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Short Communication

Toxicity of polystyrene nanoplastics in dragonfly larvae: An insight on how these pollutants can affect bentonic macroinvertebrates

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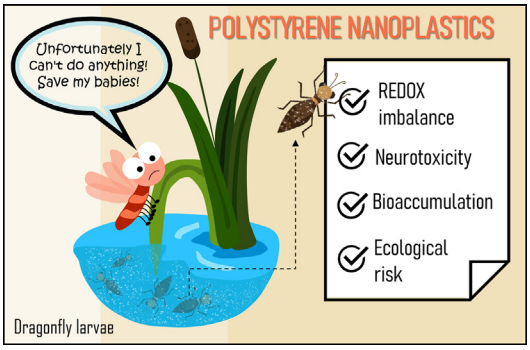
HIGHLIGHTS

- Polystyrene nanoplastic (PS NPs) represent an ecological risk to bentonic macroinvertebrates.
- PS NPs cause REDOX imbalance in *Aphylla williamsoni* larvae.
- Larvae exposed to PS NPs show decreased acetylcholinesterase activity (neurotoxic effect).
- PS NPs can accumulate in dragonfly larvae.

GRAPHICAL ABSTRACT



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ABSTRACT

Although nanoplastics (NPs) are known to be toxic to several groups of animals, the effects of such a toxicity on freshwater benthic macroinvertebrate communities remain unknown. Thus, the aim of the current study is to test the hypothesis that polystyrene nanoplastics (PS NPs) (34 μg/L - 48 h of exposure) lead to biochemical damage in *Aphylla williamsoni* larvae. Data have evidenced high bioaccumulation factor in the analyzed individuals; this finding indicates that, similar to sediments, water is also part of aquatic systems and favors PS NPs retention in dragonfly larvae. Despite the lack of evidence about the interference of these pollutants in the nutritional status of the analyzed animals, their bioaccumulation was associated with REDOX imbalance featured by concomitant increase in the number of evaluated oxidative stress biomarkers (nitric oxide and lipid peroxidation) and antioxidants (antioxidant activity against the DPPH radical and the superoxide dismutase enzyme). On the other hand, the reduced acetylcholinesterase activity observed in larvae exposed to PS NPs has suggested the neurotoxic effect of these pollutants, with potential impact on their nerve and neuromuscular functions. Therefore, the current study is pioneer in showing that PS NPs can affect the health of the investigated larvae, even at small concentrations, for short exposure-time; this outcome reinforces the ecotoxicological risk of these pollutants for freshwater benthic macroinvertebrates.

1. Introduction

Nowadays, there has been exponential increase in the production and consumption of plastic products, whose annual production has exceeded 380 Mt (Geyer et al., 2017). Therefore, the introduction of waste deriving from these products in different ecosystems, be them

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of primary or secondary origin, is almost inevitable. Plastic particles of primary origin (pellets) often derive from materials initially synthesized in small diameters (Lei et al., 2018; Anbumani and Kakkar, 2018), such as the ones seen in plastic resin pellets (Ogata et al., 2009) and/or microspheres used in hygiene and cosmetic products (Fendall and Sewell, 2009). On the other hand, particles of secondary origin derive from degradation processes (e.g., weathering) that transform larger plastic pieces into smaller ones, called microplastics (MPs) or nanoplastics (NPs). Microplastics (MPs) include plastic particles whose diameter is smaller than 5 mm (Thompson et al., 2009; Gesamp, 2016), whereas NPs encompass plastic particles smaller than 0.2 mm, according to the Working Group on Good Environmental size classification Status (WG-GES); and smaller than 100 nm, according to the general definition used for nanomaterials (Koelmans, 2015).

Regardless of their origin, both MPs and NPs have led to negative changes in aquatic ecosystems such as freshwater (Li et al., 2020) and marine (Peng et al., 2020) environments. Such particles have impact on the health of different organisms living in these environments, such as bacteria and fungi (Kettner et al., 2017; Sun et al., 2018), protists (Rillig and Bonkowski, 2018; Wiedner and Polifka, 2019), plants (Rillig et al., 2019), animals (De-Sá et al., 2018; Nelms et al., 2019) and humans (Sharma and Chatterjee, 2017; Campanale et al., 2020; Prata et al., 2020). The range of toxicological and ecological effects of these pollutants can be seen in several compilations shown in recent studies on this topic (Wang et al., 2018; Anbumani and Kakkar, 2018; Wang et al., 2020; Ma et al., 2020; Pirsaheb et al., 2020).

