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First approach to assess the effects of nanoplastics on the soil species *Folsomia candida*: A mixture design with bisphenol A and diphenhydramine

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A R T I C L E   I N F O

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A B S T R A C T

The terrestrial environment is one of the main recipients of plastic waste. However, limited research has been performed on soil contamination by plastics and even less assessing the effects of nanoplastics (NPls). Behind the potential toxicity caused per se, NPls are recognized vectors of other environmental harmful contaminants. Therefore, the main aim of the present study is to understand whether the toxicity of an industrial chemical (bisphenol A – BPA) and a pharmaceutical (diphenhydramine – DPH) changes in the presence of polystyrene NPls to the terrestrial invertebrate *Folsomia candida*. Assessed endpoints encompassed organismal (reproduction, survival and behavior) and biochemical (neurotransmission and oxidative stress) levels. BPA or DPH, 28 d single exposures (1 to 2000 mg/kg), induce no effect on organisms' survival. In terms of reproduction, the calculated EC50 (concentration that causes 50% of the effect) and determined LOEC (lowest observed effect concentration) were higher than the environmental concentrations, showing that BPA or DPH single exposure may pose no threat to the terrestrial invertebrates. Survival and reproduction effects of BPA or DPH were independent on the presence of NPls. However, for avoidance behavior (48 h exposure), the effects of the tested mixtures (BPA + NPls and DPH + NPls) were dependent on the NPls concentration (at 0.015 mg/kg – interaction: no avoidance; at 600 mg/kg – no interaction: avoidance). Glutathione S-transferase activity increased after 28 d exposure to 100 mg/kg DPH + 0.015 mg/kg NPls (synergism). The increase of lipid peroxidation levels found after the exposure to 0.015 mg/kg NPls (a predicted environmental concentration) was not detected in the mixtures (antagonism). The results showed that the effects of the binary mixtures were dependent on the assessed endpoint and the tested concentrations. The findings of the present study show the ability of NPls to alter the effects of compounds with different natures and mechanisms of toxicity towards soil organisms, showing the importance of environmental risk assessment considering mixtures of contaminants.

1. Introduction

Even though the predicted plastic release into soil is approximately 40 times higher than into aquatic ecosystems (Kawecki and Nowack, 2019), only limited research has been performed about terrestrial plastic contamination (Amorim and Scott-Fordsmand, 2021; da Costa et al., 2016). Therefore, investigating the impacts of plastic pollution on soil ecosystems is of high relevance and priority. Despite these prospects of intense plastic pollution on land, there is not yet robust and reliable techniques/methodologies for sampling, extraction, detection, quantification and characterization of the smaller plastic particles – especially the nanoplastics (NPls) (Cai et al., 2021; Wahl et al., 2021). The plastics fragmentation into NPls leads to a different and far wider environmental distribution because smaller and lighter particles are more readily spread (Ostle et al., 2019).

Plastic contamination in soil can be both intentional and unintentional (Meixner et al., 2020). Examples of intentional contamination are plastic mulching and addition of plastic to fertilizers (Nizzetto et al.,

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2016; Horton et al., 2017), while unintentional contamination can occur via composts and fermented organic waste products (Büks and Kaupenjohann, 2020), sewage sludge (de Souza Machado et al., 2018) or irrigation with water from contaminated lakes or rivers (Büks and Kaupenjohann, 2020). For example, Nizzetto et al. (2016) estimated that, per million inhabitants and per year, between 125 and 850 tons of microplastics (MPls) end up on the European agricultural soils through sewage sludge or processed biosolids (Nizzetto et al., 2016). In Europe, concentrations range from 0.3 and 3.4 mg MPls/kg soil near Malmö, Sweden, and up to 55.5 mg MPls/kg soil on floodplain sites in Switzerland (Joos and De Tender, 2022).

The presence of MPls and NPls can alter biological, chemical and physical properties of soil (Joos and De Tender, 2022; Rillig et al., 2017) and affect the valuation of carbon (C) sequestration (Meng et al., 2022). After the oceans, soil is the second largest C sink, sequestering around 80% of the global terrestrial C underground (Joos and De Tender, 2022). These changes in soil properties will consequently affect soil functions, which inevitably disturbs soil biota (Yu and Flury, 2021). MPls and NPls have been found to be taken by plant roots and incorporated by soil invertebrates moving them through the soil matrix (Rillig et al., 2017; Maaß et al., 2017; Huerta Lwanga et al., 2017). Indeed, soil invertebrates such as earthworms, collembolans and mites, can facilitate the transport of plastic particles from the surface to deeper soil layers (Zhu et al., 2018a). Considering the high abundance of collembola, its role on the mobility of MPls and NPls in soil is very relevant (Pathan et al., 2020).

It is well recognized that NPls are taken up by the organisms much more readily and rapidly than their microscale counterparts (Richards and Endres, 2017). Indeed, the cellular uptake almost exclusively happen at the nanoscale. Therefore, the focus should be on potential effects of NPls. However, few studies are available regarding the NPls effects to soil organisms (Table S1), showing that they can induce an effect on *Enchytraeus crypticus* reproduction, microbial community (Zhu et al., 2018b) and behavior (avoidance response) (Barreto et al., 2020).

The higher reactivity surface of NPls compared with MPls makes NPls more prone (up to 100 times more) to adsorb other environmental toxic contaminants (Pathan et al., 2020). NPls can serve as vectors for diverse intrinsic (e.g. plastic additives) and extrinsic (e.g. pharmaceuticals) contaminants, consequently affecting their dissemination in the environment and availability, incorporation and toxicity to biota (Wang et al., 2022).

Bisphenol A (BPA) is an industrial chemical incorporated in several hard plastics used every day. The presence of BPA in agricultural soils is of high concern (Kinney et al., 2006; Flint et al., 2012; Careghini et al., 2015), since it can reach the edaphic medium through sewage sludge or biosolids that are used as organic amendments to fertilize farmland (Careghini et al., 2015; Kinney et al., 2008; Lemos et al., 2009). Indeed, the most significant route of BPA to the terrestrial environment is through the application of sewage sludge from municipal wastewaters plants as soil improvers (concentrations range of 0.033 to 36.7 mg BPA/kg) (Lee and Peart, 2000). In terms of determined BPA concentrations in soil, a previous review reported that concentrations vary between, in general, <0.01 to 1000 μg/kg dependent on the amount and type of effluent or waste received (Corrales et al., 2015). Considered as an endocrine disruptor, the impact of BPA on the edaphic environment and soil life remains mostly unknown, with only limited studies on terrestrial invertebrates (Lemos et al., 2009; Verdú et al., 2018; Babić et al., 2016a; Lemos et al., 2010; Babić et al., 2016b). No study was found evaluating the impacts of BPA in combination with NPls on soil organisms.

