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Unravelling the suitability of *Branchinecta gaini* as a potential biomonitor of contaminants of emerging concern in the Antarctic Peninsula region

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Abstract: The occurrence and impact of contaminants of emerging concerns (CECs) have been investigated in Antarctica much less than in other parts of the world. Although legacy anthropogenic pollutants can reach Antarctica *via* long-range transport, CECs mainly originate from local sources. Here, we investigated the ability of a freshwater crustacean, the Antarctic fairy shrimp *Branchinecta gaini*, to cope with nanoscale titanium dioxide (n-TiO_2), a widely used pigment in consumer products (e.g. paintings), including those for personal care (e.g. sunscreens). An *in vivo* acute short-term exposure study (9 h, n-TiO_2 concentration range 50–200 µg ml^{-1}) was performed and the expression levels of several genes involved in stress response were evaluated. No effect on the expression of heat-shock protein chaperone genes was found, with the exception of *Hsp70a*, which was significantly upregulated at 200 µg ml^{-1} n-TiO_2. Similarly, cytochrome P450 was upregulated at 100 and 200 µg ml^{-1} n-TiO_2, while the expression levels of cathepsin L and of antioxidant genes such as superoxide dismutase and glutathione peroxidase were significantly reduced with increasing concentrations of n-TiO_2. This study shows for the first time the responsiveness and sensitivity of an Antarctic freshwater crustacean to n-TiO_2 exposure and supports its suitability as a biomonitor of CECs in Antarctica.

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Keywords: biomonitoring, ecotoxicity, gene expression, titanium dioxide nanoparticles

Introduction

Aquatic ecotoxicity studies on the effects of contaminants of emerging concern (CECs) such as microplastics, anthropogenic nanomaterials and pharmaceuticals have rapidly increased in number in the last decade, focusing mainly on freshwater rather than saltwater or terrestrial environments (Dulio *et al.* 2018). Pollution is one of the global anthropogenic drivers whose impact is expected to increase in the Antarctic in the near future (Morley *et al.* 2020). Legacy anthropogenic pollutants such as persistent organic pollutants can reach Antarctica *via* long-range transport (Krasnobaev *et al.* 2020), whereas CECs may also originate from local sources (e.g. activities related to fishing, tourism and scientific research), and if they are not monitored and regulated, CECs have the potential to accumulate in the environment and have adverse effects on polar wildlife.

The study of CECs in polar environments poses a challenge as few data are currently available regarding their sources, fate and impact in comparison with the intense research conducted at lower latitudes. Among CECs, anthropogenic nanoscale materials and nanoparticles (NPs) pose great concern due to their ability to cause a variety of injuries in exposed species (Blasco & Corsi 2019). Nanoscale titanium dioxide (n-TiO_2) is recognized globally as a potential hazard for humans and the environment (Sauvé & Desrosiers 2014, Slijkerman & Keur 2018). They are frequent components of commercial and industrial products (Gottschalk *et al.* 2009), and due to their photocatalytic and antibacterial properties they are widely present in commercial and consumer products including paints, sunscreens and textiles (Robichaud *et al.* 2009, Ziental *et al.* 2020). Titanium dioxide NP occurrences in lakes and coastal seawaters (top surface layer and in the water column) have been demonstrated recently, with concentrations in the range of 10–900 µg l^{-1}, probably being released from sunscreens (Gondikas *et al.* 2014, Labille *et al.* 2020). The ecotoxicity of titanium dioxide NPs has been widely investigated and several injuries have been documented in marine species belonging to different trophic levels. Titanium dioxide NPs can also be transferred to and along food webs (i.e. through bioaccumulation and biomagnification) and can exceed toxicity threshold levels for aquatic species (Corsi *et al.* 2020).

