



Short communication

Under pressure: Nanoplastics as a further stressor for sub-Antarctic pteropods already tackling ocean acidification

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ABSTRACT

In the Southern Ocean (SO), plastic debris has already been found in waters and sediments. Nanoplastics (<1 μm) are expected to be as pervasive as their larger counterparts, but more harmful to biological systems, being able to enter cells and provoke toxicity. In the SO, (nano)plastic pollution occurs concomitantly with other environmental threats such as ocean acidification (OA), but the potential cumulative impact of these two challenges on SO marine ecosystems is still overlooked. Here the single and combined effects of nanoplastics and OA on the sub-Antarctic pteropod *Limacina retroversa* are investigated under laboratory conditions, using two surface charged polystyrene nanoparticles (PS NPs) as a proxy for nanoplastics. Sub-Antarctic pteropods are threatened by OA due to the sensitivity of their shells to changes in seawater carbonate chemistry. Short-term exposure (48 h) to PS NPs compromised the ability of pteropods to counteract OA stress, resulting in a negative effect on their survival. Our results highlights the importance of addressing plastic pollution in the context of climate change to identify realistic critical thresholds of SO pteropods.

1. Introduction

Plastic pollution has become a global environmental issue, reaching even the most remote wildernesses of our planet, including the Southern Ocean (SO). Microplastics (0.001–1 mm) have been documented in SO surface waters (Jones-Williams et al., 2020; Lacerda et al., 2019; Suaria et al., 2020) and within the digestive tracts of Antarctic marine biota (Bessa et al., 2019; Eriksson and Burton, 2003; Sfriso et al., 2020). Fragmentation of microplastics down to the nanoscale (<1 μm) by the Antarctic krill has also been demonstrated under laboratory settings (Dawson et al., 2018) raising concern on the potential enhanced bioavailability of the so-called nanoplastics to polar marine organisms.

Although not documented in the SO, nanoplastics are expected to be as pervasive as their larger counterparts, as a common point source emission of all plastic debris is most likely (Waller et al., 2017). Laboratory studies conducted using polymeric nanoparticles (e.g., polystyrene nanoparticles, PS NPs) as proxy for nanoplastics have documented several negative outcomes, up to lethality, on marine plankton, as reviewed by Corsi et al. (2020). For instance, in Antarctic krill juveniles the observed sub-lethal effects were related to PS NP

functionalization and surface charge, with alteration in krill behaviour and moulting caused by positively charged aminated (–NH₂) PS NPs (Bergami et al., 2020).

In the SO, (nano)plastic pollution occurs concomitantly with other environmental and anthropogenic challenges. Considered a ‘bellwether’ for ocean acidification (OA) (Fabry et al., 2011), one of the most serious threats to marine ecosystems this century, the SO is subject to some of the most rapid declines in surface ocean pH and shoaling of the carbonate saturation horizon. In the SO region, calcium carbonate (CaCO₃) undersaturation is projected to occur by 2030 (McNeil and Matear, 2008). CaCO₃ undersaturation is exacerbated by the concentration of natural carbonate ion (CO₃²⁻) already low, higher solubility of CO₂ in cold waters and, freshwater input from melting sea ice.

Interactions between climate change-related stressors, such as OA, and emerging contaminants such as nanoplastics is therefore critical to understand the potential cumulative effect of multi-stressors on marine ecosystems. These interactions can be particularly relevant in the SO (Rowlands et al., 2021a, 2021b), where marine organisms have adapted to an extreme but stable environment (Meyer et al., 2015; Zane and Patarnello, 2000).

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The Sub-Antarctic pteropod *Limacina retroversa* is the most abundant thecosome pteropod North of the polar front in the SO (Bernard and Froneman, 2009). These holoplanktonic gastropods represent a significant component of SO pelagic zooplankton communities, are a key trophic link within SO food webs (Hunt et al., 2008) and a major conduit for the export of carbon to the ocean interior (Manno et al., 2007, 2019). Thecosome pteropods like *L. retroversa* are considered sentinels for OA due to their aragonite shells, a relatively soluble polymorph of CaCO₃, being vulnerable to changes in seawater carbonate chemistry (Mucci, 1983), particularly during early life stages (Bednaršek et al., 2012; Gardner et al., 2018; Manno et al., 2017).

In the SO region, the deleterious effects of OA on pteropods' shell morphology and embryonic development have already been reported (Bednaršek et al., 2012; Manno et al., 2016; Mekkes et al., 2021). Further, the observed combined impact of OA and other stressors (i.e., freshening, warming) on pteropods physiology (Gardner et al., 2018; Lischka and Riebesell, 2012; Manno et al., 2017) highlights how these organisms can act as excellent target species to identify thresholds in response to multiple anthropogenic stressors.