Diphenhydramine (DPH), a worldwide over the counter antihistaminic, is incompletely break down in wastewater treatment plants, being present in surface waters as parental compound (Topp et al., 2012). DPH was detected at concentrations as 1.4 and 1.8 μg/L in surface waters and wastewaters, respectively (Bartelt-Hunt et al., 2009; Li et al., 2013). When soil is irrigated by surface waters, DPH forms irreversible bonds with the soil particles (Gorrepati, 2018). According to Topp et al. (2012), following biosolid application, DPH remained in the soil even after three years, showing the DPH high persistence in this matrix (Topp et al., 2012). This can potentially have toxic effects on organisms that live in the terrestrial ecosystem (Gorrepati, 2018). Additionally, a recent study showed that DPH toxicity increased to *E. crypticus* in the presence of NPls (Mendes et al., 2022).

The combined effects of NPls and associated contaminants on terrestrial biota remain incompletely characterized (Wang et al., 2022). Therefore, the main aim of the present study is to understand if the toxicity of DPH and BPA is altered in the presence of NPls to the terrestrial model *Folsomia candida*, assessing parameters from biochemical (related with neurotransmission and oxidative stress) to organismal (reproduction, survival and avoidance behavior) levels.

2. Material and methods

2.1. Test species

The organisms used to the toxicity tests belong to the standard species *F. candida* (Collembola), a soil model organism in ecotoxicology (Fountain and Hopkin, 2004). Cultures of individuals were maintained in laboratory, on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio), at 20 ± 1 °C, under a photoperiod of 16 hours (h): 8 h (light: dark). The organisms were fed weekly with dried baker's yeast (*Saccharomyces cerevisae*). Organisms from these cultures were age synchronized to obtain juveniles with 10–12 days (d) to start the toxicity tests.

2.2. Test medium

The natural standard LUFA 2.2 soil (Speyer, Germany) was used as test medium. According to the supplier, the main characteristics were pH = 5.6, organic carbon = 1.71%, cation exchange capacity = 9.2 meq/100 g, maximum water-holding capacity (WHC) = 44.8% and grain size distribution of 8.9% clay, 13.9% silt and 77.2% sand. The soil was dried (48 h; 60 °C) before use.

2.3. Test contaminants

2.3.1. Nanoplastics

Polystyrene NPls dispersion was acquired from Bangs Laboratories, Inc. (USA). More information about the NPls dispersion can be found at the Supplementary Material. NPls dispersion was centrifuged prior to the toxicity tests using a Vivaspin® 2 mL ultrafiltration device (Bangs Laboratories, Inc) to remove sodium dodecyl sulfate and sodium azide present in the dispersion. The NPls stock dispersion (centrifuged, diluted in ultrapure water) and test dispersions were characterized by hydrodynamic size (HS), zeta potential (ZP) and polydispersity index (PdI). More information at the Supplementary Material.

2.3.2. Bisphenol A and diphenhydramine analysis

Bisphenol A (BPA, ≥ 99%) and diphenhydramine hydrochloride (DPH, ≥ 98%), used for toxicity tests (exposures), bisphenol A-D_{16} (98 atom % D) and diphenhydramine-D_3, used for analytical procedures, were all acquired from Sigma-Aldrich. Methanol and ultrapure water (both LC-MS grade) were acquired from Scharlab (Barcelona, Spain). Formic acid was purchased from Panreac (Barcelona, Spain) and ammonia solution (30%, *w/v*) from Carlo Erba (Barcelona, Spain). BPA and DPH were analyzed in LUFA 2.2 soil for all the experimental conditions following official methods (EPA Method 1694 and ASTM D7858–13). Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was carried out using an Accela (Thermo Scientific, San Jose, CA) quaternary pump, a thermostatted autosampler, a column oven and a TSQ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a HESI-II (heated electrospray ionization) operating in both positive mode (for DPH) and negative mode (for BPA). More details about BPA and DPH analysis can be found at the Supplementary Material.

2.4. Toxicity tests

2.4.1. Soil spiking procedures

For the prior reproduction tests (single exposures), a full concentration range for DPH and BPA was tested: 0–1–10–100–1000–2000 mg/kg soil dry weight (DW). This range was chosen based on the study of Verdú et al. (2018) with other soil organisms *Dendrobaena veneta* and *Eisenia fetida* assessing the effects of BPA. The concentrations 1 and 10 mg/kg can be considered environmentally relevant concentrations since concentrations range of 0.033 to 36.7 mg BPA/kg already was found in sewage sludge from municipal wastewaters plants used as soil improvers (Lee and Peart, 2000). For DPH, only a study was found with soil organisms (*E. crypticus*) testing only two concentrations (10 and 50 mg/kg soil) (Mendes et al., 2022). Therefore, to be possible the comparison, it was used the same concentration range for both contaminants. For the combined exposures, the following experimental conditions were tested: 0; 0.015 and 600 mg NPls/kg soil DW; 100 and 2000 mg BPA or DPH/kg soil DW; and the respective combinations. The concentrations chosen for the combined exposures were based on the results from the single exposures with DPH and BPA. The NPls concentrations were selected based on the previous studies with other soil species (*E. crypticus*) (Barreto et al., 2020). NPls concentrations <15 μg/L (0.015 mg/L) have been predicted to be environmentally relevant concentrations (Al-Sid-Cheikh et al., 2018). NPls, at 0.015 mg/kg, induced an avoidance behavior in the organisms whereas 600 mg/kg induced no effect in the organisms (Barreto et al., 2020). The control soil was made with deionized water considering the adequate moisture content (50% of the maximum WHC). The DPH and BPA test solutions were dissolved in methanol due to the low solubility of BPA in water (298 mg/L, at 25 °C, according to the supplier). A solvent control was performed, adding the same volume of methanol present in the treatments with BPA and DPH (corresponding to 10% of methanol). For the single exposures, test solutions of DPH and BPA and solvent control were added to the soil, which was left in a fume hood for 24 h, for the evaporation of the methanol. At day 0, deionized water was added to soil to adjust to 50% of the maximum WHC, then, soil was mixed and divided per vessels. For the NPls single exposures, the needed volumes of NPls test dispersions (prepared in ultrapure water) were added to the pre-moistened soil (in which water was added before) until 50% of the WHC maximum and mixed manually. For the combined exposures, the required volumes of NPls test dispersions were added to the pre-moistened soil previously contaminated with DPH or BPA (after 24 h for methanol evaporation). All the replicates with NPls were mixed individually and after 4 h, the toxicity test started.

