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Long-term effects of lithium and lithium-microplastic mixtures on the model species *Daphnia magna*: Toxicological interactions and implications to 'One Health'

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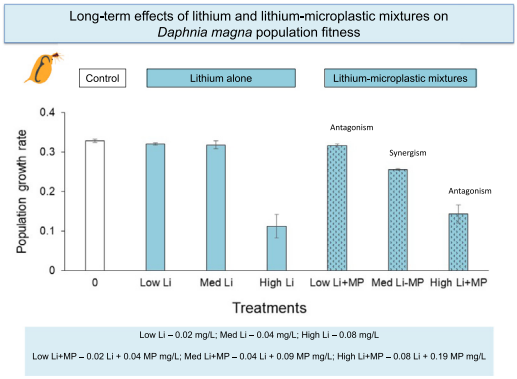
HIGHLIGHTS

- Lithium (Li) and Li-microplastic (MP) mixtures decreased *D. magna* growth and reproduction.
- 21-d exposure to 0.08 mg/L of lithium (Li) reduced *D. magna* population fitness by 67%.
- Li-microplastic (MP) mixtures ≥0.04 Li + 0.09 MP mg/L reduced *D. magna* population fitness.
- At the medium mixture concentration, Li-MP interaction in the population growth rate was synergism.
- At the lowest and highest mixture concentrations, the Li-MP interaction was antagonism.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental contamination with lithium (Li) and microplastics (MP) has been steadily increasing and this trend is expected to continue in the future. Many freshwater ecosystems, which are crucial to reach the United Nations Sustainable Development Goals, are particularly vulnerable to Li and MP contamination, and other pressures. The long-term effects of Li, either alone or combined with MP (Li-MP mixtures), were investigated using the freshwater zooplankton micro-crustacean *Daphnia magna* as model species. In the laboratory, *D. magna* females were exposed for 21 days to water concentrations of Li (0.02, 0.04, 0.08 mg/L) or Li-MP mixtures (0.02 Li + 0.04 MP, 0.04 Li + 0.09 MP mg/L, 0.08 Li + 0.19 MP mg/L). In the range of concentrations tested, Li and Li-MP mixtures caused parental mortality, and decreased the somatic growth (up to 20% and 40% reduction, respectively) and the reproductive success (up to 93% and 90% reduction, respectively). The 21-day EC_{50}s of Li and Li-MP mixtures on *D. magna* reproduction were 0.039 mg/L and 0.039 Li + 0.086 MP mg/L, respectively. Under exposure to the highest concentration of Li (0.08 mg/L) and Li-MP mixtures (0.08 Li + 0.19 MP mg/L), the mean of *D. magna* population growth rate was reduced by 67% and 58%, respectively. Based on the population growth rate and using data from a bioassay testing the same

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concentrations of MP alone and carried simultaneously, the toxicological interaction between Li and MP was antagonism under exposure to the lowest and the highest concentrations of Li-MP mixtures, and synergism under exposure to the medium concentration of Li-MP mixtures. These findings highlight the need of further investigating the combined effects of contaminants, and the threat of long-term environmental contamination with Li and MP to freshwater zooplankton, biodiversity, ecosystem services and 'One Health'.

1. Introduction

Lithium (Li) is a natural element on earth that is present in the lithosphere, atmosphere, hydrosphere and biosphere (Bolan et al., 2021; Chaves et al., 2021). The global demand for Li has been steadily increasing, mainly due to the use of Li-based batteries that are crucial for several growing industries, such as electronics (Mejame et al., 2020) and electric vehicles (Kelly et al., 2021). Li has many other industrial applications (Kszos and Stewart, 2003; Aral and Vecchio-Sadus, 2008; Bolan et al., 2021) supporting our life-style, is also widely used to treat neurological disorders, and additional therapeutic applications have been investigated (Haupt et al., 2021; Haussmann et al., 2021).