Polar regions are therefore not excluded from the potential threat posed by n-TiO_2, as personal care products, ultraviolet (UV)-filter sunscreens and pharmaceuticals have been already reported in Antarctic coastal waters in the vicinity of Antarctic research stations (Emnet *et al.* 2015, Domínguez-Morueco *et al.* 2021). Sewage can carry a significant amount of household products, increasing the risk of exposure for both fresh and marine species (Baker *et al.* 2014). Nanoparticles have sizes < 100 nm and are characterized by a colloidal behaviour in water bodies, being susceptible to agglomeration and sedimentation (Klaine *et al.* 2008). The wastewater treatment plants located in Antarctica receive various types of wastes, fats, oils and greases and also products containing NPs such as microplastics and nanometals. Depending on the type of plant and its maintenance, in some cases NPs may not be retained and may be released into the environment. Although polar species are well adapted to extreme but relatively stable environmental conditions, they are more vulnerable to environmental perturbations, including anthropogenic pollutants, compared to species from lower latitudes (Grotti *et al.* 2008).

The fairy shrimp *Branchinecta gaini* (Daday 1910) is the largest freshwater invertebrate species in Antarctica and the only species of Anostraca present in the South Shetland Islands (Maritime Antarctica) and the Antarctic Peninsula (Nedbalová *et al.* 2017). Although the biology and ecology of *B. gaini* are known (Peck 2004, Hawes 2008, Pociecha & Dumont 2008), its suitability as a biomonitor of anthropogenic pollutants and CECs has not been investigated thus far. Other species of Anostraca (e.g. brine shrimp *Artemia* spp.) have been widely used as models in ecotoxicity studies and recognized as suitably sensitive species for water pollution monitoring and impact assessment (Ates *et al.* 2013, Libralato *et al.* 2016, Bergami *et al.* 2017).

Here, for the first time, we investigated the ability the Antarctic fairy shrimp *B. gaini* to cope with n-TiO_2 exposure by analysing the expression of genes involved in stress response (e.g. antioxidant, moulting and xenobiotic responses) with acute, short-term (9 h) *in vivo* n-TiO_2 exposure (in the concentration range of 50–200 µg ml^{-1}). Some of these stress-response genes as well as those involved in moulting have already been shown to be regulated by other nanoscale particles (i.e. functionalized polystyrene NPs) in aquatic crustaceans in both acute short-term and long-term exposure conditions (Bergami *et al.* 2016, 2017, 2020, Varó *et al.* 2019).

Methods

Sample collection and experiment

A total of 80 specimens of *B. gaini* were collected during the 2018–2019 summer (January–February) from a lake in Fildes Bay near Professor Julio Escudero scientific base (Instituto Antartico Chileno; INACH) on King George Island. Individual specimens were placed in polypropylene plastic containers filled with rock pool water from Laguna INACH (62°12.128'S, 58°57.992'W) at an ambient temperature of 4 ± 2°C.

Titanium (IV) oxide (n-TiO_2) anatase nanopowders (< 25 nm particle size, 99.7% trace metal basis) were purchased from Sigma-Aldrich (St Louis, MO, USA). Working solutions of n-TiO_2 were prepared in 0.2 µm filtered rock pool waters from a stock of 1 mg ml^{-1} in Milli-Q® water (mQW; ELGA LabWater, Sartorius, UK). Suspensions were sonicated for 20 m in an ultrasonic bath (Branson, Inc., Danbury, CT, USA) before and after dilution to prevent aggregation.

Three individual fairy shrimp specimens were placed in each 50 ml polypropylene graduated plastic container and exposed to 50, 100 and 200 µg ml^{-1} suspensions of n-TiO_2 in rock pool water; a control group was exposed to rock pool water only. Experiments were run for 9 h according to the 202 OECD acute toxicity protocol (OECD 2004). After 9 h, fairy shrimps were removed and placed in RNAlater™ (Sigma-Aldrich) following the protocol of the manufacturer and stored at -80°C for gene expression analysis. Each experiment was repeated twice. Mortality was assessed by behaviour change and immobility of animals after 9 h.