To reinforce this aim, here the single and combined effects of OA and surface charged PS NPs as a proxy for nanoplastics have been investigated on the sub-Antarctic pteropod *L. retroversa* under controlled laboratory conditions.

2. Methods

(a) Sampling

Pteropods were collected from the upper 50 m of the water column of the Scotia Sea in December 2016 (RRS James Clark Ross, cruise JR16003). Sampling was performed using a motion-compensated bongo net (200 µm mesh), which limits shell mechanical damage and minimises stress of pteropods. Specimens of the Sub-Antarctic pteropod *L. retroversa* were sorted using a stereomicroscope and only individuals (Lalli and Gilmer, 1989) of similar diameter (size range from 1200 to 1350 µm), actively swimming and bearing an intact shell were selected for the study.

(b) Short-term in vivo exposure

Fluorescently labelled PS-COOH and unlabelled PS-NH₂ NPs, with a nominal diameter of 60 and 50 nm, respectively (see the supplementary material for further details) were added to 50 mL incubation jars filled with 0.2 µm filtered sea water (FSW, see Table S1 for physico-chemical parameters) to reach the final concentration of 1 µg mL⁻¹. This concentration corresponds to about 8.35·10⁹ NPs mL⁻¹ and 1.46·10¹⁰ NPs mL⁻¹ for PS-COOH and PS-NH₂, respectively ("Bangs Laboratories Inc. TechNote 206") and was chosen based on half maximal effect concentration (EC₅₀) values, i.e., the concentration of PS NPs causing 50% negative effect, estimated for marine zooplankton in previous studies (Della Torre et al., 2014; Eliso et al., 2020; Manfra et al., 2017) and based on our previous findings on Antarctic species (Bergami et al., 2020).

Low pH (7.8) was set up to simulate equilibrium with atmospheric pCO₂ of 750 ppm in OA treatments. FSW chemistry was manipulated adding a combination of Na₂CO₃ and acid (HCl) to alter pH while maintaining total alkalinity (TA) (EUR 24872 – Guide to best practices for ocean acidification and data reporting, 2011), as described in the supplementary material. Sub-samples of SW were fixed with 2% Mercuric Chloride for shore-based carbonate chemistry analysis (see Table S1).

Pteropods were gently transferred into the jars (*n* = 12 individuals per jar), which were then sealed to prevent CO₂ exchange with the atmosphere. The 6 treatments (control, OA, PS-COOH, PS-NH₂, combined OA and PS NPs) were run in triplicate, in the dark, at constant temperature (+2 °C). At 24 and 48 h, the jars were inspected to record pteropods' mortality and at the end of the incubation (48 h), specimens were stored in 70% buffered Ethanol for further analysis.

(c) Detection of PS NPs and analysis of shell condition

The presence of PS NPs on the external surface of pteropods' shells

was determined through optical fluorescent microscopy (Olympus BX51 with a Canon EOS 70D camera) for fluorescently labelled PS-COOH and scanning electron microscope (Zeiss Gemini SEM 500) for both PS NPs. Specimens from the control group were included as reference (absence of PS NPs). Individuals were carefully washed in mQW before imaging.

The investigation of shell morphology was performed using a variable pressure SEM (VP-SEM, TM3000, Hitachi). For each treatment, 12 individuals were imaged (*n* = 4 per replicate). Pteropod preserved shells were dehydrated through a series of ethanol solutions (Gardner et al., 2018), mounted on carbon conductive double-faced adhesive tapes and imaged at 1200× magnification. The outer organic layer of the shells (i.e., the periostracum) was not removed prior SEM (Peck et al., 2016).

(d) Data analysis

Data of mortality are reported as mean ± Standard Deviation (SD). Statistical analysis was performed using GraphPad Prism 9.1.2 software for Windows. Data of mortality (%) were arcsin transformed and analysed by Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons. Data were considered statistically significant at *p*-value < 0.05.

3. Results

The behaviour of PS NPs in FSW from the Scotia Sea where pteropods were collected is reported in Bergami et al. (2020). Both PS NPs kept their nominal size in FSW (+2 °C) (Fig. S1) and negative (PS-COOH) and positive (PS-NH₂) surface charges. Any potential change in PS NP behaviour related to the difference in pH in natural (pH of 8.1) and manipulated FSW (pH of 7.8) was considered negligible based on the literature (see supplementary material).

