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Polystyrene nanoplastics in the marine mussel Mytilus galloprovincialis.

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Abstract:	Concerns about plastic pollution and its toxicity towards animals and people are growing. Polystyrene (PS) is a plastic polymer highly produced in Europe for packaging purposes and building insulation amongst others. The marine environment is inevitably the fate of PS products, whether originating from illegal dumping, waste mismanagement or lack in treatment for removal of plastic debris from wastewater treatment plants. Nanoplastics (< 1000 nm) is the new focus for plastic pollution gaining a wide array of interest. Whether primary or secondary, their relatively small size permits nanoparticles to cross cellular boundaries, and consequently lead to adverse toxic effects. An in vitro assay of Mytilus galloprovincialis haemocytes exposed to 10 µg/L of polystyrene nanoplastics (nPS; 50 nm) for 24 h was used to test cellular viability along with the luminescence inhibition (LC50) of Aliivibrio fischeri bacteria to evaluate acute toxicity. Cellular viability of mussel haemocytes decreased significantly after a 24 h exposure and nPS LC50 range from 180 and 217, µg/L. Moreover, a 28-day exposure of the marine mussel M. galloprovincialis to nPS (10 µg/L; 50 nm) was carried out with the aim to evaluate neurotoxic effects and the ingestion of these plastic particles in three mussel tissues (gills, digestive gland, and gonads). The ingestion of nPS was time and tissue specific, suggesting that nPS are ingested through the gills and then translocated through the mussel bloodstream, to the digestive gland and gonads where the highest amount of ingested nPS was found. Once nPS enters these plastic particles are transported to mussels tissues, with the gonads and digestive glands being the tissue-deposits for these particles. Data on acetylcholinesterase inhibition and those previously obtained on a wide range of cellular biomarkers were elaborated through weighted criteria providing a synthetic assessment of cellular hazard from nPS.
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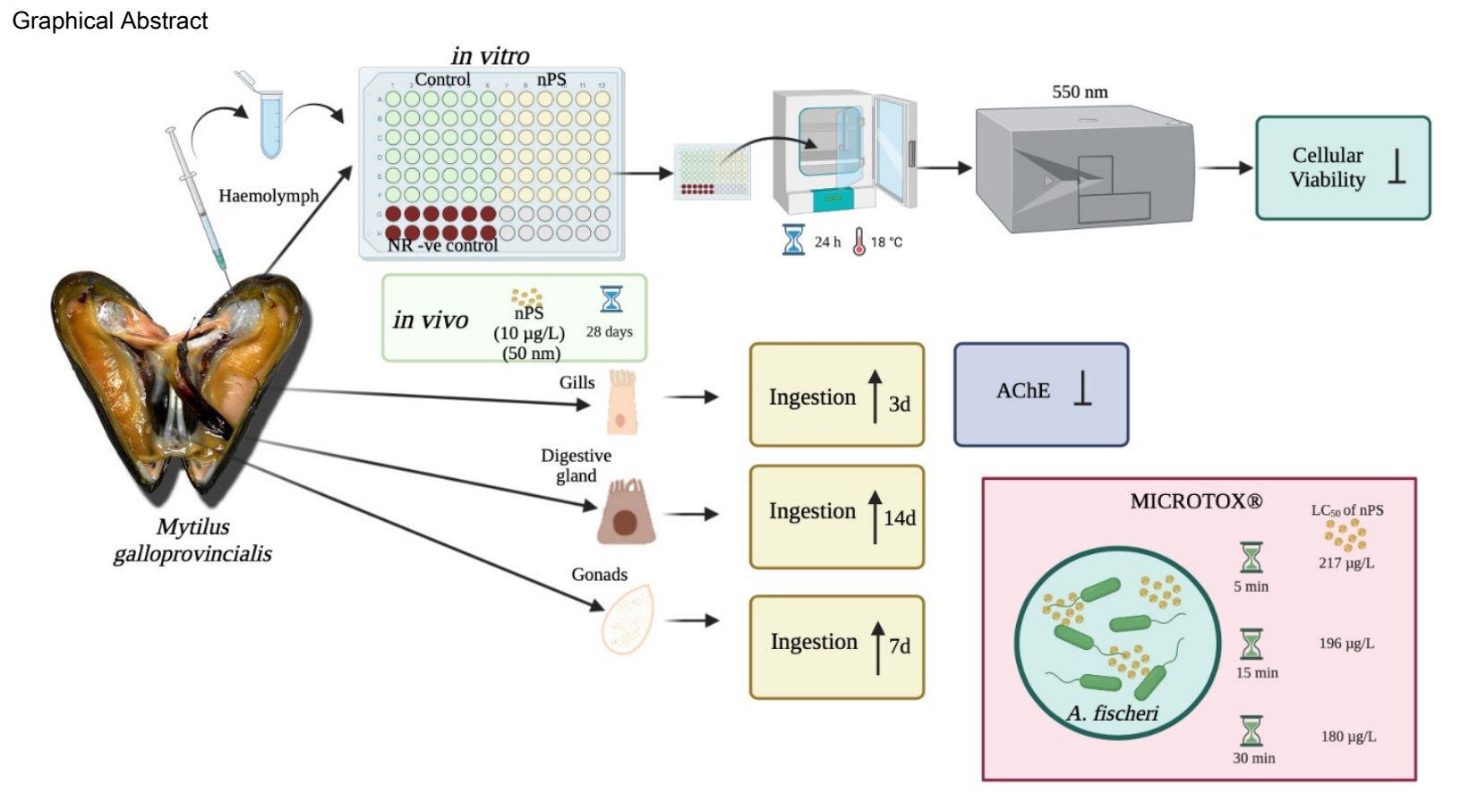
Highlights

- LC_{50} of nPS range from, 180 and 217 µg/L.
- Ingestion of nPS is tissue and time specific.
- Mussel gonads present the highest ingested µg of nPS per wet weight.
- Mussel haemocytes cellular viability decreased after *in vitro* exposure to 10 µg/L of nPS.
- Neurotoxicity occurs in mussel gills.

Graphical Abstract



Polystyrene nanoplastics in the marine mussel *Mytilus galloprovincialis*.