However, the current knowledge about the impacts of these pollutants on freshwater organisms is significantly incipient (Ma et al., 2019) in comparison to that about marine organisms (Ma et al., 2020). Another gap in the literature refers to the incipient knowledge about the toxicity of these pollutants in some animal groups, such as freshwater benthic macroinvertebrates (Haegerbaeumer et al., 2019). If one takes into consideration that several processes can lead to the aggregation of plastic particles (e.g.: adsorption to other pollutants and in organic material or bacteria), the density of MPs and NPs deposited in sediments of aquatic ecosystems can correspond to several orders of magnitude higher than that of the surrounding water (Lattin et al., 2004). Therefore, such processes increase the bioavailability of MPs and NPs for organisms living in sediments, which can occasionally ingest these pollutants due to the similarity between their size and that of sediment grains and to animals' inability to differentiate these particles from their food sources (Moore, 2008; Wright et al., 2013).

Previous studies have shown that pollutants' impact on benthic invertebrates is a worrisome factor, both for marine and freshwater habitats, since these organisms represent up to 90% of fish prey biomass (Schindler and Scheuerell, 2002; Weber and Traunspurger, 2015). Therefore, the risk posed by plastic pollution to benthic fauna is considerably high. However, most studies conducted with invertebrates and plastic particles have, so far, focused on pelagic organisms, rather than on the benthic ones. The review carried out by Haegerbaeumer et al. (2019) has shown that 80% of the analyzed studies referred to marine organisms, whereas only 20% of them focused on freshwater organisms. Thus, it is of paramount importance evaluating the likely toxicity of nanomaterials in benthic macroinvertebrates in order to help better understanding the magnitude of impacts caused by these materials on the cycling of matter, energy flow and primary production of freshwater ecosystems. Small changes in these organisms may be enough to cause ecosystem disturbances that go beyond the impact observed on individuals (Ma et al., 2019).

Insects belonging to order Odonata are a taxonomic group of great ecological importance, whose effects deriving from their exposure to NPs remain unknown. Because they are essentially carnivores, these animals play an important role in controlling populations of other organisms, such as mosquitoes and different agricultural pests (Barzoki et al., 2020; Cudera et al., 2020). According to Dijkstra et al. (2014) and Juen et al. (2014), Odonata is one of the orders of insects presenting the largest number of aquatic species ever recorded. Thus, the aim of the current study was to test the hypothesis that polystyrene NPs (PS NPs), at environmentally relevant concentration, are capable of causing biochemical disorders predictive of oxidative stress, deficit in antioxidant defenses and neurotoxicity in dragonfly larvae (*Aphylla williamsoni*). To the best of our knowledge, this study is the first to report the impact of PS NPs on these organisms; therefore, it is a warning to environmental authorities about the ecotoxicological risks posed by these nanomaterials to the fauna of benthic macroinvertebrates living in freshwater ecosystems.

2. Material and methods

2.1.1. Polystyrene nanoplastics

The current study used yellow-green fluorescent polystyrene plastics (PS NP) as representatives of nanoplastics (λex = 470 nm and λem = 505 nm, obtained at Sigma Aldrich, USA (product L5155)). Polystyrene plastics can be used to produce several materials, depending on their type. Crystal (or common) PS is widely used in CD covers, but it can also be used in disposable laboratory pipettes, disposable cups, scissors, combs, brushes and yogurt pots. High-impact PS is generated when more than 10% polybutadiene or styrene-butadiene is added to the PS used to manufacture food-storage pots, internal coatings for refrigerators, circuit breakers, combs, hangers, folder packaging and food trays. However, Styrofoam is one of the most well-known PS types; it is widely used as insulator or in packaging containers such as cups and other disposable items used to hold hot food and drinks. Therefore, it justifies the option made for this material in the present study. Moreover, particles deriving from this polymer are often found in aquatic environments due to its low reuse rate (Eriksen et al., 2014; Sá et al., 2018).