Despite the progresses in the technology aiming at reducing the environmental impact of Li, large-scale recycling, recovery and removal of Li from contaminated areas and accumulated waste, particularly e-waste, is still a challenge (Bolan et al., 2021; Chandran et al., 2021). Moreover, Li background levels in natural waters, wastewater and tap water can rise with the augment of population density, as documented in an urbanized area (Choi et al., 2019). Therefore, the environmental contamination by Li is expected to further increase in the coming years (Thibon et al., 2021) with the need to move vast sectors into more 'green' technologies, human population growth and increasing use of electronic products and vehicles as major drivers. The growing trend of Li demand and environmental contamination has been raising high concern, sometimes with strong protests of citizens against Li extraction and the opening of new exploration areas. A recent study in the Salar de Atacama, Chile (Jerez et al., 2021), illustrated diverse angles of the Li paradigm and highlighted the urgency of addressing them and come up with adequate solutions.

Li is up taken by living organisms, being accumulated by several species (Tkatcheva et al., 2015; Viana et al., 2020), and is present in terrestrial and aquatic trophic webs (Aral and Vecchio-Sadus, 2008; Bolan et al., 2021; Thibon et al., 2021). Adverse effects in animals exposed to Li at concentrations in the ppb or low ppm ranges were described, such as neurotoxicity (Viana et al., 2020; Oliveira et al., 2011), hepatotoxicity (Pinto-Vidal et al., 2021), nephrotoxicity (Jing et al., 2021), reproductive toxicity (Kszos et al., 2003), influence in the structure of wild communities (García-Seoane et al., 2016), among others (e.g., Tkatcheva et al., 2015; Pinto-Vidal et al., 2021; Vidal et al., 2021). In persons under Li therapy, neurotoxicity and several other adverse effects were documented (Diserens et al., 2021; Jacob et al., 2020; Verdoux et al., 2021). The toxicity of this metal is very complex, influenced by several factors, and the mechanisms involved are not yet completely understood (Krull et al., 2022).

In several industries and resulting applications used daily by millions of persons across the globe, where Li is an important resource, plastics are also widely used, sometimes in the form of microplastics (MP, plastic particles with size <5 mm). MP have many other uses and are also formed in the environment through the progressive fragmentation of larger plastic pieces into smaller sized ones (Andrady, 2017). MP occur worldwide in the environment and in the biota (e.g., Barboza et al., 2020; Ali et al., 2021; Vital et al., 2021; Wang et al., 2021b; Talbot and Chang, 2022). Therefore, Li and MP are present simultaneously in many ecosystems and trophic webs across the globe. Due to the high mobility of Li and MP in terrestrial compartments, freshwater and coastal ecosystems are particularly vulnerable to their contamination.

Bioaccumulation and adverse effects of MP have been described in a high diversity of aquatic species (Ali et al., 2021; Gonçalves and Bebianno, 2021; Kukkola et al., 2021; Castro-Castellon et al., 2021) at sub-individual (e.g., Barboza et al., 2018b, 2018c; Gonçalves et al., 2022), individual (e.g., Oliveira et al., 2018; Guimarães et al., 2021) and population levels (e.g., Pacheco et al., 2018; Eltemsah and Bøhn, 2019; Zimmermann et al., 2020; Trotter et al., 2021). Some adverse effects persist for generations (Martins and Guilhermino, 2018; Schür et al., 2020). Nevertheless, no significant adverse effects of MP were also documented (e.g., Rist et al., 2017; Coady et al., 2020). MP can modulate the bioaccumulation and toxicity of several other contaminants, including different metals (e.g., Barboza et al., 2018b, 2018c; Santos et al., 2020; Eom et al., 2021), and are suspect of interacting with Li toxicity (Costa et al., 2021). Moreover, MP may influence ecological and other large-scale processes (Agathokleous et al., 2021), and may have a negative impact on human health and wellbeing (Barboza et al., 2018a; Prata et al., 2020). Therefore, MP deserve further research (Barboza et al., 2018a; Eder et al., 2021; Gonçalves and Bebianno, 2021), with the effects resulting from the simultaneously exposure to MP and other emergent contaminants of high concern, such as Li, requiring special attention.