2.4.2. Reproduction test

The experimental procedures followed the standard guidelines for Collembolan Reproduction Test in Soil (OECD, 2016), with adaptations. In short, 10 individuals of synchronized age (10–12 d) were put in each test vessel, containing 30 g of moist soil and food (dried baker's yeast). Test conditions were 20 ± 1 °C and 16 h: 8 h photoperiod. Test ran for 28 d and food and water loss was replenished on the soil surface weekly. Four replicates per experimental condition ($n = 4$) were applied. An extra replicate per condition (not including organisms) was made to the measurement of the pH values.

At day 28, each test vessel was flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further counting of the organisms applying the software ImageJ. Survival (number of adults) and reproduction (number of juveniles) were assessed. After the image capture, 300 juveniles per replicate were sampled in microtubes, snap frozen in liquid nitrogen and stored at −80 °C, until further biochemical analysis.

2.4.3. Avoidance test

The ISO avoidance test guidelines was followed (ISO Soil Quality, 2011), using the 2 chamber option. Half of each box was filled with 30 g of the control soil and the other half with 30 g of spiked soil. Per replicate, twenty juveniles (10–12 d old) were utilized. At the end of the test (48 h), the soil from each half of the box was divided and placed into vessels, inundated with water and the number of floating organisms was counted directly.

2.5. Biochemical markers analysis

Procedures followed the previously optimized methodologies by Maria et al. (2014) for *F. candida*. Catalase (CAT), glutathione S-transferases (GST), acetylcholinesterase (AChE) activities and lipid peroxidation (LPO) levels were assessed. More details at the Supplementary Material. Protein concentration was determined using bovine γ-globuline as a standard (Bradford, 1976). For CAT and GST activities, Clairborne (1985) and Habig et al. (1974) were followed, respectively. LPO levels were assessed according to Ohkawa et al. (1979) and Bird et al. (1984), adapted by Wilhelm Filho et al. (2001). Acetylcholinesterase (AChE) activity was determined according to Ellman et al. (1961), adapted by Guilhermino et al. (1996).

2.6. Data analysis

The avoidance response, express as percentage of affected *F. candida* (i.e., those that avoided the spiked soil), was calculated following the ISO guidelines for avoidance test (ISO Soil Quality, 2011). The used formula can be found at the Supplementary Material. Graphics and statistics analysis were made applying the Sigma Plot 12.5 software package. Significant differences were considered for a significance level $(p) < 0.05$. The Effect Concentrations (ECx) were estimated using the Toxicity Relationship Analysis Program (TRAP v1.22). More details about statistical analysis can be found at the Supplementary Material.

3. Results and discussion

3.1. Characterization of nanoplastics

At 0.015 mg/kg it was not possible to characterize the HS and ZP of NPls due to the technique detection limits. NPls test dispersion, at 600 mg/kg, presented the expected HS (45 ± 0.1 nm) with lower PdI (0.1), being similar to the one measured at the NPls stock dispersion (44 ± 0.1 nm). NPls ZP test dispersion was highly negative (−26.8 mV) and also in concordance with the one measured at the NPls stock dispersion (−26.7 mV). In the combination with BPA, higher NPls HS (286 ± 23 nm) and PdI (0.6) were found. The same for the combination with DPH (HS: 309 ± 45 nm and PdI: 0.5). The NPls ZP were less negative in the combinations than when NPls were alone (−18.8 mV). Mendes et al. (2022) also showed that the presence of DPH induced an increase in NPls HS and a less negative ZP value. The results from the characterization of NPls showed that the presence of other contaminants seemed to promote NPls agglomeration/aggregation processes, independent on the concentration of BPA or DPH. Moreover, a possible adsorption or linking of NPls and BPA or DPH may also justify the increased HS, PdI and ZP values of NPls at the mixtures. The detection and characterization of NPls in complex matrices as soil remains a challenge (Barreto et al., 2020), not being yet possible with accuracy and robustness. However, this is a gap that urgently needs to be addressed once it is crucial to correlate the behavior/characteristics of NPls in soil and their toxic effects to terrestrial organisms. Once in soil matrix, NPls may interact with the organisms and soil constituents and this may change their chemical and physical properties with relevant effects on their reactivity and potential toxicity to the organisms (Pathan et al., 2020). Moreover, the interactions between contaminants in the soil matrix can be different from the ones occurring in a simple system as ultrapure water.

3.2. Bisphenol A and diphenhydramine quantification

In general, the measured BPA and DPH concentrations were in accordance with the nominal concentrations (Table 1). For BPA, recoveries ranged from 76% to 101.6%, while for DPH ranged from 90.7% to 110.5%. MPls/NPls can be considered as sources of hydrophobic organic contaminants as well as sinks. In fact, the hydrophobic nature and high surface area-to-volume ratio of micro(nano)plastics facilitate the accumulation of organic contaminants onto their surface (Yang et al., 2022). Although the recoveries observed for BPA are within the acceptable levels (80%–120%), slightly lower recoveries were observed for BPA (76%–101.6%) compared to DPH (90.7%–110.5%). This can be explained by the higher hydrophobicity of BPA which results in a higher sorption capacity to NPls and soil organic matter (Liu et al., 2019). The partitioning of an organic chemical to plastic is usually governed by its hydrophobicity, which is related to its log*P* (or Kow). Thus, if a certain organic chemical has a high log*P*, a high affinity for plastic is expected to exist, and thus, a higher accumulation on this material (Atugoda et al., 2021). While this stands true for non-ionizable compounds, for ionizable compounds (such as BPA and DPH) a different descriptor should be used since these compounds may exist as different species at a given pH. In this case, the distribution coefficient log*D* is the appropriate descriptor to be used. Considering a pH of 5.6 (pH of the tested soil), DPH presented a significantly lower log*D* value (0.58) compared to BPA (4.04),[1] which could explain the higher affinity of BPA for both NPls and soil organic matter. We hypothesized that this significant difference in log*D* values between BPA and DPH could be a critical factor behind the lower recovery rates observed for BPA compared to DPH. There is a clear lack of knowledge in relation to the interaction of hydrophobic organic contaminants (especially ionizable) and MPls/NPls. Yet, the available literature states that hydrophobic organic contaminants have a high tendency to get adsorbed on non-polar surfaces, displaying a greater affinity for plastic surfaces compared to soil organic matter (Carbery et al., 2018).