After 48 h of exposure, the control group (FSW only) exhibited a mortality rate of 8.3 ± 0%, used as reference for the validity of the test. A slight increase in mortality (11.1 ± 4.8%) vs control was observed in pteropods exposed to OA and to PS-COOH, while those exposed to PS-NH₂ showed a higher mortality rate of 36.1 ± 4.8%. However, these mortality rates were not significantly different with respect to the control (*p*-value = 0.2370). The combined exposure to OA and PS NPs further increased pteropods' mortality to 22.2 ± 4.8% in those exposed to OA + PS-COOH and to 47.2 ± 4.8%, in OA + PS-NH₂ treatment, the latter found significantly different from the control group (*p*-value = 0.0347, *Z* = 3.046) (Fig. 1).

Optical fluorescent microscopy showed the presence of small agglomerates of fluorescently labelled PS-COOH (1 µg mL⁻¹) on pteropods' shell surface, mostly localised at the shell aperture and apex (Fig. S2). SEM analysis further confirmed the presence of both PS NPs (PS-COOH and PS-NH₂) onto pteropods' shells (Fig. S3).

Transmitted and oblique light microscopy revealed transparent shells in controls as well as in specimens exposed to PS NPs regardless of their surface charges, while pteropods exposed to reduced pH conditions (i.e., OA treatments) showed shells of uniform opacity (Fig. 2a). VP-SEM analysis of the same specimens revealed that the external surface of shells exposed to ambient pH conditions was pristine, with the typical surface ornamentation characteristic of *L. retroversa* preserved specimens. Investigation at higher magnification showed that the periostracum had a smooth, taut surface, suggesting that the underlying prismatic crystals were fully intact. The external surface of shells exposed to reduced pH conditions also maintained the surface ornamentation, but high magnification revealed a rough texture of the shells surface (Fig. 2b). The periostracum no longer formed a smooth outer surface, appearing to have collapsed with the absence of prismatic crystals, indicating that the outer crystalline layer of the shell was compromised by dissolution.

4. Discussion

In this study we investigated the single and combined effects of nanoplastics and OA on the sub-Antarctic pteropod *L. retroversa*. In line

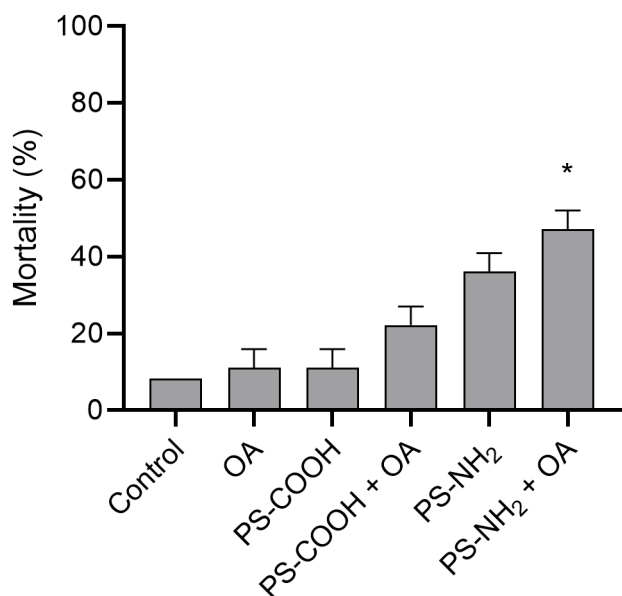


Fig. 1. Mortality (%; Mean \pm SD) of *L. retroversa* pteropods upon 48 h exposure to PS NPs ($1 \mu\text{g mL}^{-1}$) and/or OA treatment at reduced pH (n per experimental group = 36). Control group showed an average mortality of $8.3 \pm 0\%$. The combined OA + PS-NH₂ group showed an average mortality of $47.2 \pm 4.8\%$, significantly different from the control group. (single-column fitting image).

with previous findings on this species (Mekkes et al., 2021), exposure to low pH (7.8) conditions caused no significant mortality but evident sublethal effects on pteropod shells, which exhibited signs of degradation. Mekkes and co-workers reported a reduced active calcification over their entire shell in *L. retroversa* juveniles incubated for 3 days at near-future pH (7.93), suggesting that pteropods were allocate energy elsewhere to counteract unfavourable conditions.

Undersaturation with respect to aragonite (i.e., when aragonite saturation state, Ω_{ar} , is below 1) thermodynamically promotes carbonate dissolution over precipitation in SW. As a result, the exposed aragonite dissolves, with concurrent decline in pteropods shell calcification (Comeau et al., 2009; Lischka and Riebesell, 2012). These OA-driven processes are particularly relevant for vulnerable Antarctic marine calcifiers (Byrne et al., 2013), including pteropods (Bednaršek et al., 2012; Gardner et al., 2018), whose shells dissolve rapidly at elevated pCO₂.