The goals of the present study were to investigate the long-term toxicity of Li, alone and in mixture with MP (Li-MP mixtures), and the potential toxicological interactions between the two stressors, using the freshwater zooplankton crustacean *Daphnia magna* as model species. To the best of our knowledge, the long-term effects of Li-MP mixtures were not investigated before, and this knowledge is needed to protect 'One Health' under increasing Li and MP exposure.

2. Material and methods

2.1. Model species and test organisms

D. magna was selected for the present study mainly because it has been widely used in ecotoxicology (e.g., Martins et al., 2013; Sengupta et al., 2016; Cardoso et al., 2020; Serra et al., 2020; An et al., 2021) and in the aquatic risk assessment of chemicals (Gustavsson et al., 2017), has high ecological relevance in numerous freshwater ecosystems (Guilhermino et al., 2021), and is an adequate model for several types of environmental, animal and human health research (e.g., Guilhermino et al., 2000; Fuertes and Barata, 2021; Kim et al., 2017). Moreover, a study in *D. magna* short-term exposed to lithium hydroxide (LiOH) identified several genomic changes with potential to be used as biomarkers in relation to Li in animal species, including humans (Kim et al., 2017).

The organisms used in the present study were *D. magna* females (clone A, Baird et al., 1989). Briefly, *D. magna* group cultures have been maintained in parthenogenetic reproduction in our laboratory for many years in culture chambers with control of temperature (20 ± 1 °C), photoperiod [16 h light (L) and 8 h dark (D)], and other constant experimental conditions, as previously described (Pacheco et al., 2018; Martins and Guilhermino, 2018). From these cultures, juvenile females from the 3rd brood (> 6 h and < 24 h old) were used to start individual cultures. They were maintained in a chamber (Bronson PGC 1400, Netherlands), with control of photoperiod (16 h L: 8 h D), light intensity (10,830 lx, provided by Sylvania Lightning, Lynx CF-LE 55 W/840 lamps), temperature (20 ± 1 °C, water temperature). Each female was maintained in a glass beaker with 100 mL of the American Society for Testing and Materials hard water – ASTM (ASTM, 1980), with vitamins and 4 mL/L of an algae extract, as detailed elsewhere (Guilhermino et al., 2021), hereafter indicated as test medium. Females were feed every day (Monday to Friday) with *Chlorella vulgaris* (3×10^5 cells/mL/daphnia) obtained from laboratory cultures, provided immediately after test medium renewal. From these cultures, juvenile females (3rd brood, > 6 h and < 24 h old) were isolated and

maintained for 3 generations in the same conditions of water temperature, photoperiod, and light intensity. Each female was maintained in a 100 mL glass beaker, with 50 mL of test medium, with food regime and other conditions as previously indicated.

2.2. Chemicals

The source of Li used in the experiments was lithium chloride (LiCl), p. a., purchased from Merck, Germany. The MP were fluorescent plastic microspheres, provided as dry powder (Cospheric Innovations in Microtechnology, U.S.A., company reference product: FMR-1.3). As indicated by the supplier, 1 mg of the product contains approximately $1.836E^{+8}$ polymer microspheres (1.3 g/cm^3 density, 1–5 μm diameter, excitation and emission wavelength of 575 nm and 607 nm, respectively). These MP were tested mainly because they are up taken by *D. magna* and they cause long-term effects in this species (Martins and Guilhermino, 2018; Pacheco et al., 2018; Guilhermino et al., 2021). Moreover, their basic characterization and behaviour in the test medium used in this study are available (Pacheco et al., 2018), they were fluorescent, and their size was in the low μm-range.

The chemicals used to prepare *D. magna* test medium and *C. vulgaris* culture medium (MBL, Stein, 1973) were from Merck (Germany) or Sigma-Aldrich (Germany). The chemicals used to determine the actual concentrations of Li in test medium are indicated in the Section 2.4.