3.3. Toxicity tests

No differences were found between the control and solvent control ($p > 0.05$). Therefore, all the replicates from control plus solvent control were considered a "unique" control and represented as 0 mg/kg in the figures. All the treatments were compared with this control.

Table 1
Concentration of bisphenol A and diphenhydramine in the exposure media (LUFA 2.2 soil) at the beginning of the toxicity tests (day 0). Results are expressed as mean ± standard error ($n = 3$). NPls – Nanoplastics.

Nominal concentrations (mg/kg)	Measured concentrations (mg/kg)	
	Bisphenol A	Diphenhydramine
0	<LOQ	<LOQ
1	0.84 ± 0.0042	1.0 ± 0.04
10	7.8 ± 0.09	10.2 ± 0.08
100	90.3 ± 0.52	96.3 ± 1.18
1000	908.5 ± 8.32	945.1 ± 6.51
2000	1980.8 ± 22.53	1989 ± 23.42
100 + 0.015 NPls	97.7 ± 0.90	104.2 ± 0.91
100 + 600 NPls	91.01 ± 0.012	103.6 ± 1.472
2000 + 0.015 NPls	1958.8 ± 22.35	1937.6 ± 4.10
2000 + 600 NPls	2021.7 ± 6.81	1837. 9 ± 13.39

Limit of quantification (LOQ) for bisphenol A: 0.1 mg/kg and for diphenhydramine: 0.01 mg/kg; Limit of detection (LOD) for bisphenol A: 0.01 mg/kg and for diphenhydramine: 0.001 mg/kg.

3.3.1. Reproduction tests – single exposures of bisphenol A and diphenhydramine

After 28 d, although neither BPA nor DPH induced an effect on the survival of *F. candida* ($p > 0.05$; Fig. 1), an effect on the organisms reproduction was detected. BPA, at 1000 and 2000 mg/kg, and DPH, at 2000 mg/Kg, decreased the reproduction (number of juveniles) of *F. candida* ($p < 0.05$; Fig. 1).

Lemos et al. (2009) showed that 10 weeks exposure of the terrestrial isopod *Porcellio scaber* to 1000 mg BPA/kg was lethal for 50% of organisms. However, BPA induced no significant mortality for 28 d of exposure (Lemos et al., 2009), as in the present study. The previous result shows than with the increase of exposure time, the sensitivity of the organisms to BPA (and their degradation products) can increase (increasing the organisms mortality). Perhaps, in the present study, if the exposure time was increased, an effect on *F. candida* survival could be found. Therefore, further long-term BPA exposure studies are highly recommended. Indeed, although BPA has a short half-life in soil (between 2.5 and 5 d), its long-term toxic effects on terrestrial invertebrates could still be significant because some environments are constantly exposed to new BPA contaminations by breakdown and leaching from polycarbonate plastic, releasing of BPA-contaminated wastewaters, disposal of sewage sludges, among others (Babić et al., 2016b). On the other hand, as previously reported, BPA degradation products (e.g. 2,2-bis(4-hydroxyphenyl)-1-propanol and 1,2-bis(4-hydroxyphenyl)-2-propanol) can be more toxic with the increasing time (Lemos et al., 2009).

A previous study showed that the number of juveniles decreased in *E. fetida* exposed to 1000 and 2000 mg/kg BPA (Verdú et al., 2018), which corroborates with the present data. Babić et al. (2016) reported that, in *E. fetida*, BPA acted on the ovaries as target organs, causing severe alterations in their structure that would lead to a reduction of fertility (Babić et al., 2016b). In line with this, but using *P. scaber*, a decrease in reproduction with increasing BPA concentration was found (Lemos et al., 2010). *P. scaber* exposed to BPA started to molt sooner than the unexposed organisms (Lemos et al., 2009). The referred studies evidence the endocrine disruptor role of BPA on soil organisms. Different effects of endocrine-disrupting pesticides during mating, interfering with reproduction were already reported (Yasmin and D'Souza, 2010). In particular, the insecticide malathion decreased sperm viability within the spermathecae in *E. fetida* (Espinoza-Navarro and Bustos-Obregón, 2005). Further studies are needed to elucidate which mechanisms occur in *F. candida* once the reproduction was affected by the presence of BPA.

In accordance with the current apical effects on *F. candida*, Mendes et al. (2022) showed that DPH at 10 and 50 mg/Kg induced no effects on survival and reproduction of *E. crypticus* exposed during 21 d. For the aquatic invertebrate *Daphnia magna*, 100% mortality occurred at lower DPH concentrations of 27.8, 46.1 and 273.4 μg/L (compared to our tested concentrations) at days 7, 5 and 4, respectively (Berninger et al., 2011). Moreover, the lowest observed effect concentration (LOEC) for reproduction was 3.4 μg/L after 10 d DPH exposure (Berninger et al., 2011). These results show the higher sensitivity of *D. magna* to DPH compared with *F. candida* (no DPH effect on survival and LOEC for reproduction = 2000 mg/kg).

Overall, the *F. candida* reproduction endpoint was more sensitive compared with the survival endpoint, corroborating with previous ecotoxicology studies including BPA (Verdú et al., 2018), DPH (Mendes et al., 2022) and other soil organisms. Yasmin and D'Souza (2010) reported that endocrine disruptors compounds as BPA altered organisms growth and reproduction without inducing mortality, showing that these chemicals act on target organs and did not affect the whole body. Berninger et al. (2011) also showed for aquatic organisms that DPH induced effects in the reproduction, growth and behavior at lower concentrations than the effects in the survival.

