.48 |
| **Assembly** | |
| Total trinity transcripts | 603954 |
| Total trinity genes | 279798 |
| GC (%) | 38.93 |
| Total trinity transcripts After CD-Hit | 251294 |
| Total trinity genes After CD-Hit | 222734 |
| GC (%) After CD-Hit | 38.26 |
| **Assembly statistics After CD-Hit** | |
| N50 | 955 |
| Median length (bp) | 382 |
| Average length (bp) | 675.17 |
| **BUSCO statistics After CD-Hit** | |
| Total completeness (%) | **99.4** |
| Complete and single-copy (%) | 92.4 |
| Complete and duplicated (%) | 7.0 |
| Fragmented (%) | **0.6** |
| Missing (%) | **0.0** |

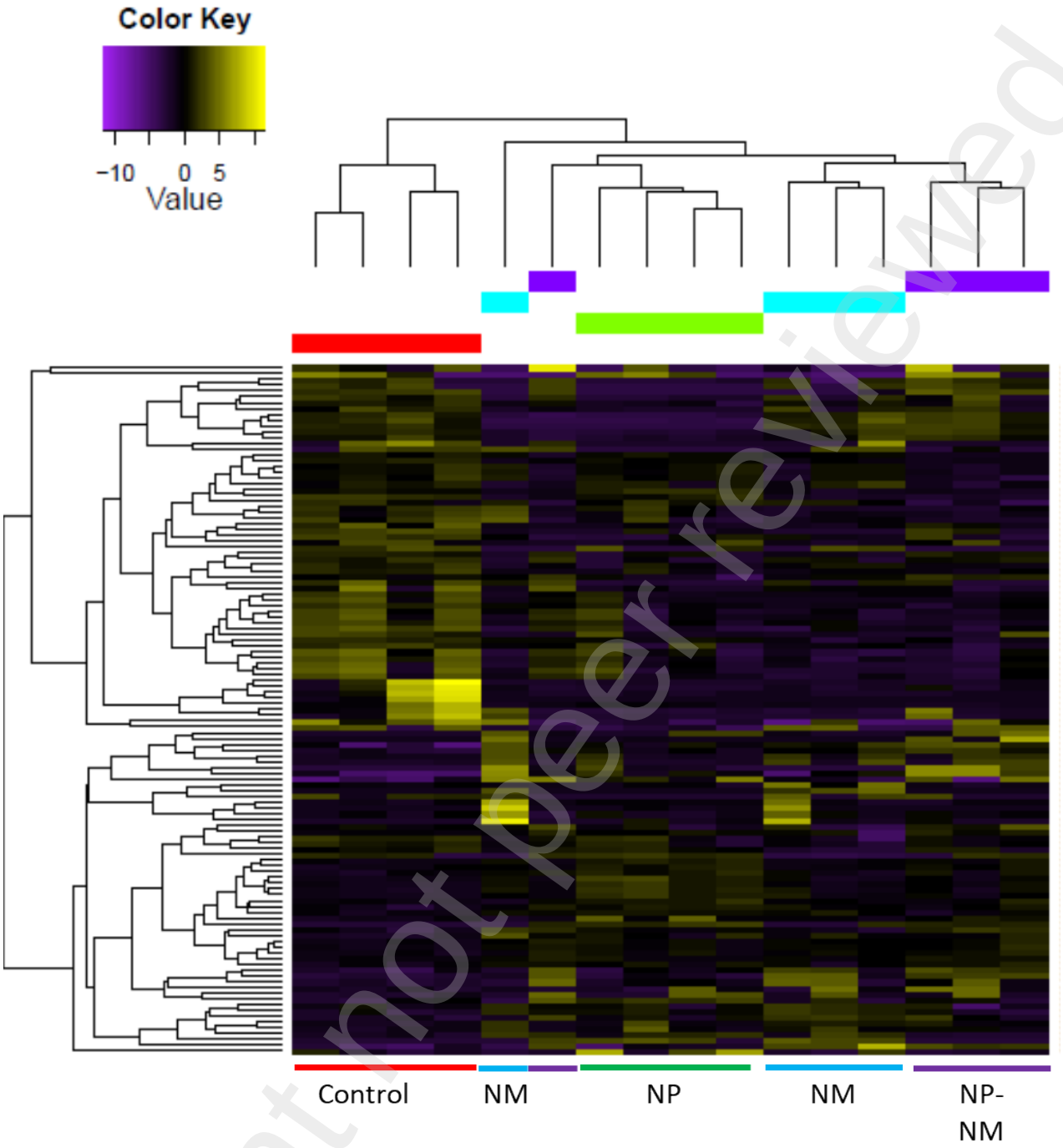
853




854

855 **Figure 2.** Heatmap of Differentially Expressed Genes revealing the clustering of the control group
856 (Control, in red), and treatments exposed to 5 µg/ml nanoparticles: PS-COOH NP (NP, in green), n-
857 $TiO_2$ (NM, in blue) and combined exposure (NP-NM, in violet).

858




859

860 **Figure 3.** Heatmap of Differentially Expressed Genes revealing the clustering of control group
861 (Control, in red), and treatments exposed to 50 µg/ml nanoparticles: PS-COOH NP (NP, in green),
862 n-$TiO_2$ (NM, in blue) and combined exposure (NP-NM, in violet).