Concerning nanoplastics, no effect on pteropod mortality was found regardless of PS NP surface charge. However, the exposure to PS-NH₂ did increase pteropods' mortality by 28% and 25% compared to control and PS-COOH, respectively. These findings are consistent with previous studies showing a high toxicity of PS-NH₂ on marine zooplankton compared to their negatively charged counterparts (PS-COOH) (Corsi et al., 2020; Venâncio et al., 2019) and also documented in Antarctic species (Bergami et al., 2020). Heightened toxicity of PS NH₂ has been related to the "proton sponge" hypothesis by which the positive charge of -NH₂ groups can disrupt cell membranes bearing negative charges (Nel et al., 2009).

Co-exposure to both PS NPs and OA led to a general increase in pteropod mortality, suggesting an additive effect of the two stressors. Such increase was significant in the OA + PS-NH₂ group compared to the control, indicating the more detrimental effect of positively charged PS-NH₂ compared to PS-COOH. To our knowledge, this is the first study to investigate the impact of the combined OA and nanoplastic stress on a polar calcifying organism.

Plastic pollution as a further anthropogenic stressor for calcifying organisms already facing the challenges of OA has only drawn attention in recent years. The Korean mussel (*Mytilus coruscus*) exposed to PS microspheres ($2 \mu\text{m}$; 10^4 and 10^6 particles L⁻¹) and OA (pH 7.7) for 14

d showed a significant inhibition in the activity of digestive enzymes (additive effect), resulting in a lower filtration rate of the mussels, though they fully recovered after 7 d in clean SW (Wang et al., 2020). No changes in the gut microbiome of *M. coruscus* were found upon 21 d exposure to PS microspheres ($10 \mu\text{m}$; 10^3 particles L⁻¹) and OA (pH 7.3) (Yang et al., 2020), suggesting a lower vulnerability of hard-shelled bivalves to these multi-stressors compared to other marine mollusks having thin shells. Differently, antagonistic effects of PS microspheres ($\approx 10 \mu\text{m}$; 10^3 and $3 \cdot 10^3$ particles L⁻¹) and low pH (7.6) have been reported in pluteus larvae of the sea urchin *Paracentrotus lividus*, in which exposure to microplastics did not exacerbate OA impact on larval growth (Bertucci and Bellas, 2021).

Our findings indicate that short-term exposure to nanoplastics may compromise the ability of Sub-Antarctic pteropods to counteract OA stress, resulting in a negative effect on their chance of survival. The tolerance and resilience of pteropods to near-future OA conditions is related to the metabolic cost that these organisms require to repair and maintain their shells when their habitat is undersaturated with respect to aragonite ($\Omega_{\text{ar}} \leq 1$) (Lischka et al., 2011; Peck et al., 2016). However, changes in the energy budget allocation for shell production to counteract OA would likely alter ecological trade-offs between calcification and physiological functions (Watson et al., 2017). Here we suggest that when aragonite undersaturation stress is interacting with other in situ stressors, such as nanoplastics, the total metabolic cost can exceed the available energy budget necessary to compensate other vital physiology activity resulting in an impact on pteropod survival.

Thecosome pteropods graze on phytoplankton and smaller zooplankton, down to $0.5 \mu\text{m}$ in size (Howes et al., 2014), using their mucus webs or through passive flux feeding (Hunt et al., 2008; Jackson, 1993). The presence of PS NP agglomerates both attached onto and inside pteropod shells indicates that these nanoplastics are bioavailable for this species upon short-term exposure. Attachment and ingestion of NPs, as recently observed in juvenile Antarctic krill (Bergami et al., 2020) suggesting that NP be transferred up trophic levels upon predation. Such potential biomagnification is particularly relevant as pteropods constitute an efficient link between phytoplankton and higher trophic levels in the SO (Hunt et al., 2008; Lalli and Gilmer, 1989), including ecologically and/or economically relevant fish, seabirds, and whales (Armstrong et al., 2008; Karpenko et al., 2007).

Our results provide a first insight on the importance of addressing plastic pollution in the context of global climate change to understand the full impact of anthropogenic and environmental stressors on SO pteropods. Future studies should focus on a deeper understanding of the mechanisms of action of nanoplastics and their interaction with OA targets at higher levels of complexity using an interdisciplinary effort able to integrate empirical, experimental, and modelling approaches.

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CRedit authorship contribution statement

C.M. and E.B. designed the experiment, carried out the fieldwork and analyses, and discussed the data. V.L.P. provided support and expertise with microscope analyses. I.C. provided support and expertise with data discussion and theoretical overviews on nanoplastics pollution. All authors contributed to the writing of the manuscript.