[1] Chemicalize was used for prediction of logD values for bisphenol A and diphenhydramine, November 2022, https://chemicalize.com/, developed by ChemAxon

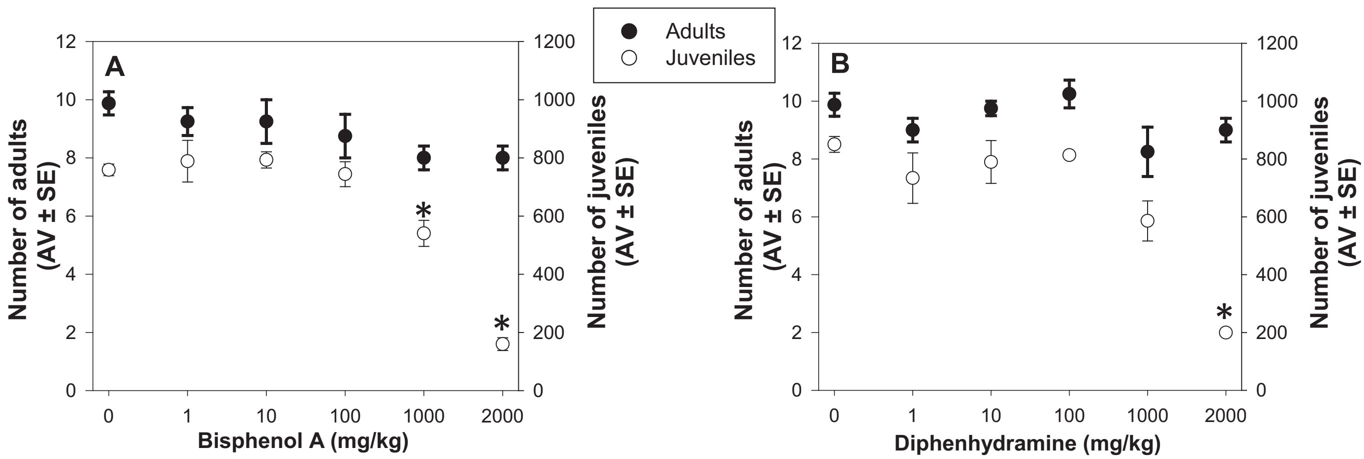

Fig. 1. Effects on survival (number of adults) and reproduction (number of juveniles) of *Folsomia candida* after 28 days exposed to bisphenol A (A) and diphenhydramine (B) in LUFA 2.2 soil. Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$).

The EC50 (concentration that causes 50% of the effect), after 28 d, for reproduction of *F. candida* was 1399.3 and 1498.2 mg/kg for BPA and DPH, respectively (Table 2). BPA was more toxic to *F. candida* with EC50 (1399 vs 1498 mg/kg) and LOEC (1000 vs 2000 mg/kg) values for reproduction lower than the ones for DPH (Table 2). The calculated EC50 and determined LOEC for both contaminants were higher than the environmental concentrations. Indeed, the adverse effects (decreased reproduction) were found at concentrations above the environmental levels, showing that BPA and DPH may pose no threat to the terrestrial invertebrates. However, caution should be taken in their environmental risk assessment since these chemicals might become more toxic in mixtures of contaminants than at single exposures.

3.3.2. Reproduction tests – combined exposures of nanoplastics with bisphenol A or diphenhydramine

After 28 d, both BPA and DPH at 2000 mg/kg, combined with NPls (0.015 or 600 mg/kg) decreased the reproduction of *F. candida* ($p < 0.05$; Fig. 2). No significant effects were found on organisms survival ($p > 0.05$; Fig. 2), similar to what happened at the single exposures of BPA or DPH. These results showed that the adverse (or absent) effects of these compounds were independent on the presence of NPls, since the observed (or no) effects in reproduction and survival were similar at single and combined exposures.

The adsorption of BPA on NPls is dominated by saturated single-layer adsorption and the introduction of electrolytes inhibited the adsorption of BPA onto NPls (Berninger et al., 2011). The adsorption process of DPH on NPls is dependent, among others, in the environmental pH. Considering the pH of the soil (5.6) used in the present study it is expected that the adsorption of the pharmaceutical by NPls mainly occurs through hydrophobic interactions (Wang et al., 2022).

Chen et al. (2017) showed that NPls may facilitate BPA uptake in zebrafish (*Danio rerio*) which may induce more effects to the organisms. This seems not occurring in the present study. Mendes et al. (2022) showed that, after 21 d, co-contamination of NPls (300 mg/kg) and DPH (10 and 50 mg/kg) resulted in a higher degree of effect in *E. crypticus* reproduction (decreased) when compared with the single exposures (where no effect was observed). This showed an interaction between NPls and DPH (synergism) indicating a combined effect of the mixture. In the present study, DPH decreased *F. candida* reproduction in the same way with and without NPls. These dissimilar effects between the studies can be due to the difference in the DPH and NPls tested concentrations, suggesting that the interaction effects of DPH and NPls might be dependent on the compounds concentration. On the other hand, despite to be two soil species, they have distinct ecological niches, leading to dissimilar contaminant exposure route and, consequently, leading to species-specific sensitivities. Collembolans (*F. candida*) are mainly exposed to soil pore water through their ventral tube, while earthworms (*E. crypticus*) to both pore water and soil particles by dermal and oral contact (Santos et al., 2020).

Table 2
Concentration that causes 50% of the effect (EC50), applying the 2-parameters Logistic model, and lowest observed effect concentration (LOEC) for *Folsomia candida* reproduction, assessed after 28 days exposure to bisphenol A or diphenhydramine in LUFA 2.2 soil.

Chemicals	EC50 (mg/kg)	SE	95% LCL	95% UCL	LOEC (mg/kg)
Bisphenol A	1399.3	79.3	1236.2	1562.4	1000
Diphenhydramine	1498.2	110.4	1271.3	1725.1	2000

SE: Standard error. 95% LCL: 95% lower confidence limit. 95% UCL: 95% upper confidence limit.

3.3.3. Avoidance test

After 48 h, both BPA and DPH, at 2000 mg/kg, alone and combined with NPls (600 mg/kg) induced a *F. candida* avoidance behavior ($p < 0.05$; Fig. 3). Interesting, 2000 mg/kg of BPA or DPH combined with 0.015 mg/kg NPls did not induce the same avoidance response. This effect was distinct from the expected based on the single exposures (2000 mg/kg BPA or DPH induced avoidance behavior). This shows that the effects of the mixtures BPA or DPH and NPls are dependent on the NPls concentration: 2000 BPA or DPH mg/kg + 0.015 NPls mg/kg – antagonism interaction (no avoidance behavior); 2000 BPA or DPH mg/kg + 600 NPls mg/kg – no interaction (avoidance behavior similar to the single exposures). Contrary to what happened with the reproduction and survival endpoints, the mixtures effects on the organisms' avoidance behavior were dependent on the NPls presence. Hence, the interaction effects of the mixtures were dependent on the assessed endpoint, showing the dissimilar toxicity mechanisms of the mixtures.