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Abstract

Concerns about plastic pollution and its toxicity towards animals and people are growing. Polystyrene (PS) is a plastic polymer highly produced in Europe for packaging purposes and building insulation amongst others. The marine environment is inevitably the fate of PS products, whether originating from illegal dumping, waste mismanagement or lack in treatment for removal of plastic debris from wastewater treatment plants. Nanoplastics (< 1000 nm) is the new focus for plastic pollution gaining a wide array of interest. Whether primary or secondary, their relatively small size permits nanoparticles to cross cellular boundaries, and consequently lead to adverse toxic effects. An *in vitro* assay of *Mytilus galloprovincialis* haemocytes exposed to 10 µg/L of polystyrene nanoplastics (nPS; 50 nm) for 24 h was used to test cellular viability along with the luminescence inhibition (LC_{50}) of *Aliivibrio fischeri* bacteria to evaluate acute toxicity. Cellular viability of mussel haemocytes decreased significantly after a 24 h exposure and nPS LC_{50} range from 180 and 217, µg/L. Moreover, a 28-day exposure of the marine mussel *M. galloprovincialis* to nPS (10 µg/L; 50 nm) was carried out with the aim to evaluate neurotoxic effects and the ingestion of these plastic particles in three mussel tissues (gills, digestive gland, and gonads). The ingestion of nPS was time and tissue specific, suggesting that nPS are ingested through the gills and then translocated through the mussel bloodstream, to the digestive gland and gonads where the highest amount of ingested nPS was found. Once nPS enters these plastic particles are transported to mussels tissues, with the gonads and digestive glands being the tissue-deposits for these particles. Data on acetylcholinesterase inhibition and those previously obtained on a wide range of cellular biomarkers were elaborated through weighted criteria providing a synthetic assessment of cellular hazard from nPS.

1. Introduction

Concerns about plastic pollution and its toxicity towards animals and people are growing. Global plastic production (GPP) increased to 390.7 Mt in 2021 (PlasticEurope, 2022) and, among the consequences of illegal littering and dumping, waste mismanagement, as well as a low percentage of recycled plastics (8.3%) compared to fossil-based plastics (90.2%), the aquatic

environment is the inevitable fate of these materials. In Europe, plastic production was equivalent to 15% of GPP (57.2 Mt), with applications such as packaging and building and construction composing 39.1 and 21.3% of Europe's plastic demands, respectively (PlasticEurope, 2022). In 2021, the European Union adopted a directive banning all single-use plastics, except for bottles, as single-use plastic, and fishing gear which account for 70% of marine litter (Directive (EU) 2019/904).

Polystyrene (PS) is a plastic polymer highly produced for packaging purposes, building insulation, medical equipment, and toys as well as single-use plastics (PlasticEurope, 2022). Other than inexpensive and readily available, PS is known for its incredible dimensional stability and water resistance, permitting it to remain consistent in size and shape, along with being certified as safe for use in food and beverages (Block et al., 2017). In day-to-day products, for example, PS can be found in disposable cutlery, food containers, plates, cups, automotive and electrical components, and in household items. However, not all plastic products are recyclable, and PS products are not (The Waste and Resources Action Programme, 2023). With PS products placed in waste bins, waste mismanagement and illegal dumping, with the addition that wastewater treatment plants (WWTP) also lack the correct technique or method for plastic debris removal, the marine environment will be the endpoint for these PS particles. Once in the ocean, plastics undergo five main processes—hydrolysis, mechanical/physical degradation, thermal oxidative degradation, photo-degradation, and biodegradation (Andrady, 2011) — that break down and fragment plastics into smaller sizes. After much focus on microplastics (>1 and 5 mm), nanoplastics (< 1000 nm; nPS) are gradually gaining priority as nano-sized plastic particles may cross cellular boundaries, increasing their potential toxicity towards marine organisms (Peng et al., 2020). nPS in the marine environment can be either primary or secondary. Primary nPS enter the environment already in the nano-size range, and they are found in daily-use products such as in cosmetics, clothing fibres, drug delivery and in 3D ink printers (Bergami et al., 2016; Bessa et al., 2018; Canesi et al., 2015; Dong et al., 2019; Tamminga et al., 2018; Wang et al., 2018). The break down and fragmentation of macroplastics (> 5 mm) and microplastics (< 5 mm) generates secondary nPS. Although techniques to detect and quantify nPS in the ocean remains challenging, there is evidence for the presence of plastics in the nano-size range. In the North Atlantic Gyre, a nanoplastic segment was found containing PS in the nano-size range, as well as polyethylene, polyvinyl chloride, and polyethylene terephthalate (Halle et al., 2017); after simulation of coastal activities on a PS-cup and lid, nano-sized particles were liberated after 5 minutes (Ekvall et al., 2019).

The toxic effects of nPS in marine organisms have been evaluated (Manon Auguste et al., 2020; Capolupo et al., 2021; Ferreira et al., 2019; Joanna M. Gonçalves & Bebianno, 2021; Joanna M. Gonçalves et al., 2022; Kihara et al., 2021), showing that nPS mediated toxicity influences cell growth, larvae development, embryo malformation, inflammation, and also possible inactivation of photosystems in algae (Joanna M. Gonçalves & Bebianno, 2021; Kögel et al., 2020). In the marine

mussel *Mytilus galloprovincialis*, nPS provoke oxidative stress and damage (10 µg/L; 50 nm; 21-d) (Joanna M. Gonçalves et al., 2022), and lysosomal destabilization (1.5 – 150 ng/L; 50 nm; 21-d) (Capolupo et al., 2021). Although ingestion and translocation of MPs have been evaluated, the assessment of whether nPS toxicity is entirely from their ability to cross cellular boundaries, or if these nanoparticles are ingested, accumulated, and translocated between tissues remains difficult. In this study, a combination of *in vitro* and *in vivo* experiments was carried out to evaluate both acute toxicity and chronic effects of nPS on key model species. An *in vitro* assay on *M. galloprovincialis* haemocytes was performed to evaluate cellular viability after 24 h exposure to 10 µg/L of nPS (50 nm) and acute toxicity was further characterized through the Microtox® bioassay (LC_{50}). An *in vivo* 28-d exposure of mussels was performed at the same concentration of nPS to evaluate both ingestion of nanoparticles in gills, digestive gland, and gonads, as well as the onset of neurotoxicity in gills. These data were further integrated with those previously reported on a wide selection of cellular biomarkers; their overall elaboration through weighted criteria was expected to provide a better evaluation of the toxicological hazard of nPS.