PS NPs samples were analyzed in Raman spectrometer (Horiba LabRam HR Evolution) to confirm their chemical composition (Fig. 1S). In addition, nanomaterials were observed in transmission (Fig. 1A) and scanning electron microscopy (Fig. 1B).

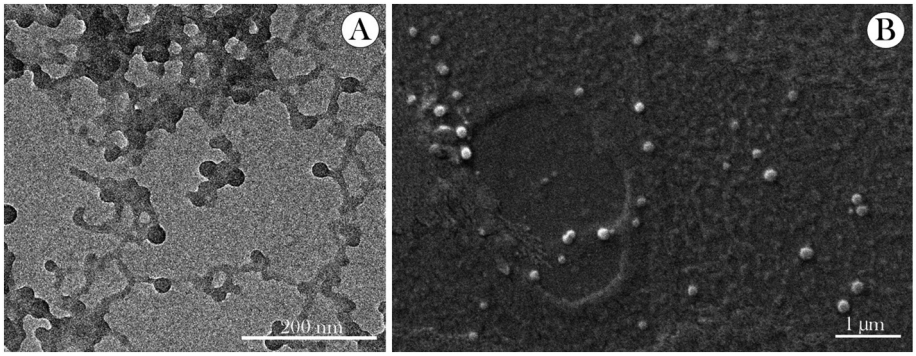

Fig. 1. Transmission (A) and scanning (B) electron microscopy applied to PS NPs *Aphylla williamsoni* larvae were exposed to.

2.2. Animals and experimental design

Aphylla williamsoni larvae (biomass: 102.5 mg ± 8.9 g; length: 12 mm to 14 mm) were collected in the permanent preservation area of Goiano Federal Institute (IF Goiano) - Urutaí Campus (GO, Brazil) (Fig. 2) in July 2020. Specimens were identified through dichotomous keys (Richardson, 2003; Bouchard, 2004). *Aphylla williamsoni*, also known as "two-striped forceptail", is a club-tailed species belonging to family Gomphidae (Class: Insecta, Order: Odonata) (Gloyd, 1936), has good adaptability to laboratory conditions, sensitivity to pollutants, resistance disease as well as being a good representative model of freshwater benthic macroinvertebrates (Cowell and Vodopich, 1981). Individuals were collected with the aid of a benthic hand dip net one-to-two feet into stagnated water, based on Samanmali et al. (2018). Collected nymphs were recorded and transferred to sampling jars filled with water from the very same waterbody. These jars were carefully taken to the laboratory and kept in a room at 27 °C ± 1 °C, under 14/10-h light/dark photoperiod, based on procedures recommended by Rice (2008).

Before experiment installation, individuals were left to acclimate in climatic chamber at the aforementioned temperature, for 10 h. The adopted acclimation period corresponds to rough natural conditions. Next, larvae were distributed into the following treatments (n = 24 replicates, each): (i) control group, whose exposure water was not added with PS NPs and (ii) PS NP group, whose exposure water was added with PS NPs at the concentration of 34 μg/L. Such a concentration simulated highly pessimistic pollution scenarios identified at points close to pollution sources (Besseling et al., 2014).

Animals were exposed to treatments for 48 h in order to simulate ephemeral exposure to pollutants in static systems (i.e., without water renewal). Each larva (i.e., each replica) was kept isolated in beaker filled with 50 mL of naturally de-chlorinated water added, or not, with pollutants. Individual exposure was adopted to avoid the cannibal behavior often observed among dragonfly larvae (Van-Buskirk, 1989). Larvae were not fed throughout the 48-h exposure period to avoid energetic carry-over effects, due to changes in food intake, from the exposure to the post-exposure period, based on procedures adopted by Tollett et al. (2009) and Jinguji et al. (2018). Larvae were weighed at the end of the experiment and separated in previously cleaned microtubes to be stored in ultra-freezer (−80 °C) until biochemical analysis and PS NPs quantification, which were carried out up to 24 h and 48 h after the end of the experiment, respectively.

2.3. Toxicity biomarkers

Predictive biomarkers of nutritional deficit, oxidative stress, interference in animals' antioxidant systems and neurotoxicity were used to assess PS NPs toxicity. Samples were prepared as previously described in Meyer et al. (1986), with some modifications. Each larva was macerated in 1 mL of PBS and centrifuged at 13,000 rpm, at 4 °C, for 5 min; supernatants were separated into aliquots, which were used in different biochemical evaluations. All tests were performed in ELISA microplate (96 wells), as detailed below.