If for both concentrations (0.015 and 600 mg/kg), NPls maintain their nano sizes, 600 mg/kg NPls should adsorbed more BPA or DPH than 0.015 mg/kg. Therefore, a greater interaction between contaminants (NPls and BPA or DPH) would be expected. However, this was not found in the present study for avoidance behavior endpoint. At low concentrations, as 0.015 mg/kg, NPls are more prone to maintain their nanosizes (Barreto et al., 2020), presenting higher probability to be incorporate (with adsorbed BPA or DPH) by the organisms than NPls at 600 mg/kg. At high NPls concentrations, agglomeration/aggregation can often occur, leading to particles with largest sizes and, consequently, less incorporation by the organisms (Barreto et al., 2020). Moreover, a previous study showed that aggregation processes can decrease the BPA

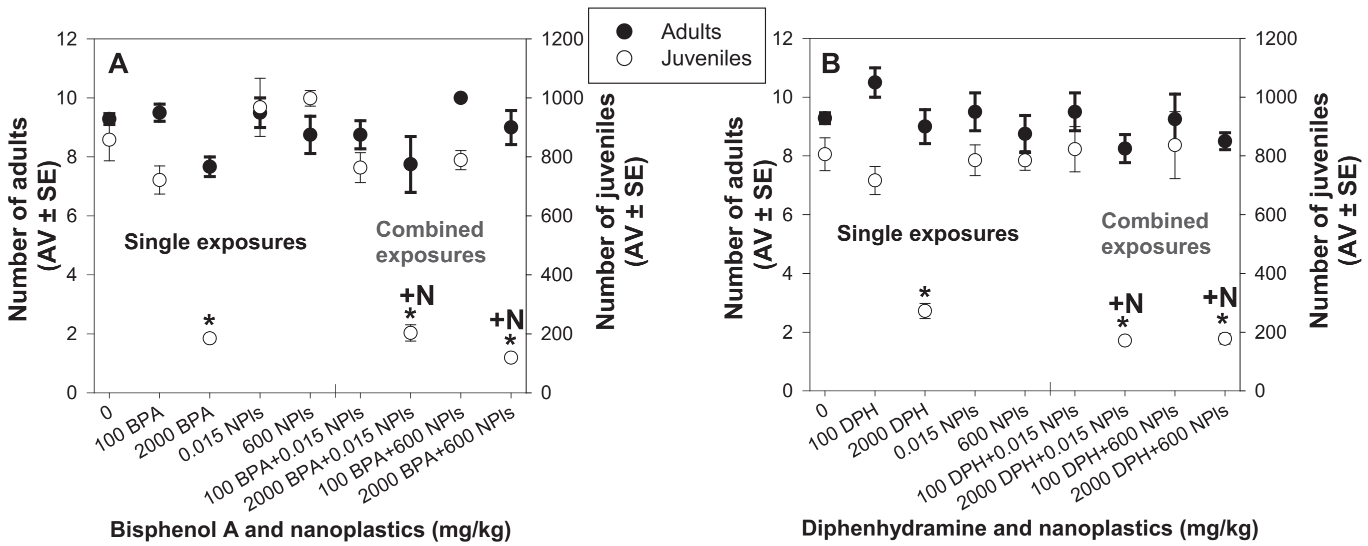

Fig. 2. Effects on survival (number of adults) and reproduction (number of juveniles) of *Folsomia candida* after 28 days exposed to bisphenol A (BPA) (A) and diphenhydramine (DPH) (B) combined with nanoplastics (NPls) in LUFA 2.2 soil. Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control (p < 0.05). [+N] Significant differences to the correspondent nanoplastics single concentration (p < 0.05).