2. Materials and Methods

2.1. Polystyrene nanoplastics (nPS)

Fluoresbrite® Plain YG spherical polystyrene nanoplastics of 50 nm in size (CAS 9003-53-6) were purchased from Polysciences, Inc. (Germany). 50 nm particles packed as 2.5% aqueous suspension, with 3.64 × 1014 particles/mL in ultrapure water (7732-18- 5) (CV = 15%, Excitation max. = 441 nm, Emission max. = 486 nm). Gonçalves et al. (2022) provides a full description of the characterization of nPS and demonstrate that the hydrodynamic diameter of nPS rises when distributed in FSW (852 103 nm), which suggests that the high salt content in seawater causes aggregation/agglomeration kinetics. A concentration of 10 µg/L was used for exposure assays.

2.2. Acute toxicity assay

The solid phase Microtox® bioassay (SPT, Azur Environmental, 1998) was used to evaluate polystyrene nanoplastics (nPS; 50 nm) acute toxicity in the bioluminescent marine bacterium *Aliivibrio fischeri* according to ISO 1134-3: 2007 and ISO 21338: 2010 according to the method described in Gambardella et al., 2019). All reagents and lyophilized *A. fischeri* bacteria (NRRL B-11177) were obtained from Modern Water Ltd (USA). Tests were carried out at 15ºC in the Microtox diluent supplied. The initial concentration of nPS used was 3724 µg/L and sequential diluted. Acute toxicity of nPS was measured in terms of relative bioluminescence by Microtox™ 500 luminometer after 5-, 15-, and 30-mins incubation. The bioluminescence inhibition was determined using the Microtox®FX equipment**,** and data was analysed using the MicrotoxOmni

software. The median lethal concentration (LC_{50}) was defined as the concentration that produced a 50% light reduction after 5-, 15-, and 30-mins of contact time for bacteria.

2.3. *In vitro* assay

Fifteen mussels *M. galloprovincialis* (60 ± 5 mm) were collected from an offshore aquaculture site in Lagos, Southeast Portugal (A: 37º04'200"N 8º42,800"W, B: 37º04'200"N 8º41'000"W, C: 37º03'400"N 8º41'000"W, D: 37º03'400"N 8º42'800"W; Testa & Cunhas Ltd) and transported alive to the laboratory. Mussels (2 mussels/L) were placed into a 10 L tank, with 7.5 L of seawater (S: 36 ± 1) and left to acclimatise for 48 hours. Then, mussel haemolymphs were extracted from the posterior adductor muscle of mussels with a sterile hypodermic syringe (1 mL) (25 G needle), under aseptic conditions in a vertical laminar airflow cabinet, and kept on ice. Cell extraction and incubation methods were based on the modifications of protocols developed by Gómez-Mendikute & Cajaraville (2003) and Katsumiti et al. (2014). To have sufficient haemolymph for *in vitro* exposures, five pools of three mussels per pool were used. Firstly, 10 µL of pooled haemolymph was diluted in anti-aggregation solution (171mM NaCl; 0.2M Tris; 0.15% v/v HCl 1 N; 24mM EDTA) to avoid clumping and aggregation of cells (Katsumiti et al., 2014). Trypan blue dye (0.4% in physiological solution; v/v) was added for cell staining in a 1:2 proportion (cell suspension: Trypan Blue 0.4%). Using a Neubauer chamber (200 cells per specimen) and with the aid of a haemocytometer and light microscopy (Compound Light Microscopy; 400x), cell viability was determined by the following equation:

$$Concentration\ (cells\ mL^{-1}) = \frac{nº\ cells\ \times\ 10\,000}{nº\ squares}\ \times\ dilution\ factor$$

Following cellular viability, cell suspensions were diluted at a density of 2 x 10^5 haemocyte cells/mL (in anti-aggregation solution), and 100 µL were seeded into 96-well microplates (6 replicated per pool) with cell culture media Dulbecco's Modified Eagle Medium (DMEM, pH 7.4) and exposed to 10 µg/L of nPS. Microplates were incubated for 24 h at 18 °C. Afterwards, culture media was discarded, and the neutral red (NR) assay was applied following an adaptation from the protocol described in Katsumiti et al. (2014). Culture medium from the wells were removed and state of cells were verified under a light microscopy (Compound Light Microscopy; 400x). 50 µL of neutral red working solution (0.4%, pH 7.3-7.4) was added to each well, including empty wells for negative control, and left to incubate for 1 hour in the dark. The microplate was then centrifuged at 270 *g* (4ºC, 10 mins) and the supernatant removed and carefully washed with PBS. The dye was then extracted from the viable cells in acetic acid/ethanol solution (1:100), following samples transference to U-bottom 96-well microplates and centrifuged at 270 *g* for 10 min, at 4 °C.

Supernatants were placed in flat bottom microplates and absorbance was measured at 550 nm (Infinite M200 Pro, TECAN®).

2.4. *In vivo* assay

Mussels *M. galloprovincialis* Lamarck, with a shell size of 60 ± 5 mm were collected from the Ria Formosa Lagoon, Southeast Portugal (37°00'30.6"N 7°59'39.6"W) and transported alive to the laboratory. Following a four-day acclimatisation period, a total of 50 mussels were placed into each 30 L tanks, with 25 L of seawater in a triplicate design, to follow the ratio of 2 mussels per litre. Mussels were contaminated with 10 µg/L of polystyrene nanoparticles (nPS; 50 nm) for 28 days. Seawater was exchanged every two days and nanoparticles were re-dosed. No mortality was encountered in either treatment.

Mussels were collected at the beginning of the experiment and after 3, 7, 14, 21 and 28 days of exposure. Mussels were weighed and gills, digestive gland and gonads were dissected and instantly frozen in liquid nitrogen and stored at -80°C for subsequent investigation of neurotoxicity (AChE) in the gills, and for the ingestion of nPS in the three different tissues.