2.3. Null and alternative hypotheses, experimental design and exposure conditions

The following null hypotheses were tested: H_{01} – in the range of concentrations tested, Li does not induce long-term adverse effects on *D. magna* population fitness; H_{02} – in the range of concentrations tested, Li-MP mixtures do not induce long-term adverse effects on *D. magna* population fitness; H_{03} – in the range of concentrations tested, toxicological interactions between Li and MP do not occur in long-term exposed *D. magna*.

The hypotheses alternative to H_{01}, H_{02} and H_{03} were, respectively: H_{A1} – in the range of concentrations tested, Li induces long-term adverse effects on *D. magna* population fitness; H_{A2} – in the range of concentrations tested, Li-MP mixtures induce long-term adverse effects on *D. magna* population fitness; H_{A3} – in the range of concentrations tested, toxicological interactions between Li and MP in long-term exposed *D. magna* occur.

A 21-day *D. magna* bioassay was carried out following in general the OECD guideline 211 (OECD, 2012) with some punctual alterations and the experimental design modified to test for Li and Li-MP mixture effects. It was carried out in a test chamber (Bronson PGC 1400, Netherlands), at water temperature of 20 ± 1 °C, photoperiod of 16 h L: 8 h D, and light intensity of 10,830 lx provided by compact fluorescent cold white lamps (Sylvania Lightning, Lynx CF-LE 55 W/840) that emit low levels of UV radiation.

The bioassay was started with 3rd brood juvenile females (>6 h, <24 h old). Females were exposed in 100 mL glass beakers (1 female per beaker) with 50 mL of test medium, which was renewed every 24 h. The test medium contained sodium (Na) that is a component of ASTM (~ 52.5 mg/L), and possibly additional traces of it from the organic extract, and from the microalgae used as food, which were cultured in MBL (Stein, 1973) that contained Na. Each female was feed daily with 3×10^5 cells of *C. vulgaris* per mL of test medium (~ 0.322 mg of carbon/daphnia/day, Guilhermino et al., 1999). Ten females per treatment were used (1 female per beaker).

The following treatments were tested: control (test medium only), three nominal concentrations of Li alone (0.02, 0.04 and 0.08 mg/L) and the following nominal concentrations of Li-MP mixtures: 0.02 Li + 0.05 MP mg/L, 0.04 Li + 0.1 MP mg/L, and 0.08 Li + 0.2 MP mg/L. These concentrations of Li and MP are lower than some documented in environmental waters, such as 2.20 mg/L (Neves et al., 2020) and 2.98 mg/L of Li (Steinmetz et al., 2021), and more than 5 mg/L of MP (Lasee et al., 2017). The tested concentrations were selected based on previous studies where the effects of Li (Kszos et al., 2003; Nagato et al., 2013; Bozich et al., 2017) and MP (Martins and Guilhermino, 2018; Pacheco et al., 2018) in aquatic species were investigated separately. Treatments containing Li alone were prepared by serial dilution (in test medium) of a stock solution with a LiCl concentration of 200 mg/L, prepared in the same day. Mixture treatments were prepared by diluting the appropriate volume of the Li stock solution and of a MP stock solution (400 mg/L) in test medium, prepared immediately before the treatments.

At the beginning of the bioassay, at the time of test medium renewal (every 24 h), and at the end of the exposure period (21 days), samples were collected to determine the actual concentrations of Li (Section 2.4) and MP (Section 2.5) in freshly prepared (fresh) and/or 24 h old (old) test medium, and test medium temperature, dissolved oxygen and pH were measured (HACH HQ40d multi probe, U.S.A.; WTW Multi 340i/SET, Germany). The light intensity was measured using a Roline RO-1332 Digital Luxmetter (Germany).