2.3.1. Nutritional status assessment

Total soluble protein, carbohydrate and triglyceride levels were evaluated to assess animals' nutritional status, based on Souza et al. (2019). Total protein concentrations were determined based on the Lowry method (Lowry et al., 1951), whereas triglyceride concentrations were determined based on the enzymatic colorimetric method by using glycerol-3-phosphate oxidase (GPO) (Kalia and Pundir, 2004). Total soluble carbohydrate concentrations were evaluated based on the methodology by Dubois et al. (1956).

2.3.2. Oxidative stress parameters

Griess colorimetric reaction test (Grisham et al., 1998) was used to measure nitric oxide concentrations; it consisted in detecting nitrite (NO2-) resulting from NO oxidation, based on Ajjuri and O'Donnell (2013). Thiobarbituric acid reactive species (TBARS) test was used to measure lipid redox state (Draper and Hadley, 1990), based on procedures described by Pothiwong et al. (2007) and Carvalho et al. (2019), with adaptations in order to be performed in microtubes and subjected to ELISA microplate reading.

2.3.3. Predictive parameters of changes in antioxidant activity and neurotoxicity

Total superoxide dismutase (SOD) activity was evaluated by following the method described by Dieterich et al. (2000). The evaluation was based on SOD's ability to scavenge superoxide radical anion (O2−), which decreased the overall pyrogallol autoxidation rate. Total thiols (nonenzymatic antioxidant) were determined based on procedures described by Santos et al. (2015); diphenyl −1-picrylhydrazyl (DPPH) radicals' scavenging activity was performed in compliance with Brand-Williams et al. (1995). Acetylcholinesterase (AChE) enzyme

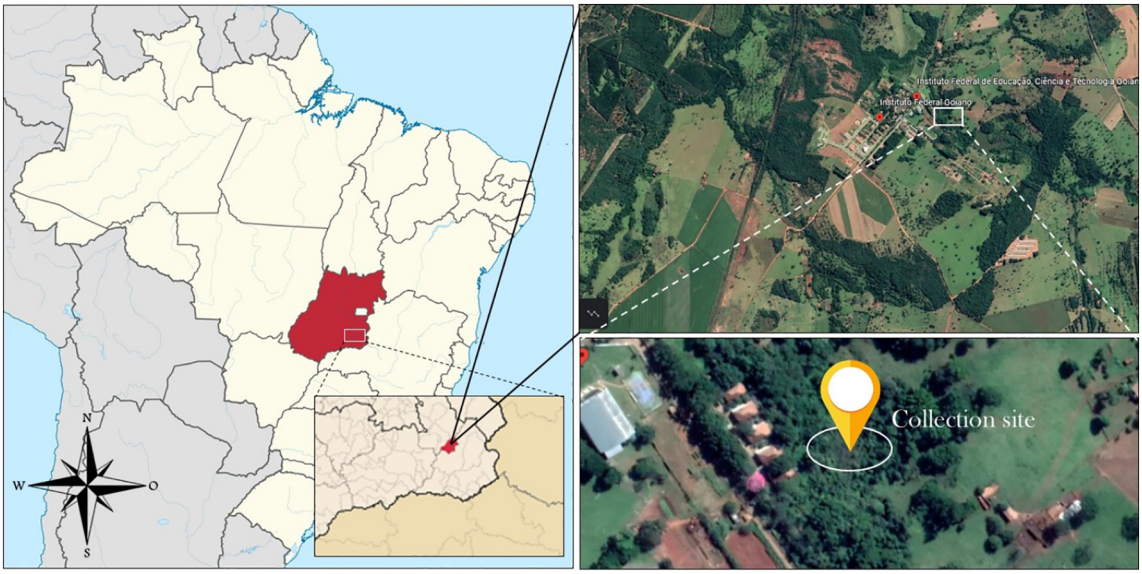

Fig. 2. Geographic location of the collection site (*Aphylla williamsoni* larvae), *Instituto Federal Goiano* - Campus Urutaí, Goiás, Brazil.

activity was defined as predictive neurotoxicity parameter; it was determined based on the spectrophotometric method by Ellman et al. (1961).