2.5. Quality control and assessment

Each tank was covered in glass to reduce airborne pollution, and glass pipettes were used to administer aeration to prevent plastic contamination. No gloves, plastic instruments, or plastic materials were used during tissue dissection to prevent further plastic contamination.

2.6. Acetylcholinesterase activity (AChE)

AChE activity was only assessed in the gills of *M. galloprovincialis* (n=5 per treatment and time of exposure) following a modification of the Ellman's colorimetric method (Ellman et al., 1961). Firstly, gills were individually homogenized in 5 mL of Tris-HCl buffer (100 mM, pH 8) and 50 µL of Triton – X 100 (0.1%). Following centrifugation (12 000 x *g*, 4ºC, 30 min), the supernatant was collected, and stored at -80ºC until further analysis.

For the determination of AChE activity, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, 0.75 mM) was added to samples and left to incubate at room temperature for 5 mins. Then, acetylthiocholine solution (ATC, 3 mM) was added to trigger the reaction, and the absorbance was read at 412 nm using a Tecan (Infinite 200 Pro) microplate reader, for 5 mins with 30 sec intervals. AChE activity is expressed as nmol ATC min^{-1} mg $protein^{-1}$.

2.7. Total protein

AChE activity was standardized with the determination of total protein concentrations, using the method defined by Bradford (1976). Total protein concentrations (mg protein g^{-1} tissue) were calculated using bovine serum albumin (BSA)**,** as a standard, and optimized for microplate reader.

2.8. Ingestion

Following the method described by Gagné (2019), ingestion of nPS was evaluated using a fluorescence-based methodology using the molecular rotor probe 9-(dicyanovinyl)-julolidine (DCVJ). First and foremost, individual tissues (gills, digestive glands, and gonads) (n=6 per treatment, per time of exposure) of mussels were homogenized at 20% (w/v) in an ice-cold buffer solution (50 mM NaCl, 10 mM Hepes – NaOH [pH 7.4], 1 mM EDTA, and 1mM DTT), using a VWR Star-Beater (5 min, 20/s shaking, with grinding balls). Samples were centrifuged for 20 mins (2ºC, 15 000 *g*) to isolate the cytosolic fraction, and the resulting supernatant was immediately frozen (-80ºC) until further analysis. Samples were then analysed using the spectrofluorometric microplate reader (Berthold Tristar 5) with excitation at 450 nm and emission spectra ranging from 400 – 800 nm. The wavelength equalling to nPS was 510 nm. Results are expressed as nPS µg/g wet weight in relation to controls.

2.9. Statistical Analysis

The significance of the differences between treatments and time were determined using the Shapiro-Wilk test for data distribution and variance homogeneity and either parametric tests (ANOVA, followed by Tukey's Post-hoc test) or non-parametric equivalent tests (Kruskal-Wallis and a two-tailed multiple comparisons test). The results were significant if $p < 0.05$. For the statistical analysis, GraphPad Prism version 9.4.1 (GraphPad software, Inc. CA) was utilized. Additionally, the relationship between treatments (not exposed and exposed to nPS) and the examined tissues (gills, digestive gland, and gonads) was assessed using a Principal Component Analysis (PCA) (Statistica 7.0 software (Statsoft Inc., 2005; USA).

2.10. Weight of Evidence (WOE)

In addition to results on acetylcholinesterase, data on cellular biomarkers (superoxide dismutase SOD, catalase CAT, glutathione peroxidase GPx, glutathione S-transferases GST, lipid peroxidation LPO and DNA damage) were also obtained from Gonçalves et al. (2022) who exposed mussels to the same exposure to nPS as described in the present work (10 µg/L of nPS 50 nm for 21 days). The overall biomarkers results were elaborated through a quantitative Weight Of Evidence model (WOE, Sediqualsoft) that provides a synthetic hazard index based on a specifically developed algorithm and mathematical procedure (Regoli et al., 2019). For each analysed

biomarker, the magnitude of observed variation is compared to a specific threshold, corrected for the toxicological relevance of biological endpoint (weight) and the statistical significance of the difference in respect to controls. Whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgements have been previously described in detail (Regoli et al., 2019).

3. Results

3.1. nPS Acute Toxicity

The luminescence inhibition EC_{50} (µg/L) of *A. fischeri* after 5, 15 and 30 minutes of exposure to nPS was 217, 196, and 180 µg/L, respectively (Table 1).

3.2. *In vitro* assay

In *M. galloprovincialis* haemocytes, the 24 h exposure to nPS (10 µg/L) caused a significant reduction in cell viability compared to unexposed ones ($p < 0.05$) (Fig. 1).

3.3. AChE activity

Significant differences in unexposed mussels were observed on days 14, 21 and 28 compared to the beginning, while the effects of nPS were particularly evident after 3 and 7 days of the exposure ($p < 0.05$; Fig 2). In the gills of nPS exposed mussels, a significant decrease in AChE activity occurred after 3 days of exposure ($p < 0.05$), and a further 2-fold decrease was measured between 3 and 28 days ($p < 0.05$).

3.4. Ingestion

Mussel gills presented a significant ingestion of nPS (1.7 ± 0.01 µg/w.w.), equivalent to 6.75 x 10^8 particles/mL (Fig. 3A) after 3 days of exposure compared to other exposure times ($p < 0.05$). In the digestive glands, a significant increase in nPS ingestion occurred after 14 days of exposure ($p < 0.05$), equalling to 5.5 x 10^{10} particles/mL (Fig. 3B). Compared to gills and digestive glands, the highest levels of nPS ingestion were found in gonads after 7 and 14 days of exposure, with 2.6 ± 0.5 and 2.0 ± 0.6 µg nPS/g w.w., representing 2.9 x 10^{10} and 2.1 x 10^{10} particles/mL, respectively ($p < 0.05$), (Fig. 3C).