Characterization of n-TiO_2

The stability and agglomeration of n-TiO_2 suspensions (at 200 µg ml^{-1}) in mQW and rock pool waters (exposure medium) were determined using dynamic light scattering (DLS; Malvern Instruments, Malvern, UK) combined with the *Zetasizer Nano Series* software, version 7.02 (Particular Sciences, Dublin, Ireland). The Z-average (nm), polydispersity index (PDI; dimensionless) and ζ-potential (mV) were measured at 4°C as key parameters describing NP behaviour in complex environmental media (SCENIHR 2007, Stone *et al.* 2010). Measurements were carried out at different time intervals (0, 3, 6 and 12 h) and were performed in triplicate, with each measurement set as 11 runs of 10 s (Z-average) or 20 runs (ζ-potential) according to Bergami *et al.* (2017). The initial aggregation kinetics of n-TiO_2 within 1.2 h after suspension in mQW and rock pool water were further investigated as individual sequential measurements at 6 min intervals.

RNA extraction, sequencing and assembly

RNAlater was removed from samples and RNA extracted according to the protocol of the E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). RNA quality and quantity were determined on a 2100

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Table I. Results from the *Blastx* sequence similarity searches of the target genes in the *Branchinecta gaini* transcriptome.

Contig ID	Description	Organism accession number	Expected value
DN10391_c0_g1_i1	Cytochrome P450 4C1-like (*Daphnia magna*)	XP_032793845.1	8e-172
DN9320_c0_g1_i1	Superoxide dismutase (Cu-Zn) (*Amphibalanus amphitrite*)	KAF0297955.1	2e-73
DN11024_c5_g1_i1	Glutathione peroxidase (*Hypsibius dujardini*)	OWA51519.1	2e-75
DN10381_c0_g1_i2	Cathepsin L precursor (Clap) (*Artemia franciscana*)	AAV63977.1	0.0
DN11391_c0_g1_i6	Caspase-1 (*Artemia sinica*)	AGB84766.1	6e-160
DN9765_c0_g1_i1	Heat shock 70 kDa protein cognate 5 (*Artemia franciscana*)	QDA02045.1	0.0
DN11037_c0_g2_i1	Major heat shock 70 kDa protein Bbb (*Daphnia magna*)	KZS19529.1	0.0
DN11037_c0_g1_i1	HSC70 (*Artemia franciscana*)	QDA02044	0.0
DN6849_c0_g1_i1	Heat shock 90 kDa protein (*Artemia franciscana*)	QEQ13288.1	0.0
DN11637_c0_gi_i1	Endoplasmin homologue isoform X1 (*Daphnia magna*)	XP_032781722.1	0.0

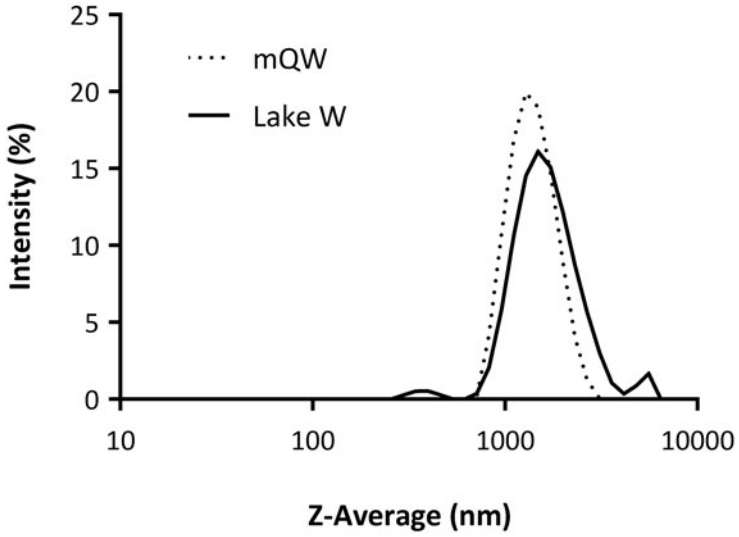

Fig. 1. Physicochemical characterization of nano-TiO_2 (200 µg ml^{-1}) in Milli-Q water (mQW; as reference medium) and rock pool water (Lake W; 0.20 µm filtered, pH 8.22, 4°C) *via* dynamic light scattering. Intensity-weighted size distributions of Z-averages (nm) are shown on a logarithmic scale (*x*-axis) starting at 10 nm, representing averages of three measurements for each medium.

Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quality control assessment gave a RNA integrity number (RIN) of > 7.5. An RNA library was produced using 1 µg of total RNA with a TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA). High-throughput sequencing was performed on a HiSeq 2500 sequencer provided by Macrogen (Seoul, South Korea) using ~40 million and a 2 × 100 base pair (bp) paired-end strategy. Trimmed reads were assembled using *Trinity* version 2.4.0 including an *in silico* read normalization for sequences with a minimum length of 200 bp. The resulting assembly contained 43 243 transcripts, a length observed for 50% of the contigs assembled (contig N50) of 1253 bp and an average length of 85 339 bp.

RNA sequence database annotation and identification of candidate genes

The nucleotide sequences of the genes coding for several heat-shock proteins (Hsps; Hsp83, Hsp90a, Hsp90b, Hsp70m, Hsp70a, Hsp70b), caspase 1, glutathione peroxidase (GPx), superoxide dismutase (Sod), cytochrome P450 4C1-like (P450) and cathepsin L precursor (Clap) were obtained from the transcriptome of *B. gaini* (Bioproject ID PRJNA661774). The degree of homology of these sequences was confirmed by similarity search using the *Blastx* program (NCBI, Bethesda, MD, USA), where amino acid sequences were extracted for multiple alignment using the CLC Main Workbench (QIAGEN, Hilden, Germany) to determine conserved areas. Subsequently, the primers were designed from the conserved areas using the software *Primer 3.0*.

Gene expression analysis through real-time quantitative polymerase chain reaction

In order to protect Antarctic ecosystems and to encourage preventative mitigation strategies against the potential negative effects from CEC exposure, here we investigated the ability of an Antarctic freshwater crustacean (*B. gaini*) to cope with n-TiO_2 exposure by analysing the expression of several genes involved in stress response (Table I). The candidate genes were investigated through quantitative real-time quantitative polymerase chain reaction (RT q-PCR), using the mean expression of

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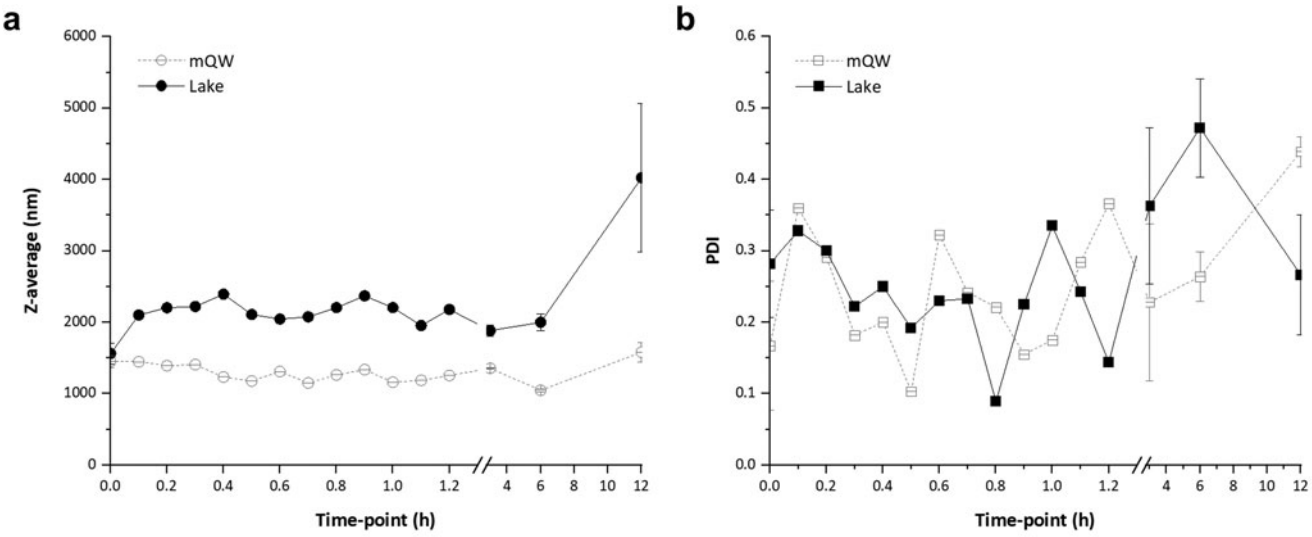