2.4. PS NPs quantification in Aphylla williamsoni larvae

PS NPs quantification was estimated based on Katzenberger and Thorpe (2015), Boyero et al. (2020) and Sarasamma et al. (2020), with some modifications. The adopted methodology consisted in the following steps: macerate of *A. williamsoni* larvae were collected; next, sample aliquots (200 μL) were added to microplate wells for subsequent reading in microplate reader (Heales, model MB-580) at 450 nm. Background fluorescence of non-exposed larvae - which were processed in the same way as exposed fish tissues - was detected and subtracted from that of treated samples. Standard sample absorbance was generated by using fluorescence PS-NP suspensions (34 μg/L) processed under the same conditions as those applied to other samples. Each detection procedure was run in triplicate. Bioaccumulation factors (BAF) were computed through eq. 1 (where units were expressed as g/mL) in order to investigate the likely translocation of PS NPs found in the exposure water, based on Nelson et al. (2019):

$$\text{Bioaccumulation factors (BAF)} : \frac{\text{Dragonfly PS NP (μg/g)}}{\text{Aqueous PS NP (μg/mL)}} \quad (1)$$

2.5. Statistical analysis

Shapiro-Wilk test was used to analyze the normality of all collected data, whereas Bartlet-test was used to analyze homoscedasticity of variances. Pairwise comparisons were performed through Student's *t*-test (parametric data) or Mann-Whitney *U* test (non-parametric data), at 5% probability level. DPPH radicals' scavenging activity data were subjected to one-way ANOVA, with Tukey's post-test, at 5% probability level. All analyses were performed, and graphs were generated, in GraphPad Prism software (version 7.0).

3. Results

Exposure period was enough to enable PS NPs accumulation in *A. williamsoni* larvae, whose BAF values ranged from 36,965 to 255,133 per individual (mean: 134,260 ± 29,626). However, there was no difference in the evaluated nutritional parameters (proteins and total soluble carbohydrates and triglycerides) between experimental groups (Fig. 3A–C, respectively); this outcome suggests that the investigated pollutant did not change animals' energy reserves.

However, PS NPs accumulation was directly associated with increased nitric oxide levels (Fig. 4A), thiobarbituric acid reactive species (Fig. 4B), SOD activity (Fig. 4C) and DPPH radical scavenging activity (%) (Fig. 4D). This outcome suggests that the pollutant was able to induce increased oxidative stress and to stimulate animals' antioxidant activity. On the other hand, total thiol concentrations did not differ between treatments (Fig. 2S). In addition, PS NPs have suppressed AChE activity (Fig. 4E), which confirmed the hypothesis about the neurotoxicity of these pollutants.

4. Discussion

Assessing the impact of nanomaterials on aquatic organisms is essential to anticipate more far-reaching harmful effects. Thus, it is possible subsidizing the elaboration of strategies or measures as well as to mitigate or remedy plastic pollution in sweet ecosystems, to predict how its potential impacts on individuals can cause unprecedented ecosystem imbalances. Accordingly, the current study provides scientific support on how PS NPs can affect the health of freshwater benthic macroinvertebrates.

PS NPs can be absorbed by, and cause unprecedent biochemical damage in, *A. williamsoni* larvae, which were herein used as representative organisms of benthic macroinvertebrates living in polluted areas. High BAF value (134,260 ± 29,626) has indicated high pollutant accumulation level in body tissues of the investigated larvae and confirmed that, similarly to sediments, water is also part of aquatic systems and