3.5. Principal Component Analysis (PCA)

The ingestion of nPS in the three mussel tissues (gills, digestive gland, and gonads) was described using a PCA, which was applied to all the data collected at each time point throughout the 28-d of exposure (Fig. 4). The two principal components accounted for 74.2% of the overall variation (PC1 = 50.37 %, PC2 = 23.82 %; Fig. 3). There is a clear separation between mussel gills and the other mussel tissues evaluated, as well as a distinction between unexposed and exposed to

polystyrene nanoplastics. The ingestion in mussel digestive gland and gonads are positively related to the principal component, and support the results observed in Fig. 3B and 3C. Moreover, PCA implies that although gills are the first tissue to contact with the nanoplastic, this tissue does not accumulate it within its tissues. This suggests that the translocation of the ingested polystyrene nanoplastics in mussels is distributed, once filtered by the gills, to the other tissues, being the digestive gland and gonads the destination of these nano-sized particles. PCA supports that at days 7, 14 and 21 of exposure that ingestions of nPS are most critical for mussels, as they are positively related with the 1st component. Therefore, PCA confirms that ingestion of nPS in *M. galloprovincialis* is tissue-dependent and time-dependent. Furthermore, PCA corroborates the different time-point increase in nPS ingestion observed in individual tissues.

3.6. WOE

The overall WOE elaboration of biomarker results (Table 2) indicated an increase of the cellular hazard after 3 days (Moderate) and even more after 7 and 14 days (Major), while it returned to a Slight level at day 21. The parameters that mostly contributed to the time-course increase of hazard were the antioxidant enzymes (SOD, CAT, GPx and GST) further reflected by severe variations of oxidative damages in terms DNA and lipid peroxidation.

4. Discussion

The haemolymph of mussels reports the functional condition of the organs it perfuses as well as extensive information on the animals' overall physiological status (Digilio et al., 2016). The viability of haemocyte cells in *M. galloprovincialis* haemolymph was affected by the exposure to nPS (10 µg/L; 50 nm; Fig. 1). Functionalized – nPS (PS-COOH and PS-NH_2), alter immune parameters, such as phagocytosis, lysosome activity, ROS and NO production (Auguste et al., 2018; Auguste et al., 2020; Canesi et al., 2015). Additionally, an increase in DNA damage in *M. galloprovincialis* haemolymph was caused by exposure to nPS [10 µg/L; 50 nm; 14-d (Gonçalves et al., 2022) and 0.05 – 50 mg/L; 106 ± 10 nm; 96 h (Brandts et al., 2018)], and a protein corona formation with PS-NH_2 was noticeable in the haemolymph serum of *M. galloprovincialis* [50 nm; 50 µg/mL; 30 mins (Canesi et al., 2015)]. The translocation of nPS from tissues to cells was evaluated, demonstrating that internalization of nPS in haemocytes is size-dependent, being 50 nm nPS mostly internalized compared to 100 nm and 1 µm polystyrene particles (Sendra et al., 2020a). Therefore, nPS of 50 nm in size cause cytotoxicity and can have more severe implications on the overall physiological status of *M. galloprovincialis*. Regarding acute toxicity, no effects of microplastics were demonstrated at environmentally relevant concentrations (Booth et al., 2016; Gagné, 2017; Gambardella et al., 2019), while exposure of the luminescent bacteria to nPS (50 nm)

caused toxic effects after 5, 15 and 30 mins of exposure (Table 1). Gambardella et al. (2019) suggested that particles smaller than 1µm would be toxic as the pore size of cell walls prevent larger particles from entering (PE-MPs; 1 – 500 µm; 25 mg/L), thus confirming the ability of nano-sized particles to cross cellular boundaries; nano-sized plastics also affected the growth of the bacterium *Halomonas alkaliphile* [nPS; 20 mg/L; 55 nm; (Sun et al., 2018)]. With impacts on cellular viability, growth, and their possible internalization of nPS, nano-sized particles certainly pose a greater risk than micro-sized ones, with still unpredictable consequences on trophic transfer and impacts on the whole ecosystem.

In the gills of mussels, neurotoxicity occurred after 3 days exposure to 10 µg/L of nPS (50 nm). Despite the differences observed in controls, a decrease in AChE activity was noticeable throughout the 21-d exposure. A 15 ng/L exposure of nPS (50 nm) for 21 days also caused a decrease in AChE activity in *M. galloprovincialis* gills (Capolupo et al., 2021), while no neurotoxicity was observed in the freshwater clam *Corbicula fluminea* after exposure to nPS (80 nm; 0.1 – 5 mg/L; 96 h) (Li et al., 2020). Microfibres of polyethylene terephthalate caused a dose-dependent increase of AChE activity in the gills of *M. galloprovincialis* (PET-MFs; 100 µm; 0.0005 – 100 mg/L; Choi et al., 2021). Acetylcholine, a key neurotransmitter in the neurological and sensory systems, is broken down by AChE in order to convey impulses at cholinergic synapses, which is essential for neurotransmitter release and synaptic plasticity (Picciotto et al., 2012). Therefore, from all available data it is deducible that the smaller are the plastic particles, the more neurotoxic they are towards mussels, being more effective in seawater compared to freshwater.

To the best of our knowledge, this is the first data set on the ingestion of polystyrene nanoplastics (50 nm) in three tissues of *M. galloprovincialis* after a 10 µg/L exposure for 28 days. Considering the pathways of NPs intake for a filter-feeder organism like *M. galloprovincialis*, due to their small size these particles can directly enter the organism through the gills, or their ability in crossing cellular boundaries. With biological reactivity increasing with decreasing particle size (Peng et al., 2020), the relation of NPs ingestion and toxicity towards organism is a crucial issue. After 28-days of exposure to 10 µg/L of nPS (50 nm), *M. galloprovincialis* gills, digestive gland and gonads presented time-specific ingestion rates. As filter-feeding organisms, the increase in nPS ingestion by the gills after 3 days of exposure is expected, as this is the first tissue to encounter contaminants in the surrounding environment. Looking at the same experimental conditions (10 µg/L nPS; 50 nm; 21-d), the gills of *M. galloprovincialis* after 3 days of exposure led to a significant decrease of antioxidant enzymes activity until the 14th day, leading to oxidative damage (Joanna M. Gonçalves et al., 2022). Results suggested that the internalization of nPS in the gills have a prolonged toxic effect, although an adaptive response was observed after 21-days. In another study, the presence of nPS (50 nm) was also found in *M. galloprovincialis* gills, although larger nPS (100 nm and 1µm) were more abundant after 24h of exposure (Sendra et al., 2020b); this was partly

expected as larger particles have similar sizes to food sources and are often mistaken as non or low nutritional food (nPS; 30 nm; 0.1 – 0.3 g/L; 8 h) (Wegner et al., 2012).