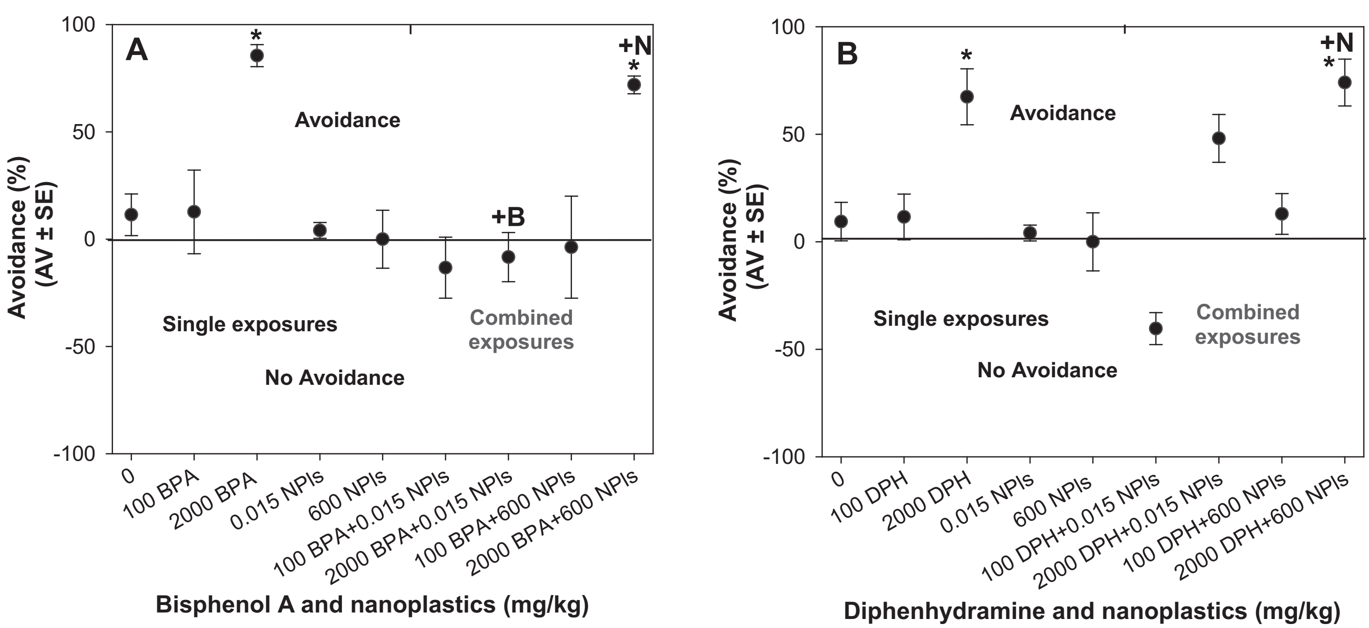

Fig. 3. Avoidance responses of *Folsomia candida* after 48 h exposed to bisphenol A (BPA) (A) and diphenhydramine (DPH) (B) combined with nanoplastics (NPls) in LUFA 2.2 soil. Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control (p < 0.05). [+N] Significant differences to the correspondent nanoplastics single concentration (p < 0.05). [+B] Significant differences to the correspondent bisphenol A single concentration (p < 0.05).

adsorption on NPls (Li et al., 2022). Therefore, at 600 mg/kg, less BPA or DPH is adsorbed to NPls, being "free" as when it is alone, presenting similar effects at single versus combined exposures (avoidance behavior). It seems that these possible processes/occurrences were not preponderant for the effects (or not) detected on the reproduction and survival, once the effects were similar at single and combined exposures.

Another explanation could be that at 0.015 mg/kg NPls, the possible higher incorporation of NPls (with adsorbed BPA or DPH) by the organisms, can induce a direct or indirect neurotoxic effect leading to the incapacity of the organisms to be able to avoid the contaminated soil. Co-exposure of NPls (1 mg/L) and BPA (1 μg/L) led to a significant increment of BPA uptake in the head and viscera of adult zebrafish when compared with BPA alone treatment (Chen et al., 2017). Further studies assessing the internal levels of BPA and DPH in exposed *F. candida* (single versus combined exposures) are needed to complement the obtained results and corroborate the previous explanation.

The avoidance behavior must be understood as a protective mechanism. When organisms are not capable to get away from contaminated environments, they can get intoxicated, and this can affect other biological responses (Barreto et al., 2020). However, in the other hand, *F. candida* exposed to 2000 mg/kg BPA present the highest percentage of avoidance (86%), being higher than 80%, suggesting that the habitat function will be compromised (ISO Soil Quality, 2011). The percentage of avoidance for organisms exposed to 2000 mg BPA/kg + 600 mg NPls/kg was 72%. Regarding DPH, the percentages of avoidance were similar for single (67%) versus combined exposures (2000 mg BPA/kg + 600 mg NPls/kg: 63%).

3.4. Biochemical markers analysis

At the experimental conditions with 2000 mg/kg BPA or DPH, it was not possible to collect the 300 juveniles needed to the biochemical analysis due to the reproduction effect (decrease) of both contaminants (alone and combined with NPls) on the organisms. *F. candida* CAT activity was not altered after 28 d exposure to the tested experimental conditions ($p > 0.05$; Fig. 4A). However, 100 mg/kg DPH + 0.015 mg/kg NPls increased the activity of GST ($p < 0.05$; Fig. 4B) and 0.015 mg/kg NPls increased the LPO levels ($p < 0.05$; Fig. 4C). NPls induced oxidative damage at a predicted environmental concentration. Previous studies reported that oxidative stress and its corresponding pathways are key events in NPls toxicity (Hu and Palić, 2020). In the present study, it was not found an activation of detoxification (GST) or oxidative defense (CAT) related enzymes by NPls but other enzymes must be further analyzed to understand the cascade induced by NPls before the oxidative damage. Another important aspect is to analyze the oxidative stress at different periods of time, i.e., at the first days of exposure. CAT and GST could be activated earlier during the exposure period and, then, their activities returned to basal values after 28 d. But if so, it seems that this induction was not enough to avoid the increase of LPO levels that persisted even after 28 d exposure. A previous study with *E. fetida* showed that 3 d BPA exposure increased lipid oxidation indicating oxidative stress (Babić et al., 2016b). No effect of 28 d BPA exposure was detected in LPO for *F. candida.*

Regarding GST, there was an interaction effect of the mixture NPls and DPH (synergism) since at the single exposures no effect was detected. For LPO, it seems that the presence of BPA and DPH cancelled the negative effects of 0.015 mg/kg NPls (antagonism effect). These results showed (again) that the effects of the mixtures are dependent on the assessed endpoint and the tested concentrations.

AChE activity was not altered after the exposure to the tested experimental conditions ($p > 0.05$; Fig. 4D). However, an effect on AChE activity could be an expected result due to the non-avoidance behavior found for some tested experimental conditions. A correlation between non-avoidance of *F. candida* and AChE inhibition was previously described (Pereira et al., 2013). Moreover, previous studies with zebrafish already reported an alteration on the AChE activity for organisms exposed to DPH (increase) (Barreto et al., 2022), and to BPA and NPls (decrease) (Chen et al., 2017). Chen et al. (2017) reported that the decrease of zebrafish AChE activity found for BPA and NPls single exposures (leading to subsequent neurotoxicity), was not detected for