The effect criteria were: the total somatic growth (somatic growth); the day of the first brood release (first brood release); the total number of broods released (brood number); the number of total offspring (total offspring); the number of living offspring (living offspring); number of dead offspring (dead offspring); the number of aborted eggs; and the intrinsic rate of population increase (population growth rate) that was used as indicative of population fitness. The effect criteria were determined as indicated in other studies (Martins et al., 2013; Guilhermino et al., 2021), and expressed as the mean per parental female that survived until the end of the bioassay. Data from dead parental females were only analysed regarding parental mortality. Females were observed at least twice a day. Offspring, dead organisms and moults were removed as soon as possible. Parental females and offspring were considered dead when they did not show any movement for 15 s under a brilliant light.

The bioassay was carried out simultaneously to another one assessing the long-term effects of MP alone (0.04, 0.09 and 0.19 mg/L) in *D. magna* at 20 °C and 10,830 lx, which is described and discussed elsewhere (Guilhermino et al., 2021). The juvenile females used to start the two bioassays were from the same parental females. In each day of test medium preparation, the treatments containing MP alone and Li-MP mixture treatments were prepared in parallel using the same stock solution of MP. The two bioassays were carried simultaneously for 21 days, in the same test chamber, under the same experimental conditions, and the control group was the same for the two bioassays.

2.4. Lithium concentrations in test medium

The actual concentrations of Li were determined in samples of fresh and old (24 h) test medium. Briefly, all chemicals used in the analyses were of Suprapure grade and solutions were prepared in double deionized water. Water purification systems were a Seralpur PRO 90 CN and Seradest LFM 20. All the procedures were carried out in a dust-free area, using powder-free gloves. A rigorous decontamination of all polytetrafluoroethylene materials (Teflon vessels, micropipette tips, and autosampler cups) was performed by immersing them in freshly prepared 15% pro-analysis HNO_3 (v/v, Merck, Germany) for 24 h, and then rinsing thoroughly with double deionized water, previous to drying. Li standard solutions were daily prepared from a 1000 mg/L stock solution (Spectrosol, BDH) in 0.2% HNO_3 (v/v) at the linearity range 0–12.5 μg/L. Metal quantifications were carried out in the water samples, after a 10× dilution in 0.2% HNO_3 (v/v), in a Perkin-Elmer HGA-850 furnace installed in a model AAnalyst 600 spectrometer with Zeeman background correction, equipped with an AS-800 autosampler. The spectrometer settings and furnace programs used with pyrolytic graphite-coated Perkin-Elmer HGA tubes with integrated platform were as follows: drying temperature, 110 °C (15 s for ramp time and 30 s for hold time) followed by 130 °C (15 s for ramp time and 30 s for hold time); pyrolysis temperature, 900 °C (20 s for ramp time and 20 s for hold time); atomization temperature, 2100 °C (5 s for hold time); cleaning temperature, 2450 °C (1 s for ramp time and 3 s for hold time). All data were taken at 670.8 nm. The slit width was 0.7 nm, and the purge gas

was argon, with an internal flow rate of 250 mL/min, except during the atomization step, where gas flow was stopped. The auto-sampler was programmed to pipet 20 μL of the standard/sample solution. Readings on the spectrometer were taken by using the peak area mode (integrated absorbance). The detection (LOD) and quantification limits (LOQ) of the instrumental method were 2.20 and 6.70 μg/L, respectively, as determined by carrying out at least 20 determinations of blank samples [0.2% HNO_3 (v/v)] and calculating the values by the equations LOD = 3.3 s/m and LOQ = m10s/m, respectively, where *s* is the standard deviation of the measurements of the blank and *m* the slope of the calibration curve.

2.5. Microplastic actual concentrations in test medium

The actual concentrations of MP in test medium of treatments with MP nominal concentrations of 0.1 and 0.2 mg/L were determined by spectrofluorimetry (575/607 nm excitation/emission wavelengths, Jasco FP-6200 spectrofluorometer, Japan) using the following model fitted to a calibration curve done immediately before the bioassays (Guilhermino et al., 2021):

$$\text{MP actual concentration (mg/L)} = -0.033 + 0.026 \times \text{fluorescence (F units)}, R = 99.9\%$$