Fig. 2. Aggregation kinetics of nano-TiO_2 (at 200 µg ml^{-1}) in rock pool water (Lake; 0.20 µm filtered, pH 8.22, 4°C) and Milli-Q water (mQW). **a.** Z-average values (nm) and **b.** polydispersity index (PDI; dimensionless) obtained *via* dynamic light scattering are reported as means ± SDs of three measurements each at 0, 3, 6 and 12 h after preparation and as single measurements at 6 min intervals within 1.2 h of suspension.

glyceraldehyde 3-phosphatase dehydrogenase (*Gapdh*) and low-density lipoprotein receptor-related protein 8 (*Lrp8*) as housekeeping genes. The retrotranscription was performed using 1 µg of RNA extracted by means of the M-MLV Reverse Transcriptase kit (Invitrogen, Waltham, MA, USA), using random hexamers and oligo(dT) as primers for the synthesis of cDNA. RT q-PCR was carried out using the Brilliant II SYBR® Green QPCR Master Mix kit (Agilent Technologies) in an Mx3005P QPCR System thermocycler (Agilent Technologies), analysing each gene in triplicate for each sample. The validation of *Lrp8* and *Gapdh* as constitutive genes was carried out using the *BestKeeper* software (Pfaffl *et al.* 2004). The comparison of the expression levels of the targets genes was carried out by the $2^{-\Delta\Delta C(T)}$ method (Livak & Schmittgen 2001). Further details on primer sequences are presented in Table S1.

Statistical analysis

Gene expression datasets were tested for Gaussian distribution using the Shapiro-Wilk test. Homogeneity of variance was assessed using the Brown-Forsythe test for equal variances. Expression data of the reference genes were tested for differences between treatments using one-way analysis of variance and Tukey's *post hoc* test. In case of non-normally distributed data and non-equal variances, the analysis was performed using Mann-Whitney tests. All statistical analyses were conducted using the *GraphPad Prism* version 7.0 software for Windows (GraphPad Software, San Diego, CA, USA).

Results

None of the tested concentrations (range 50–200 µg ml^{-1}) cause mortality in the Antarctic fairy shrimps. The stability and particle size distribution of n-TiO_2 in mQW and rock pool waters were investigated at time 0 and after 3, 6 and 12 h. The DLS results showed similar patterns for n-TiO_2 in rock pool waters and in mQW soon after preparation of the suspensions (Fig. 1 & Table S2). The aggregation kinetics within the first hour after dispersion confirmed the presence of stable populations of n-TiO_2 aggregates with sizes of ~1500 and 1800 nm in mQW and rock pool waters, respectively (Fig. 2a).

However, in the exposure medium (i.e. rock pool water), a strong agglomeration and a broader size distribution of n-TiO_2 occurred with time, as is shown by the PDI values (Fig. 2b), with an increase in hydrodynamic diameter observed at 6 and 12 h corresponding to Z-averages of 2000 ± 120 and 4024 ± 1042, respectively (Fig. 2 & Table S2). The agglomeration of n-TiO_2 thus appeared to be time-dependent.

All genes involved in antioxidant response capacity, moulting and xenobiotic responses were upregulated after n-TiO_2 exposure (Fig. 3). The very short exposure time (9 h) allowed us to observe the expression of the genes at an early stage of the toxicity response.