The digestive gland of mussels is known as being responsible for key metabolic functions, such as food intracellular uptake/digestion and as a storage for reserve substances (Faggio et al., 2018). Here, mussels digestive gland shows significant intake of nPS after 14 days of exposure ($p < 0.05$). This suggests that after an initial ingestion of the gills at day 3, nPS translocate to the digestive gland, and remains stored until the end of the experiment. Observing toxicity of nPS in the digestive gland after a 21-d exposure to 10 µg/L (50 nm), the 7th day was the most critical, causing inhibition of CAT and GPx activity and consequently leading to oxidative damage (Joanna M. Gonçalves et al., 2022). In this case, results imply that although not significant, the presence of nPS at 7-d is most critical for this tissue, and that the increase in ingestion after this time-point truly overwhelm the mussels antioxidant defence system. Moreover, Sendra et al. (2020) found the digestive gland of *M. galloprovincialis* as the common destination for nPS (50 nm; 3 and 24h), despite the difficulties in observing these particles using histological assessments. Also, glitter particles (62.5 particles/L; 7-d) ingested by *M. galloprovincialis* accumulated in the digestive tract of mussels, whereby ingested glitter increased with the decrease in particle size (Provenza et al., 2022). Findings confirm that the smaller the size of the nanoplastic, the higher the accumulation of these particles in the digestive system of mussels.

Gonads of mussels showed the highest levels of nPS among those measured in the three tissues, particularly after 7 and 14 days of exposure (2.6 ± 0.5 and 2.0 ± 0.6 µg nPS/g w.w., respectively; $p < 0.05$). Considering the anatomy of mussels, a hypothesis is that nPS, due to their small size and ability to cross cellular boundaries, may 'diffuse' across tissues besides being transported to different tissues via the haemolymph. A translocation study from cells to tissue showed that internalization of nPS (50 nm, 100 nm and 1µm; 3 – 24 h) into haemocytes was size – dependent, with nPS of 50 nm being the most internalized (Sendra et al., 2020b). Therefore, with the same size of particles, the observed ingestion of nPS in gonads may be due to their translocation in the haemocytes, despite their ability to cross cellular boundaries should not be excluded. Looking at the toxic effects 10 µg/L of nPS pursue in *M. galloprovincialis* gonads after 21 days of exposure, a similar pattern to the digestive gland is clear **(**Gonçalves & Bebianno, 2023). The most critical time-point for gonads is at 3 days of exposure, as nPS exposure leads to an 18.6-fold increase in oxidative damage, although highest ingestion is seen on days 7 and 14. Considering the antioxidant enzyme activities at both 7 and 14 days of exposure, CAT and GPx activity increased, whereas GST activity decreased **(**Gonçalves & Bebianno, 2023), suggesting that with the increase of ingested nPS in gonads of mussels, the organisms antioxidant defence mechanisms are hindered. The gonads of mussels and their development are crucial for reproductive success and during the gametogenesis cycle, lipid content has shown to enhance with the ripening of female gonads, as well as been used as an energy source during gametogenesis and embryo-larval development (Martínez-Pita et al.,

2012). More concerning, as nPS of 50 nm internalize haemocytes, spermatozoa and oocytes may also be at risk. In *Crassostrea gigas*, fertilization rate decreased after gametes were exposed to nPS (50 nm; 1.5 h) (Tallec et al., 2018), and sperm mobility and fertilization success were impaired, as agglomerates of nanoparticles were found attached to spermatozoa and to the jelly coating of oocytes (PS-COOH and PS-NH_2; 0.1 to 100 mg/L; 1, 3 and 5 h) (González-Fernández et al., 2018). In *M. galloprovincialis*, malformations of D-larvae were found after embryo's were exposed to nPS (50 nm; 10 µg/L; 48 hpf) (Auguste et al., 2021), suggesting the high content of ingested nPS in mussel gonads may pose a serious threat for the reproduction of these organisms.

In the present study, the biological significance of the results observed in this and in a previous study (Gonçalves et al., 2022) was summarized in specific hazard indices allowing an easier qualitative and quantitative comparison of the effects of nPS at different exposure times. The applied elaboration considers the toxicological relevance (weight) of measured end points and both the number and magnitude of variations normalized to specific thresholds. This model has been validated in several case studies aimed to evaluate biological and environmental risk assessment providing the possibility to integrate large dataset of heterogeneous data and to better interpret asynchronous variations of complex pathways (Mezzelani et al., 2021; Nardi et al., 2022; Pittura et al., 2018; Regoli et al., 2014). The weighted elaboration of biomarker results confirmed the involvement of oxidative stress in modulating toxicity of nanoparticles with a clear time-dependent trend as already outlined by discussion of individual results (Table 2). The cellular hazard raised to Moderate after 3 days further increasing at days 7 and 14 when the observed variations were summarized in a Major hazard. After this peak, a counteracting response to oxidative challenge was evidenced by the return to a Slight hazard condition.

5. Conclusion

This dataset provides evidence for the ingestion of polystyrene nanoplastics in the marine mussel *M. galloprovincialis*, as well as neurotoxicity and *in vitro* effects on mussel haemolymph. nPS of 50 nm cause neurotoxicity in mussel gills and lead to a reduction of viable haemocytes in mussels haemolymph. Ingestion in mussels is tissue and time specific, whereby gonads present the highest ingested nPS. Biochemical alterations of mussel tissues may possibly be explained by the quantity of ingested nPS, however further investigation is necessary. Moreover, the effects of ingested nPS in the gonads of mussels needs more insight into how it can affect gametogenesis, fertilization success, and embryo-larval development. Lastly, the impacts of eating nPS contaminated shellfish batches can cause adverse effects on human health and should be considered for future analysis.