The deviation of the MP actual concentration relatively to the nominal ones (MP deviation) and the decrease of MP concentration in test medium (MP decay) were calculated per beaker (replicate) at the time of each test medium renewal, as indicated in Guilhermino et al. (2021). Because in some beakers the MP decay was higher than 20%, the time-weighted mean concentration (TWM) in each replicate was calculated, and the estimated exposure concentrations (EEC) per treatment were determined (OECD, 2012). The actual MP concentrations in replicates of treatments containing the lowest nominal concentration of the particles (0.05 mg/L) could not be determined from fluorescence readings due to low sensitivity of the method in this range. Therefore, the EEC in these treatments were estimated as follows (Guilhermino et al., 2021):

$$\text{EEC (mg/L)} = \text{A–b} \times \text{A}$$

where: A – nominal concentration of the treatment containing the lowest amount of MP (0.05 mg/L); b – mean decay in treatments with a nominal MP concentration of 0.1 mg/L (percentage/100).

2.6. Data analyses

Each data set was tested for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). Because these conditions were not achieved even after data transformation, different treatments were compared using the Kruskal-Wallis test (H). When significant differences were found, pairwise comparisons were carried out with significance values adjusted by the Bonferroni correction for multiple tests, to compare treatments and determine the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) when applicable. The Mann-Whitney test (U) was used to compare two data sets.

The concentrations of Li and Li-MP mixtures (based on the concentration of Li or MP) that induced 10%, 20% and 50% of effect (reduction) on living offspring after 21-d exposure (21-d EC_{10}, 21-d EC_{20}, and 21-d EC_{50}, respectively, 21-d EC_xs indicating all them) were calculated from a logistic model fitted to each data set, with lower limit of zero, as previously detailed and used for MP alone (Guilhermino et al., 2021).

D. magna population growth rate was used to investigate the type of toxicological interaction between Li and MP using the conceptual approach described in Crain et al. (2008). Briefly, the approach was based on the comparison of the effects caused by the simultaneous exposure to Li and MP (combined effect, mixture effect) and the sum of the effects caused by each of the environmental contaminants alone (independent effects), using the effect sizes measured with Hedge's *d* (Crain et al., 2008). The individual and combined effects of stressors, the sampling variance and the 95% confidence interval (95% CI) for each Hedge's *d* were calculated as described in Guilhermino et al. (2021), based on previously developed methods (Gurevitch et al., 1992, 2000). Three exposure scenarios were considered: S1 – low concentrations of Li and MP; S2 – medium concentrations of Li and MP; and S3 – high concentrations of Li and MP. Data on the effects of MP alone were from Guilhermino et al. (2021). For each interaction scenario, working with no weighted Hedge's *d*, the type of interaction was identified based on the direction of individual effect sizes of the stressors, tested independently, and the interaction effect size with the corresponding 95% CI, as indicated in Crain et al. (2008).

The fitting of the logistic model and the calculation of parameters and its variability was carried out using the *drc* extension package for dose-response analysis using R (Ritz et al., 2015). The calculations to assess the type of interaction among stressors were performed in Microsoft excel. All the other statistical analyses were carried out in the IBM SPSS statistical package, version 26.0. The significance level was 0.05.

3. Results

There was no parental mortality in the control group, the mean and standard deviation (SD) of living offspring number per female was 91 ± 2 and the coefficient of variation was 2.5% (Guilhermino et al., 2021). The means (± SD) of test medium temperature per treatment were 20.2 ± 0.2 °C (maximum variation in individual beakers: 0.9 °C), the pH means ranged from 8.36 ± 0.14 to 8.44 ± 0.12 pH units (maximum variation in individual beakers: 0.52 pH units), and the dissolved oxygen means ranged from 8.12 ± 0.06 to 8.14 ± 0.06 mg/L (minimum value in individual beakers: 7.99 mg/L). Therefore, the validity criteria and recommendations of the OECD guideline 211 (OECD, 2012) regarding these parameters were achieved.