Our study investigated such responses at the molecular level in *B. gaini*, where the gene expression levels of *Sod* (50 µg ml^{-1}, $P = 0.0260$; 200 µg ml^{-1}, $P = 0.0087$) and *GPx* (50 µg ml^{-1}, $P = 0.0033$; 100 µg ml^{-1}, $P = 0.0187$; 200 µg ml^{-1}, $P = 0.0432$) showed significant downregulation following n-TiO_2 exposure, suggesting a decreased capacity

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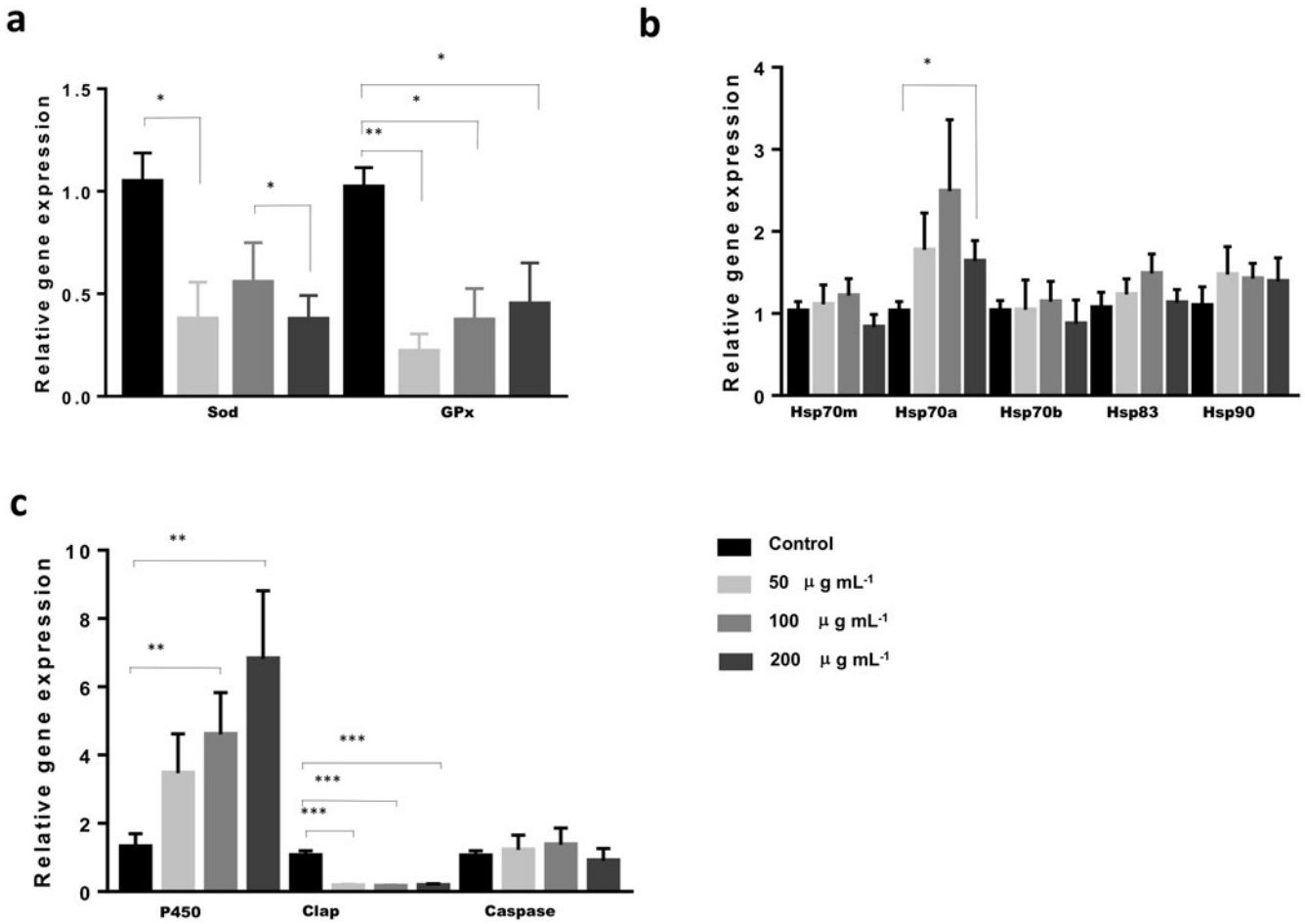

Fig. 3. Gene expression in *Branchinecta gaini* after exposure to nano-TiO_2 determined using real-time quantitative polymerase chain reaction. Cell stress responses are shown for genes involved in **a.** xenobiotic response, **b.** moulting and apoptosis processes and **c.** antioxidant activity. All data were obtained from two independent experiments and each experiment was performed in triplicate. Significant differences in the *Hsp70a* and *P450* expression levels in terms of two-group comparisons were assessed using Mann-Whitney tests. Asterisks represent Tukey's *post hoc* test results following one-way analysis of variance, expressed as means ± standard errors: $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$.

to reduce reactive oxygen species (ROS) in exposed specimens (Fig. 3a).