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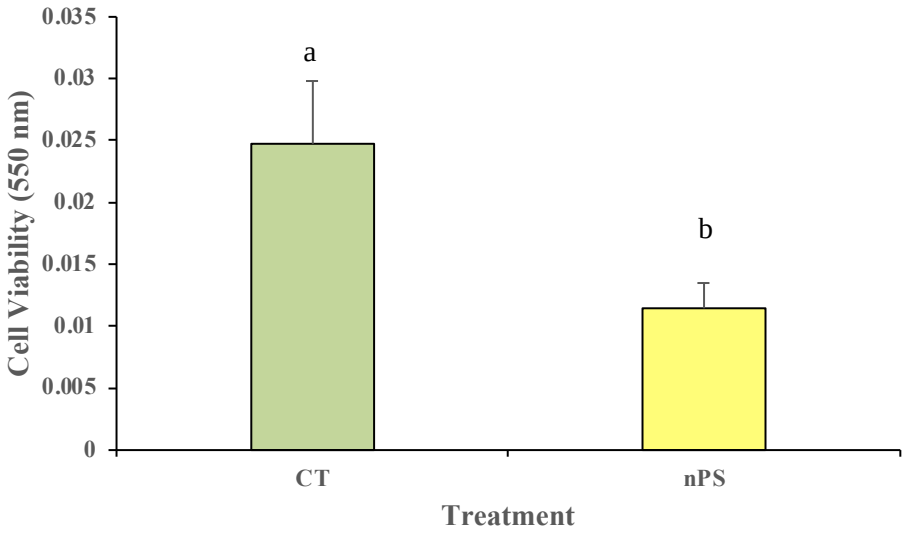


Figure 1. *M. galloprovincialis* cell viability (mean ± sd) using Neutral Red dye in unexposed haemocytes and those exposed to 10 µg/L of nPS after 24 h. Different letters indicate significant differences between treatments ($p < 0.05$).

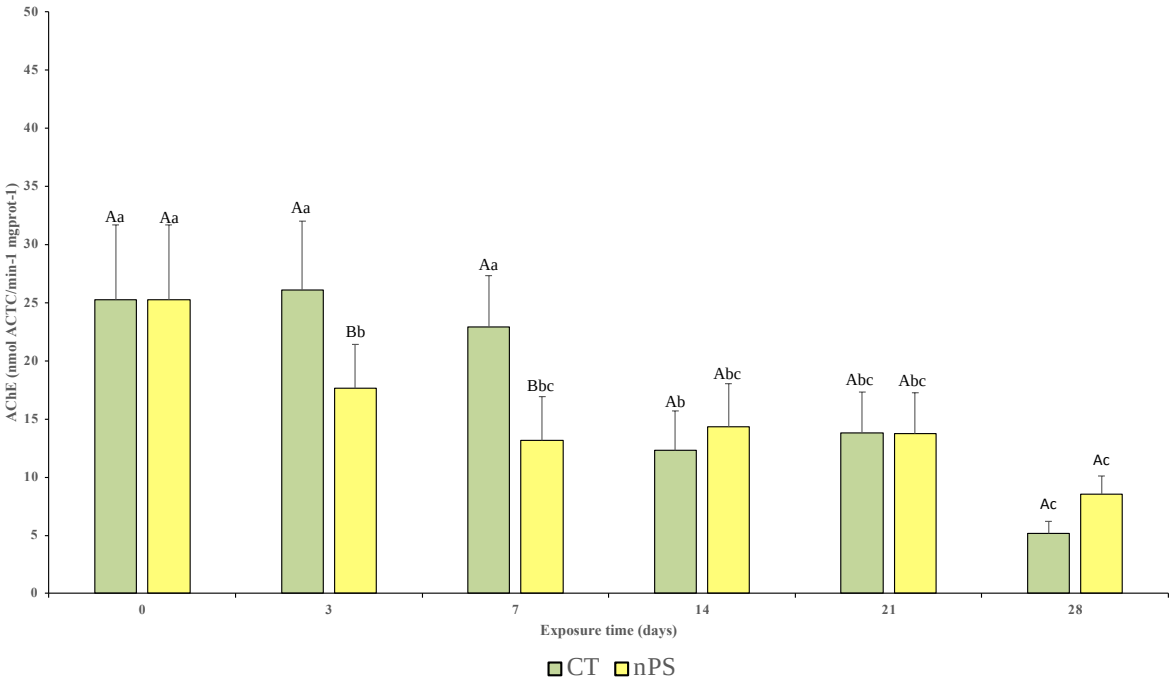


Figure 2. Acetylcholinesterase activity (AChE) in gills of *M. galloprovincialis* (mean ± sd) exposed to 10 µg/L of nPS after 28-d. Different upper- and lower-case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