3.1. Lithium and microplastic concentrations in test medium

The concentrations of Li in samples of test medium from the control were lower than the LOD of the method. Regarding the other samples, no significant differences between the actual concentrations of Li determined in fresh and old test medium were found ($U = 1446$, $p = 0.063$, $N = 120$), therefore the Li concentrations remained relative stable for 24 h. The total means per treatment are indicated in Table 1. Significant differences in the actual concentrations of Li among treatments were obtained ($H_5 = 105.911$, $p < 0.001$) but the treatments with the same nominal concentration of Li (with and without MP) were not significantly different (Table 1). Therefore, the exposure concentrations of Li along the bioassay were indicated as the total mean of actual Li concentrations in treatments with the same nominal concentration of the metal. Hereafter, they will be indicated as the lowest concentration of Li (0.02 mg/L), medium concentration of Li (0.04 mg/L) and highest concentration of Li (0.08 mg/L).

Table 1
– Mean (± standard deviation – SD) of the actual concentrations of lithium determined in test medium along the bioassay. Li nom conc – nominal concentrations of lithium. MP nom conc – nominal concentrations of microplastics. N – number of samples analysed. Mean Li actual conc – mean and SD of the actual concentrations of lithium determined per treatment. Comp – distinct letters indicate significant differences in the actual concentrations of lithium (Kruskal-Wallis test and pairwise comparisons, $p \leq 0.05$).

Li nom conc (mg/L)	MP nom conc (mg/L)	N	Mean Li actual conc (mg/L)	Comp.
0.02	0	20	0.021 ± 0.001	a
	0.05	20	0.021 ± 0.002	a
0.04	0	20	0.040 ± 0.003	b
	0.10	20	0.040 ± 0.002	b
0.08	0	20	0.079 ± 0.005	c
	0.20	20	0.079 ± 0.006	c

Table 2
– Mean (± standard deviation - SD) of actual concentrations (Actual Conc) of microplastics (MP) in freshly prepared (0 h) and old (24 h) test media, deviation (Dev) of MP actual concentrations at 0 h from nominal ones, decay of MP actual concentrations in test media over 24 h (Decay), and mean of time weighted mean concentration (TWM) per treatment. MP nom conc – nominal concentrations of MP. Li nom conc – nominal concentrations of lithium. N – number of replicates per treatment (replicate: 1 beaker with 1 female that survived until the end of the bioassay; mean of 21 samples of each type of test medium per beaker along the exposure period, except for TWM that were calculated as indicated in OECD (2012). Different letters after the TWM mean indicate statistical significant differences (Mann-Whitney test, $p \leq 0.05$).

MP nom conc (mg/L)	Li nom conc (mg/L)	N	MP actual conc 0 h (mg/L)	MP actual conc 24 h (mg/L)	Dev (%)	Decay (%)	TWM (mg/L)
0.1	0.04	10	0.1034 ± 0.0003	0.0800 ± 0.0005	3.4 ± 0.3	22.7 ± 0.5	0.0911 ± 0.0003 a
0.2	0.08	6	0.2072 ± 0.0009	0.1798 ± 0.0006	3.6 ± 0.4	13.2 ± 0.5	0.1933 ± 0.0008 b

The actual concentrations of MP in fresh and old test medium of the mixture treatments with the highest nominal concentrations of MP are indicated in Table 2. There was a decay of MP concentrations during the interval of test medium renewal higher than 20% in replicates of the treatment with the medium nominal concentration of MP, and there were significant differences in the TWM between treatments (Table 2). The TWM means were used as EECs, namely 0.09 mg/L (medium) and 0.19 mg/L (highest). The EEC of MP in the treatment containing the lowest nominal concentration of the substance was 0.04 mg/L. Therefore, the EECs of Li-MP mixtures along the bioassay were: 0.02 Li + 0.04 MP mg/L (lowest concentration), 0.04 Li + 0.09 MP mg/L (medium concentration) and 0.08 Li + 0.19 MP mg/L (highest concentration). The EECs of MP in the Li-MP mixtures were equal to those estimated in Guilhermino et al. (2021) allowing the comparison of the effects on *D. magna* results under exposure to Li-MP mixtures and MP alone.