863

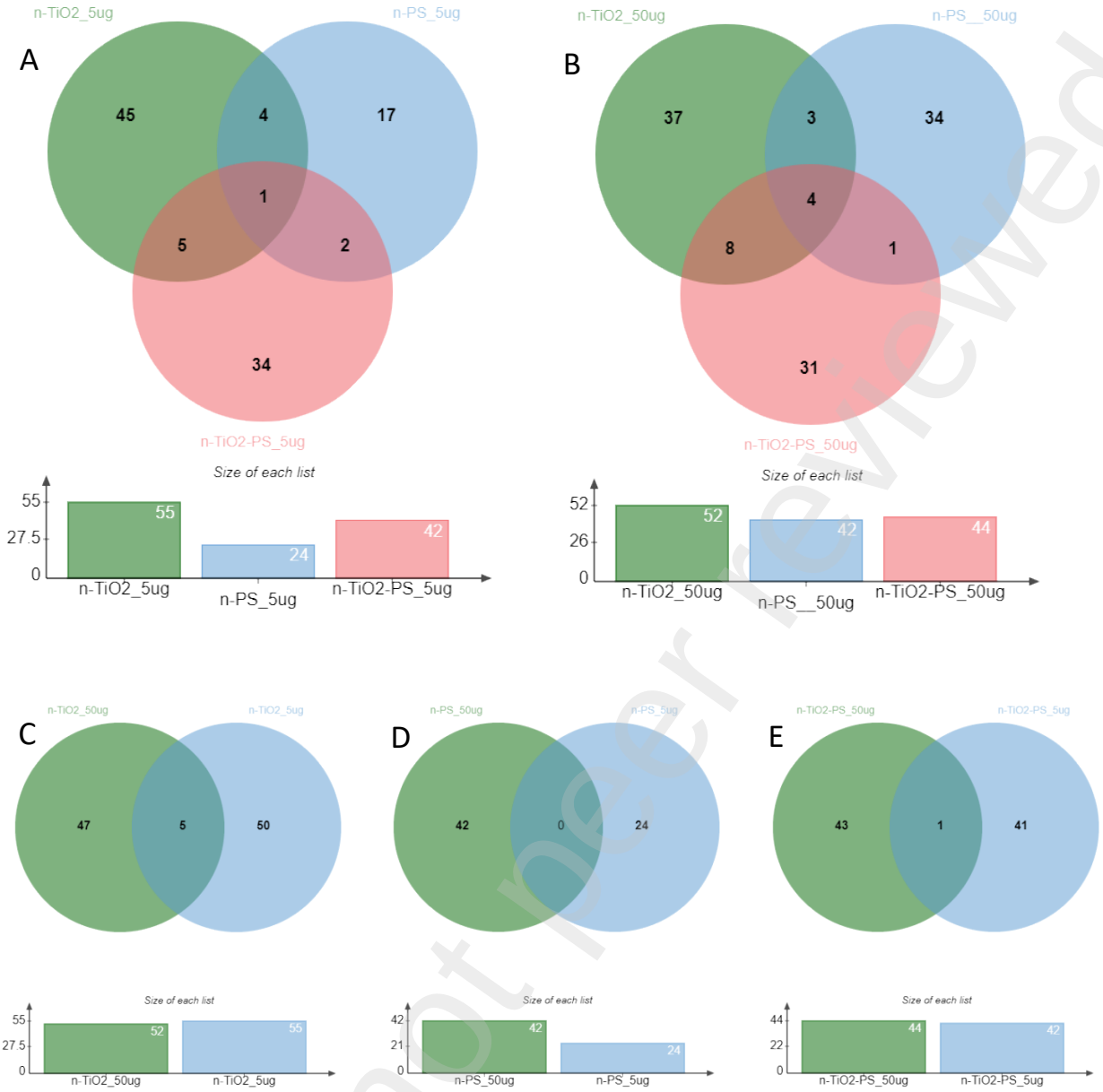
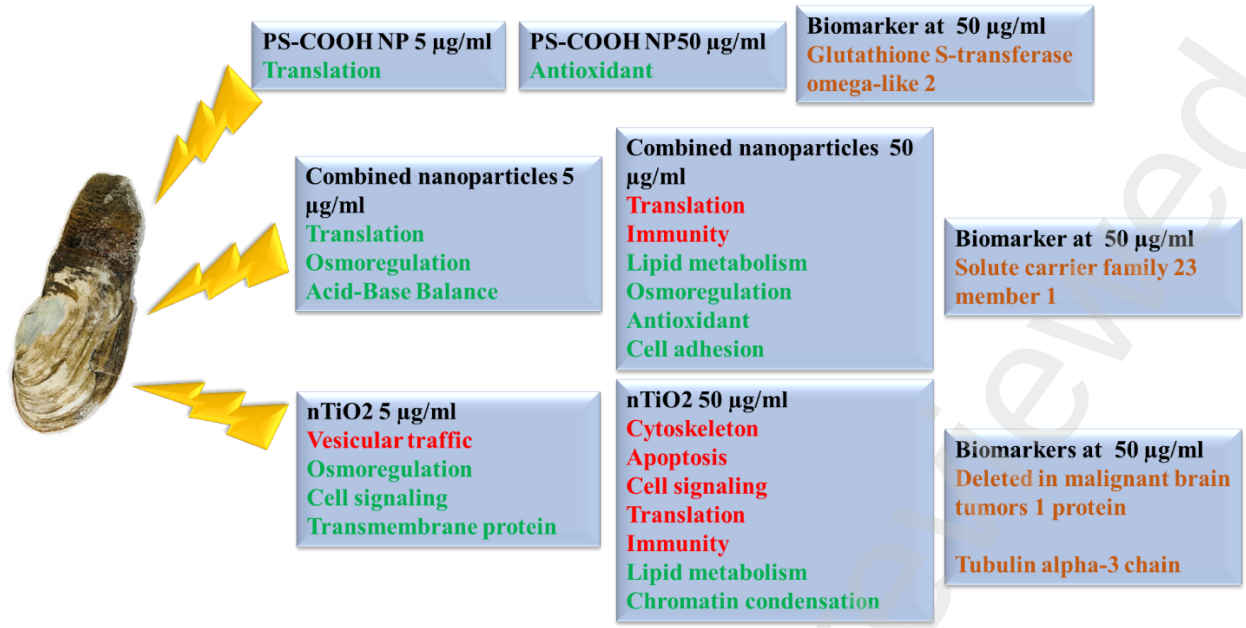



**Figure 4.** Venn diagrams showing shared Differentially Expressed Genes (DEG) when comparing individuals exposed to control end experimental conditions at 5 µg/ml (A) and 50 µg/ml (B). The green circles represent the n-$TiO_2$ conditions, the blue ones the PS-COOH NP conditions and the pink ones the 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-$TiO_2$ (A), PS-COOH NP (B) and combined exposure (C). Green circles represent the 50 µg/ml treatments for each pollutant and the blue ones represent the 5 µg/ml treatments. The overlaps of the circles represent the shared transcripts. Barplots show transcript numbers by conditions.




878

879 **Figure 5.** Molecular effects of the exposure to PS-COOH NP and/or n-$TiO_2$ in the Antarctic soft clam
880 *L. elliptica* at 5 and 50 µg/ml. Molecular functions with up-regulated transcripts are shown in green
881 and down-regulated ones in green. The molecular biomarkers validated with eight clams are marked
882 in brown.

883

884