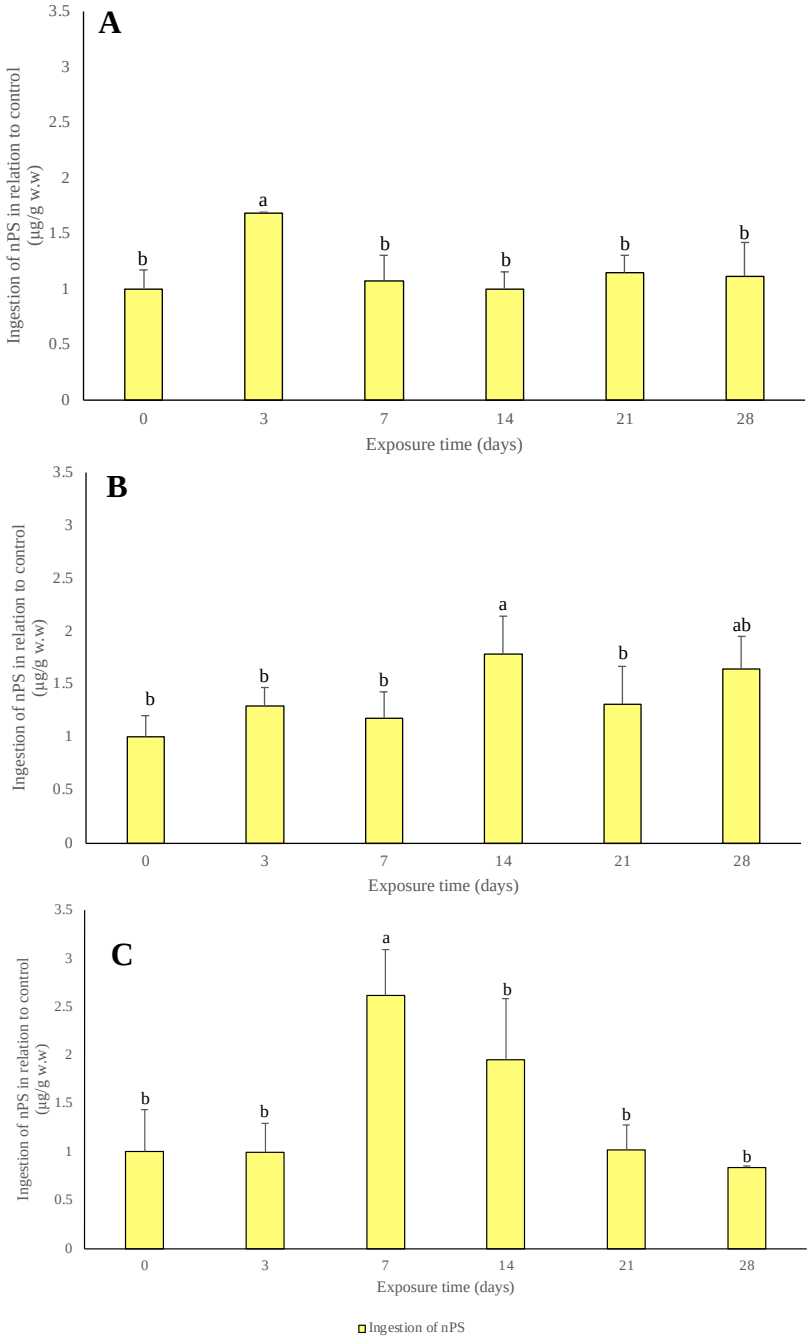


Figure 3. Ingestion of nPS in relation to controls (µg/g w.w) in *M. galloprovincialis* **(A)** gills, **(B)** digestive glands, and **(C)** gonads (mean ± sd) after 28-d to 10 µg/L of nPS (50 nm). Different lower-case letters indicate significant differences between time for the same treatments ($p < 0.05$).

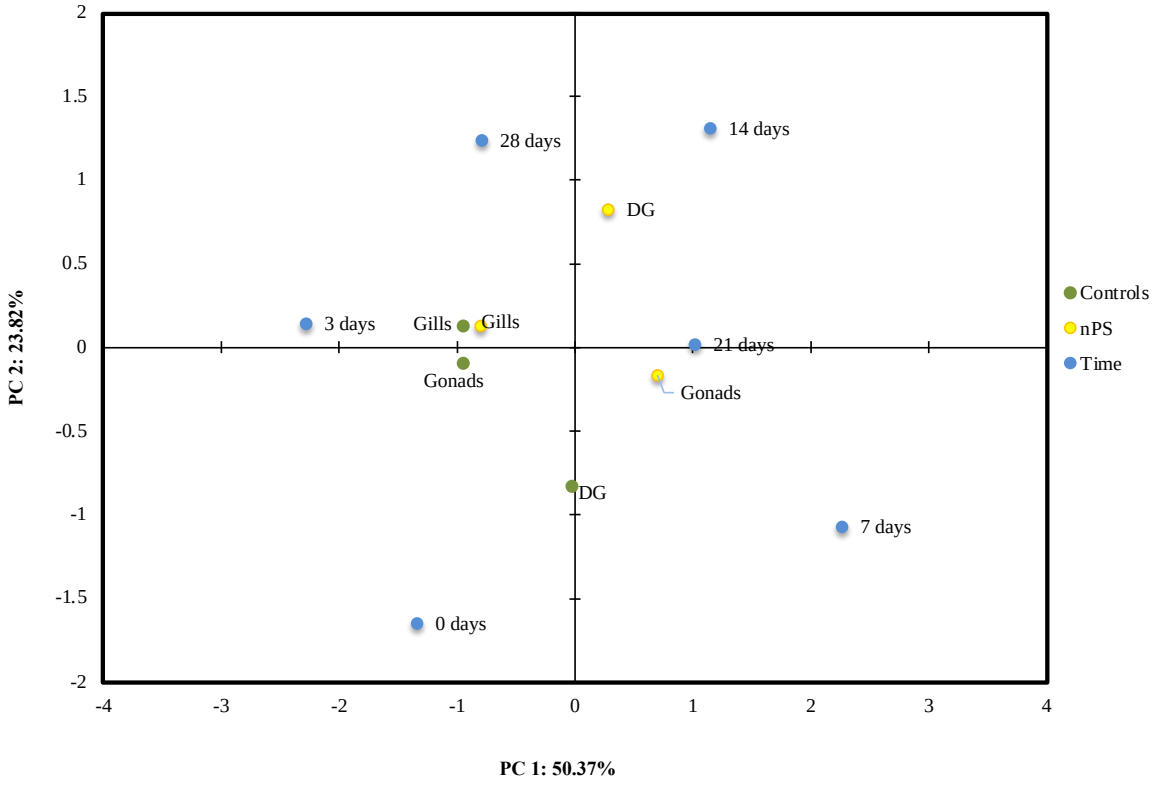
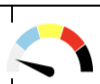
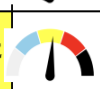
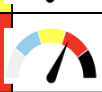


Figure 4. Principal component analysis in of ingestion in the gills, digestive glands, and gonads of *M. galloprovincialis* in unexposed and exposed to 10 µg/L of nPS for 28-d ($p < 0.05$).

Table 1. LC_{50} concentration (µg/L) of polystyrene nanoplastics

LC_{50} (µg/L)		
5 mins	15 mins	30 mins
217	196	180

Table 2. Weight of Evidence (WOE) of 10 µg/L of nPS

Sample code	N. param in class ABSENT	N. param in class SLIGHT	N. param in class MODERATE	N. param in class MAJOR	N. param in class SEVERE	Level of hazard for biomarkers
nPS-0	17	0	0	0	0	ABSENT 
nPS-3	10	3	2	1	1	MODERATE 
nPS-7	8	1	2	4	1	MAJOR 
nPS-14	10	0	3	3	1	MAJOR 
nPS-21	12	4	0	0	0	SLIGHT 

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author statement

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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