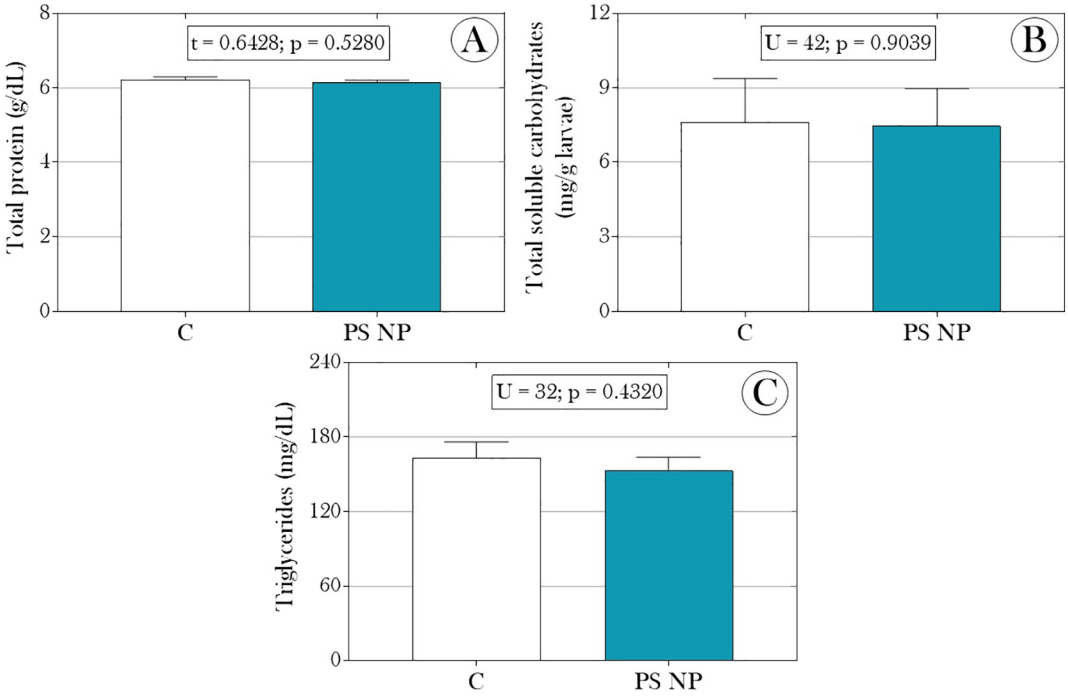

Fig. 3. Nutritional status parameters in *A. williamsoni* larvae exposed, or not, PS NPs. (A) Total protein concentrations (B) total soluble carbohydrates and (C) triglycerides. Bars indicate the mean + standard deviation of data. Statistical summaries are shown at the top of the graphs. C: control group; and PS NP: group exposed to PS NPs at the concentration of 34 μg/L. n = 24 larvae/group.

favors PS NPs retention in *A. williamsoni* larvae. According to Kwok et al. (2013), BAFs are an appropriate tool to highlight such an association. Therefore, these pollutants may have been absorbed through oral intake during gas exchange performed in their rectal tracheal gills or due to nanoparticles' penetration in animals' exoskeleton, as addressed by Benelli (2018).

Despite the lack of changes in the evaluated nutritional parameters (Fig. 3), PS NPs were capable of triggering oxidative stress in different tissues of *A. williamsoni* larvae (nitric oxide and TBARS levels, Fig. 4A–B). Although their defense antioxidants - which were measured through SOD and other antioxidants capable of scavenging free radicals such as DPPH (Fig. 4C–D) - have increased in comparison to those of the control group, they were not capable of mitigating oxidative reactions. These results are particularly interesting, since they indicate that oxidative stress induction by PS NPs is one of the toxicity mechanisms similar to those of other nanomaterials - such as metallic nanoparticles - evaluated in arthropods' tissues (Nair and Choi, 2011; Nair and Choi, 2012; Foldbjerg et al., 2015; Mao et al., 2018); as well as that increased antioxidant activity suggests the existence of common mechanisms (among organisms) used to initiate the system accounting for detecting and eliminating oxidative stress induced by animals' exposure to micro- and nanoplastics. Thus, PS NPs found in the intracellular space may be able to bind to protein and DNA structures and lead to fast organelle and enzyme denaturation. In addition, decreased membrane permeability and disturbances in the driving force of protons can lead to loss of cell function, cell death and, consequently, to increased oxidative stress.

On the other hand, data in the present study differ from the ones reported by Parenti et al. (2020) who exposed *Bombyx mori* larvae to PS NPs (0.4–0.6 μm) and did not observe oxidative stress induction by the tested treatments, although SOD activity has significantly decreased. In fact, the compilation of studies carried out by Prokić et al. (2019) has shown diversity of, and sometimes controversial, responses to oxidative stress by organisms exposed to plastic particles with diameter ranging from 0.05 μm to 100 μm. These responses appear to depend on several factors, such as the analyzed tissue or its composition, size and shape, as well as on exposure route and time, investigated species and the concentrations of the tested pollutants. It is important emphasizing that oxidative stress biomarkers have often shown specific tissue response to plastic particles, which may have led to interpretive bias in studies whose evaluation was not organ-specific. In this case, future investigations can significantly contribute to better explain oxidative stress induction mechanisms in different physiological systems of the investigated animals.