In order to better understand the molecular mechanisms by which n-TiO_2 could induce toxicity, the expression levels of five Hsps were investigated. Our results showed a gradual upregulation of only *Hsp70a* compared with the control when exposed to high concentrations of n-TiO_2; however, significant upregulation was only found in specimens exposed to the highest concentration of n-TiO_2 (200 µg ml^{-1}; $P = 0.0411$; Fig. 3b) when the data were analysed in a non-parametric way.

The expression of the *Cyp450* gene in *B. gaini* was upregulated at 100 and 200 µg ml^{-1} of n-TiO_2 ($P = 0.0087$ and $P = 0.087$, respectively; Fig. 3c), suggesting that this gene may be involved in the response to n-TiO_2. On the other hand, strong downregulation of *Clap* gene expression was also observed at all concentrations tested (50 µg ml^{-1}, $P = 0.0001$; 100 µg ml^{-1}, $P = 0.0001$; 200 µg ml^{-1}, $P = 0.0001$; Fig. 3c). Finally, the relative expression level of the caspase-1 transcript remained unchanged with respect to the control (Fig. 3c), indicating that n-TiO_2 did not affect the expression of this gene involved in the apoptotic process.

Discussion

Although the occurrence of n-TiO_2 in the Antarctic environment has not been reported yet, traces of pharmaceutical and personal care products, including sunscreens, have been found in sewage effluents, costal seawaters and sea ice near Antarctic research stations (Emnet *et al.* 2015). As a UV blocker, n-TiO_2 pigments are commonly used in several commercial products (e.g. paints, sunscreens and textiles), and although its release from formulations has been demonstrated (Al-Kattan

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et al. 2013, Tovar-Sánchez *et al.* 2013, Gondikas *et al.* 2014), there are still major technical limitations to accurately detecting and quantifying its occurrence in wastewater discharges and to linking such exposures to the documented effects on aquatic biota. Here, we provide the first results showing the responsiveness and sensitivity of an Antarctic freshwater crustacean to n-TiO_2 exposure and highlight its suitability as a biomonitor of CECs in Antarctica.

In aquatic environments, the agglomeration of n-TiO_2 has been found to be controlled by the presence and concentration of dissolved and particulate organic matter, pH and ionic strength (Brunelli *et al.* 2013). The resulting micron and submicron-sized n-TiO_2 agglomerates have been found to be taken up by organisms and producing toxic effects and changes in the expression of some genes, such as the suppression of antioxidant-related genes (Corsi *et al.* 2020).

To date, nano-ecotoxicity studies evaluating the acute effects of NPs on crustaceans at the molecular level have mostly considered gene expression related to antioxidant enzymes such as Sod, catalase, GPx and glutathione S-transferase (Kim *et al.* 2010, Clemente *et al.* 2014, Kögel *et al.* 2020). Recently, Thiagarajan *et al.* (2020) showed that n-TiO_2 caused reduced Sod activity in *Artemia salina*. It is well known that n-TiO_2 increases ROS production and could activate antioxidant defence mechanisms such as Sod and GPx to protect cells from oxidative stress. In some cases, the literature remains inconclusive regarding the effects of n-TiO_2, with studies reporting contradictory findings on the activity of these enzymes (Clemente *et al.* 2014).