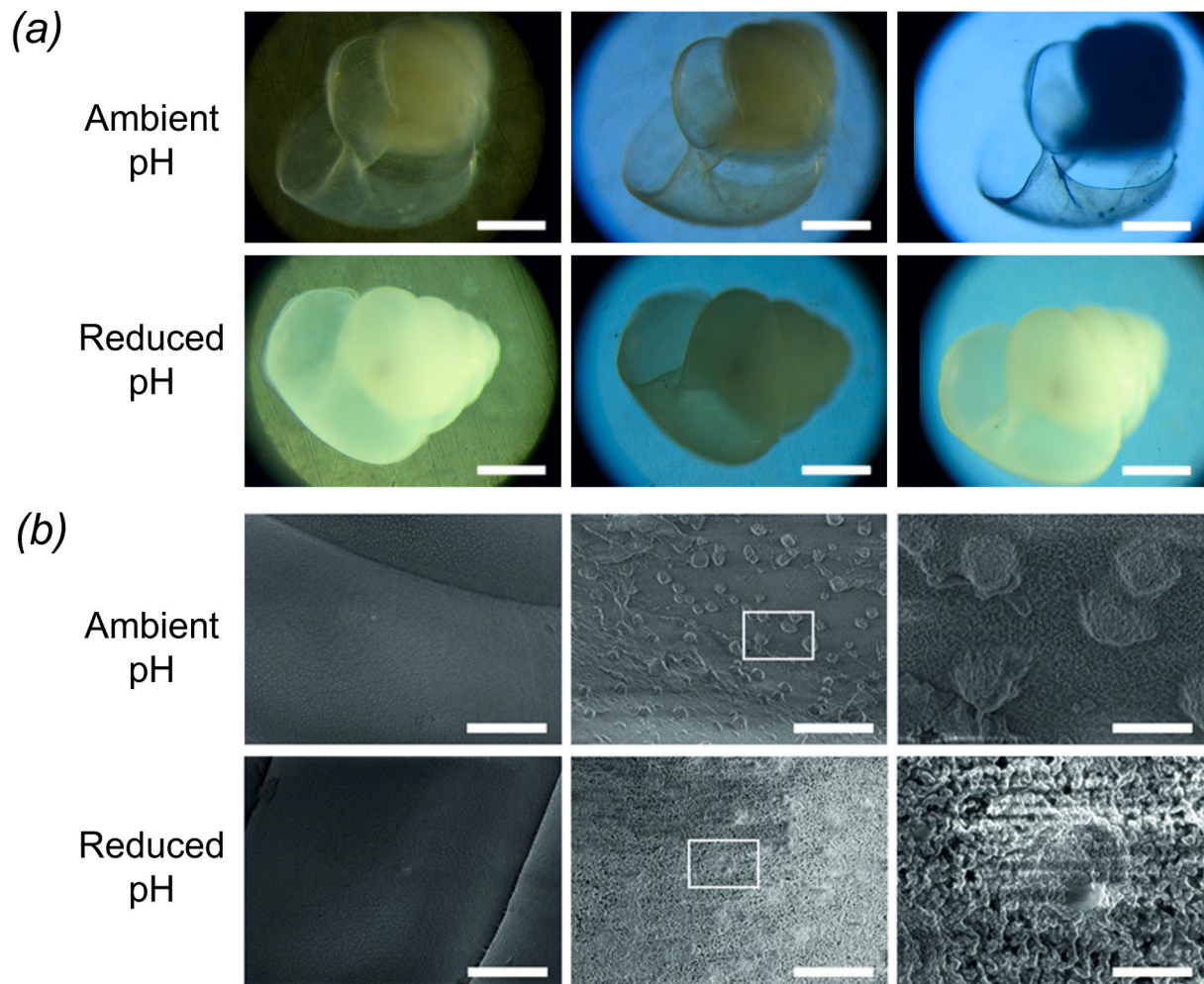


Fig. 2. Representative specimens of *L. retroversa* pteropods (a) shells observed under light microscopy, from ambient pH (8.1, top) and at reduced pH (7.8, bottom) showing transparent shells (top) and shells of uniform opacity (bottom). From left to right: incident light, transmitted light and combined incident and transmitted light. Scale bar: 500 μm ; (b) details of shells at VP-SEM microscopy from ambient pH (8.1, top) and at reduced pH (7.8, bottom) showing detailed views of a pristine shell (top) and shell crystalline layer compromised by dissolution (bottom). Scale bar: 100 μm , 10 μm and 2 μm , from left to right. (2-column fitting image).

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2021.113176>.

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