Another important result observed in the present study lies on the decreased AChE activity observed in *A. williamsoni* larvae exposed to PS NPs. As previously addressed by Pang (2014), AChE plays a crucial role in transmitting impulses between neurons, which hydrolyze the acetylcholine (ACh) neurotransmitter into cholinergic synapses. Therefore, it plays a crucial role in several physiological functions of different organisms such as benthic macroinvertebrates. Changes in AChE activity had been previously reported in dragonfly larvae exposed to other environmental stressors. Thangaraj et al. (2018) have found increased

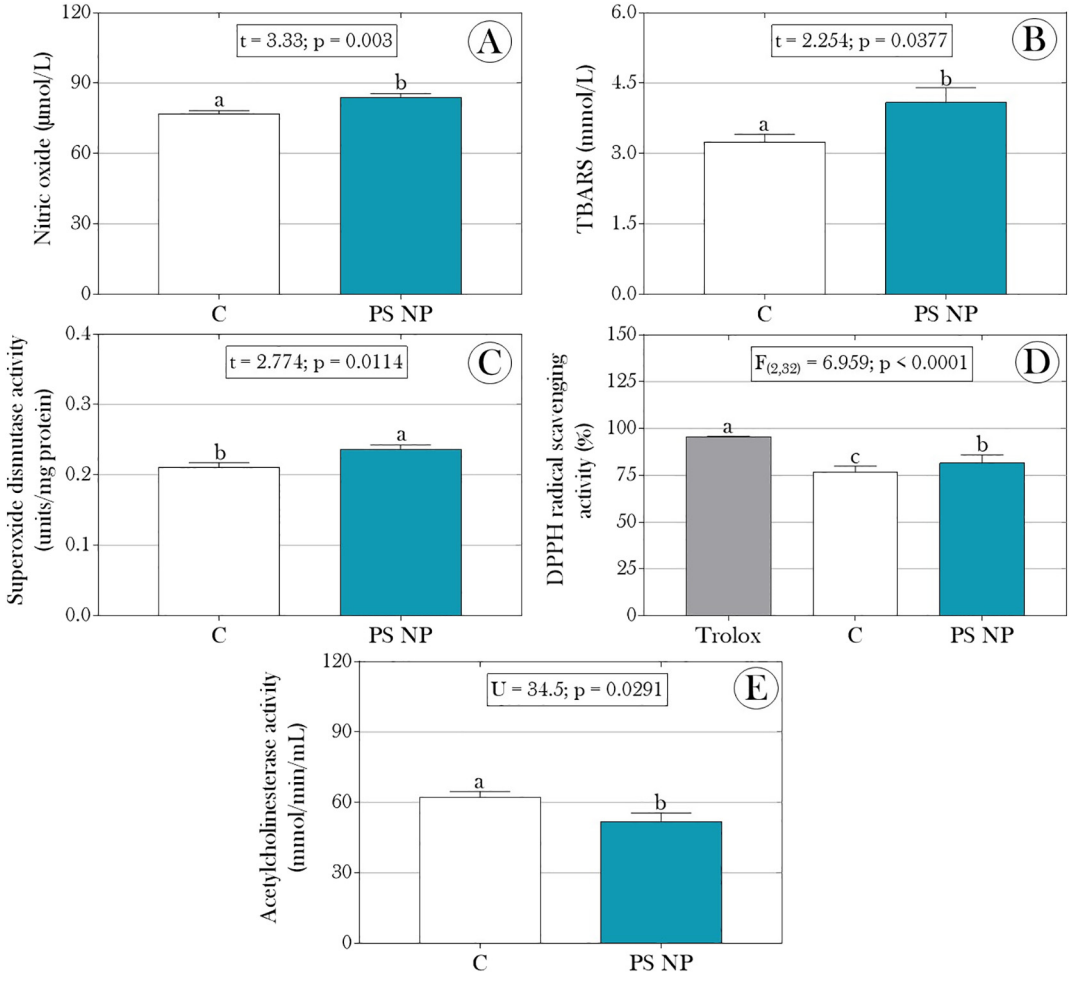

Fig. 4. (A) Nitric oxide levels, (B) thiobarbituric acid reactive species, (C) superoxide dismutase activity, (D) DPPH radical scavenging activity (%) and (E) acetylcholinesterase activity in *A. williamsoni* larvae exposed, or not, to PS NPs. Bars indicate the mean + standard deviation of data. Statistical summaries are shown at the top of the graphs. C: control group; and PS NP: group exposed to PS NPs at the concentration of 34 μg/L. n = 24 larvae/group.