Regarding cellular responses to exposures to toxicants such as nanometals, these could be used as early markers of toxicity *via* the induction of Hsp expression. The effect of n-TiO_2 on the overexpression of the *Hsp70* gene has been demonstrated previously in the human bronchial epithelial cell line (Okuda-Shimazaki *et al.* 2010). However, in the human alveolar type II-like epithelial cell line n-TiO_2 induced cytotoxicity but did not induce *Hsp70* and *Grp78* expression (Aueviriyavit *et al.* 2012). Previous research showed that the crustaceans *Artemia franciscana* and *Daphnia magna* respond to environmental stress by producing the Hsps, which play an important role in mitigating cellular damage (Kim *et al.* 2017, Gbotsyo *et al.* 2020). However, whether Hsps are involved in the biological responses to n-TiO_2 in crustaceans is currently unknown. Decreased detoxification due to Hsp declines also lead to tissue injury or cell death (Shah *et al.* 2017).

The sequence identified in the transcriptome of *B. gaini* belongs to the Cyp4 family of proteins, which are involved in ecdysteroids hormone metabolism and xenobiotic metabolism in arthropods (James & Boyle 1998, Baldwin *et al.* 2009). The increased expression of *Cyp450* may be responsible for hormone synthesis and metabolism, resulting in endocrine disruption. Therefore, the observed upregulation of this gene raises further questions regarding the ability of n-TiO_2 to disrupt hormone synthesis and metabolism in the fairy shrimp. Cytochrome P450 monooxygenases catalyse the oxidation and metabolism of a large number of endogenous compounds, including xenobiotics (Shankar & Mehendale 2014). In marine invertebrates, P450s play a key role in physiological adaptation to environmental pollutants (Rewitz *et al.* 2006).

Clap is involved in key processes related to crustacean development, particularly moulting (Le Boulay *et al.* 1998, Butler *et al.* 2001). Qiao *et al.* (2017) demonstrated moulting stage-regulated expression during the five moulting stages of a kuruma shrimp *Marsupenaeus japonicus*, indicating its role in the ontogenic development of this species. The full-length cDNA sequences of *Clap* isolated from *B. gaini* has two main domains characteristic of proteases (the cathepsin pro-peptide inhibitor domain (I29) and the papain family cysteine protease), confirming its potential functional role. Zhang *et al.* (2020) have reported a similar reduction in cathepsin gene expression in *Daphnia pulex* upon exposure to polystyrene NPs for 21 days). The contribution of caspases to the apoptotic process is evolutionarily conserved (Menze *et al.* 2010). The role of caspase 1 is well determined in vertebrates, and the activated caspase 1 has the capacity to cleave IL-1β into the mature protein form. However, reports characterizing the biochemical regulation of caspases in crustaceans are scarce and focused more on their cloning and molecular characterization. Caspase 1-mediated cell death was activated by white spot syndrome virus to counteract viral infection (Yang *et al.* 2019), and in another study, the expression of caspase-1 in *Artemia sinica* was measured at various times during embryonic development, and it was found that gene expression increased during early embryo development (Chu *et al.* 2014).

Our results provide the first assessment of the acute short-term effects of n-TiO_2 on an Antarctic freshwater crustacean species. Future studies should focus on toxicity over longer exposure times and at relevant predicted environmental concentrations, if available. Furthermore, in order to predict the consequences for early life stages, studying the effects of n-TiO_2 on the embryonic and larval stages of *B. gaini* is recommended. In this regard, the transcriptomic analysis performed on *B. gaini* recommends its use as a suitable model organism to monitor the ice-free freshwater systems of the Maritime Antarctic and the Antarctic Peninsula region, which are areas of high anthropogenic activity and hence heavily influence the current and predicted impacts of contamination and climate change (Siegert *et al.* 2019, Morley *et al.* 2020).

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Future studies assessing the risk posed by n-TiO_2 as a model CEC to Antarctic biota under relevant natural scenarios are encouraged, as well as those aiming to determine the environmental concentrations of this pollutant to facilitate environmental management.

Supplemental material

Supplemental tables can be found at https://doi.org/10.1017/S0954102022000086.

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Author contributions

MG-A, GI and EB conceived the experimental design. GI carried out the experimental assays. EB carried out the dynamic light scattering analysis. RR and AF carried out the sequence analyses and transcriptome production. GI and MG-A carried out the statistical analyses. EB, IC, CAC and MG-A wrote the manuscript with contributions from AF, RR and GI. All authors read and approved the final manuscript.

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