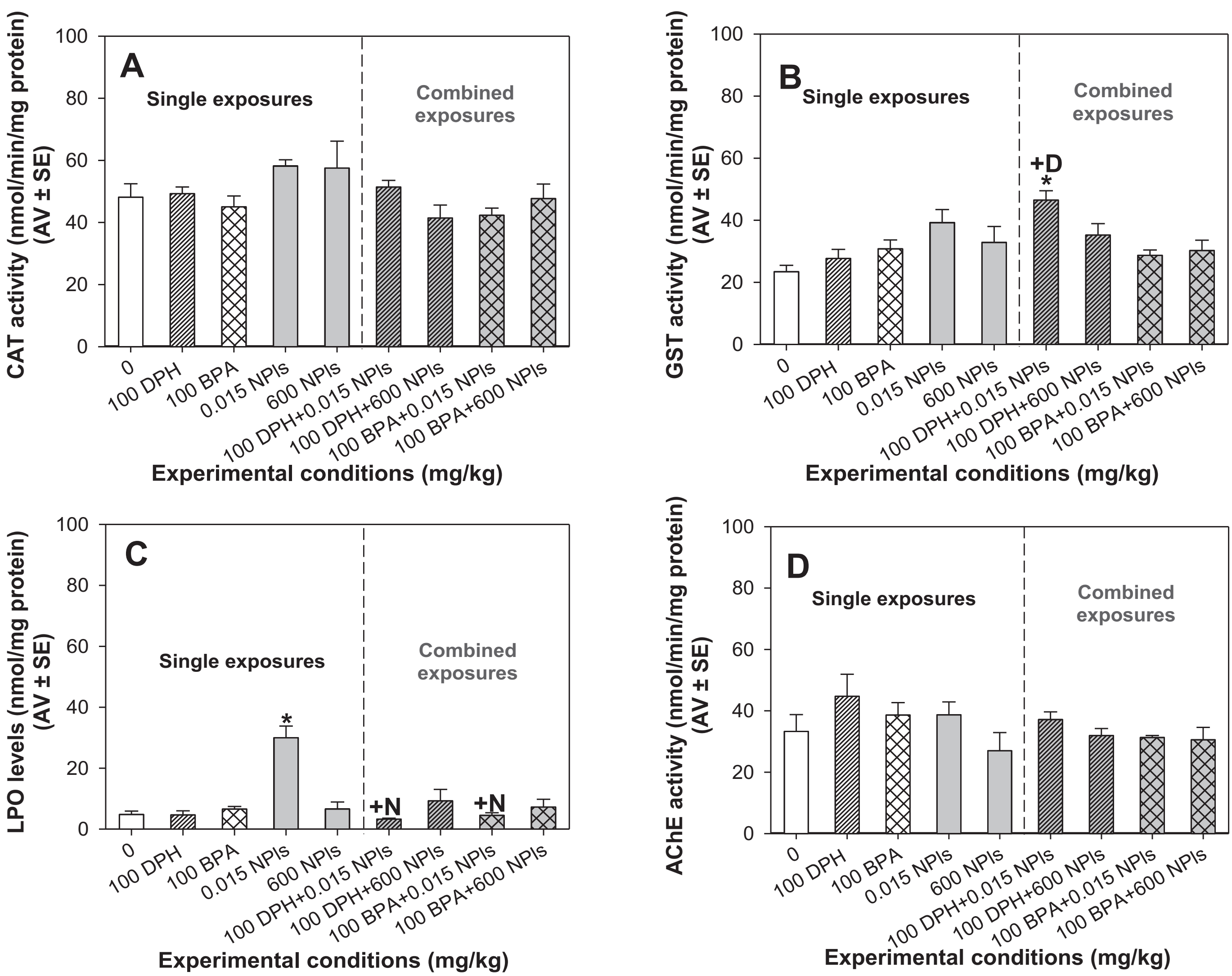

Fig. 4. Biochemical responses of *Folsomia candida* after 28 days exposed to diphenhydramine (DPH) and bisphenol A (BPA) combined with nanoplastics (NPls) in LUFA 2.2 soil, in terms of: catalase (CAT) activity (A); glutathione S-transferases (GST) activity (B); lipid peroxidation (LPO) levels (C); and acetylcholinesterase (AChE) activity (D). Data are expressed as average value (AV) ± standard error (SE). * Significant differences to control ($p < 0.05$). $^{+N}$ Significant differences to the correspondent nanoplastics single concentration ($p < 0.05$). $^{+D}$ Significant differences to the correspondent diphenhydramine single concentration ($p < 0.05$).

the combination BPA + NPls.

The NPls concentration 0.015 mg/kg induced more effects in the biochemical endpoints that 600 mg/kg. As above described, a more cellular uptake may occur at lower concentrations of NPls, inducing more effects to the organisms. The lower aggregation/agglomeration processes of NPls at low concentrations when compared with high concentrations can explain the response pattern observed (Barreto et al., 2020). The characterization of NPls in soil medium during the exposure period (0 to 28 d, in this case) would be helpful to corroborate or not this hypothesis.

The observed effects at biochemical endpoints (GST activity and LPO levels increase) occurred at concentrations lower than the effects induced at the individual levels (survival, reproduction and avoidance behavior). This is an expected result since the formation of reactive oxygen species (ROS) is considered an initiating event, leading to adverse outcomes at individual levels through oxidative stress cascades and inflammatory responses (Hu and Palić, 2020).

As occurring with many pharmaceuticals, it is possible that the toxicity mechanisms of DPH are related with its therapeutic mechanism/mode of action (MoA), rather than nonspecific narcosis responses typically seen with industrial chemicals (Berninger et al., 2011). DPH has multiple MoA targeting the histamine H1, AChE and 5-HT reuptake transporter receptors (Barreto et al., 2022). BPA may modify natural endocrine functions by binding to the estrogen receptor (ER) (Qiu et al., 2019). For the assessed endpoints, despite BPA (an industrial chemical) and DPH (a pharmaceutical) presenting distinct natures and mechanisms of toxicity, the results showed that at the tested concentrations their toxicity behavior was independent on the NPls presence. However, for some endpoints, interaction effects of the mixtures occurred. Therefore, studies that perform the evaluation risk assessment must consider, among others, the presence of NPls, longer-exposure periods and assessment of other endpoints, besides oxidative stress, for a complete understanding of NPls role on toxicity of other environmental contaminants.

4. Conclusions

In general, the effects of BPA or DPH to *F. candida*, including biochemical to organismal responses, were independent on the presence of NPls. However, for some endpoints (avoidance behavior, GST activity and LPO levels) the toxicological effects of the mixtures (BPA + NPls or DPH + NPls) were lower (antagonism) or higher (synergism) than the expected based on the results from single exposures. These findings show the ability of NPls (at predicted environmental concentrations: 0.015 mg/kg) as vector for other environmental contaminants with distinct natures (an industrial chemical and a pharmaceutical) altering their effects towards soil organisms, dependent on the contaminants concentration and the assessed endpoint.

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CRediT authorship contribution statement

Angela Barreto: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Joana Santos:** Methodology, Writing – review & editing. **Lara Almeida:** Methodology, Writing – review & editing. **Vítor Tavares:** Methodology, Writing – review & editing. **Edgar Pinto:** Methodology, Writing – review & editing. **Maria Celeiro:** Methodology, Writing – review & editing. **Carmen Garcia-Jares:** Methodology, Writing – review & editing. **Vera L. Maria:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2023.100450.

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