AChE activity in *Bradinopyga geminata* larvae exposed to effluent deriving from textile industries; this finding suggests that this change took place due to water contamination with heavy metals observed in these effluents. On the other hand, cholinesterase activity in *Anax junius* larvae exposed to chlorpyrifos did not differ from that of control groups (Brewer and Atchison, 1999), which - in this case – corroborates the idea that the neurotoxic effects observed in dragonfly larvae are directly dependent on pollutant type and on the tested concentrations of it.

The current data suggest that PS NPs have adversely affected animals' cholinergic nervous system, which may have led to potentially negative impact on their nerve and neuromuscular functions; this assumption should be further investigated. In addition, it is tempting speculating that reduced AChE activity is also associated with endocrinological changes [see details in Rattner and Fairbrother (1989)] indirectly caused by nanopollutants. However, one cannot neglect the hypothesis that PS NPs affect the expression of genes linked to AChE enzyme synthesis and release, such as ace genes (Ye et al., 2017). According to Kim and Lee (2013), the biological functions of the two ace genes were assessed in insects. Overall, AChE1 is the major enzyme found in insects and it is more abundant than AChE2. The expression of ace1 is higher than that of ace2 in some insect species (Kim et al., 2006; Jiang et al., 2009; Kim et al., 2015), whereas the opposite can be observed in other species.

Finally, it is necessary taking into consideration that, regardless of the action mechanisms adopted by PS NPs in *A. williamsoni* larvae, any anticholinesterase effect on these animals is an alarming factor if one takes into account the key role played by AChE in controlling several physiological aspects. AChE expression inhibition has led to high mortality rates, growth inhibition, malformations, significantly reduced fertility and behavioral disorders in different experimental insect models (Kumar et al., 2009; Revuelta et al., 2009; Hui et al., 2011; He et al., 2012). Besides affecting the larval development of these animals, these effects can significantly affect individuals' fitness and the dynamics of insect populations living in polluted areas.

Finally, it is important emphasizing that the present study did not exhaust the investigated topic. In addition, the current findings open room for new investigations focused on identifying and better understanding how PS NPs affect freshwater benthic macroinvertebrates. Assessing hormone production and secretion (e.g.: steroid hormones; cortisol, etc.) in individuals exposed to PS NPs can help better understanding how these pollutants affect the growth/development and behavioral response of individuals. It is worth investigating whether the effects observed in the present study are similar to those observed in other dragonfly species and whether they cause irreversible or reversible sequelae in adult individuals. Furthermore, it is questionable whether the PS NPs dye would have leached and interfered with the animals' response to treatments. In this case, future studies may be useful to assess and clarify this issue.

5. Conclusion

The current study has partly confirmed its initial hypothesis, since although the short exposure of *A. williamsoni* larvae to PS NPs has led to high pollutant accumulation in the investigated animals (inferred by the BAFs), they did not present changes indicative of nutritional deficit. On the other hand, the most relevant results emerged from REDOX imbalance observations, as inferred through increased oxidative stress biomarkers and antioxidant defenses; such an imbalance had indirect potential to have negative effects on the development and survival of the investigated animals, as well as on the dynamics of their populations. Since the current study did not exhaust the addressed topic, further investigations about the exposure of freshwater benthic macroinvertebrates to PS NPs should be carried out to help better understanding the real magnitude of impacts caused by these pollutants on these important components of the aquatic ecology.

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.

Acknowledgment

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Ethical approval

All experimental procedures were carried out in compliance with ethical guidelines set for animal experimentation. Meticulous efforts were made to assure that animals suffered the least possible and to reduce external sources of stress, pain and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data. This article is not associated with any study with human participants performed by any of the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141936.

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