MP concentrations of 0.04, 0.09 and 0.19 mg/L corresponded approximately to 7344, 16,524 and 34,884 MP particles/mL, and to ~2.4%, 5.2% and 10.4% in relation to the total number of particles (MPs + microalgae cells) per mL, respectively.

3.2. Effects of lithium and lithium-microplastic mixtures and toxicological interactions

The means of each effect criterion in females exposed to different treatments are indicated in Table 3. All the females exposed to 0.02 mg/L of Li alone survived until the end of the bioassay, and there were no significant differences in any effect criterion in relation to the control group, despite the release of a few dead juveniles. Exposure to 0.04 mg/L of Li caused parental (10%) and offspring mortality (31%), and significantly decreased the total offspring number by 32%, and the living offspring number by 53%. The highest concentration of Li (0.08 mg/L) caused parental (20%), and significantly decreased the somatic growth by 40%, increased the number of days until the first brood release by 1.3 fold, and reduced the brood number by 20%, the total offspring number by 78%, the living offspring number by 93%, and the population growth rate by 67%. The LOECs of Li were 0.04 or 0.08 mg/L depending on the effect criteria. The 21-d EC_{10}, EC_{20} and EC_{50} of Li on living offspring with the corresponding 95% confidence limits (95% CL) are indicated in Table 4.

In relation to the control group, the lowest concentration of Li-MP mixtures reduced the somatic growth by 26% (Table 3). Exposure to the medium concentration of Li-MP mixtures decreased the somatic growth by 32%, and reduced the total offspring number by 33%, the living offspring number by 54%, and the population growth rate by 22%. The highest concentration of Li-MP mixtures caused 40% of parental mortality between days 5 and 18, decreased the somatic growth by 44%, increased the number of days until the first brood release by 1.4 fold, and reduced the brood number by 20%, the total offspring number by 77%, the living offspring number by 90% and the population growth rate by 58%. The LOECs ranged from 0.02 Li + 0.04 MP mg/L to 0.08 Li + 0.19 MP mg/L depending of the effect criterion. The EC_xs are indicated in Table 4.

The individual and combined effects of Li and MP on *D. magna* population growth rate measured through Hedge's *d* are shown in Fig. 1. Under exposure to the lowest and the highest concentrations of Li and MP (scenarios S1 and S3, respectively), the 95% CI did not overlap zero, the individual effects of stressors were both negative and the interaction was positive. Therefore, following Crain et al. (2008), the toxicological interaction was antagonism. Under exposure to medium concentrations of Li and MP (scenario S2), the 95% CI did not overlap zero, the individual effects of both stressors and the interaction were negative, thus, following Crain et al. (2008), the interaction was synergism.

Table 3
– Number of females that survived until the end of the bioassay (N), parental mortality (PM), days or interval of days where parental mortality (PMD) occurred, and mean (± standard deviation) of the somatic growth, first brood day number, total brood number, total offspring number, living offspring number, dead offspring number and population growth rate per parental female in each treatment. All the concentrations are in mg/L. MP – microplastics; n – number. Different letters after the mean indicate statistical significant differences (Kruwkal-Wallis test and pairwise comparisons, $p \leq 0.05$) among treatments per effect criterion. The data of the control treatments were first published in Guilhermino et al. (2021).

Concentrations (mg/L)	N	PM (%)	PMD (days)	Growth ($\times 10^{-2}$ mm)	1st brood (day number)	Brood number (n)	Total offspring (n)	Living offspring (n)	Dead offspring (n)	Population growth rate
Control	10	0	–	0.206 ± 0.005 a	8.8 ± 0.4 a	5 ± 0 a	91 ± 2 a	91 ± 2 a	0 ± 0 a	0.33 ± 0.01 a
0.02 Li	10	0	–	0.192 ± 0.008 a	9 ± 0 a	5 ± 0 a	88 ± 3 a,b	87 ± 3 a	0.7 ± 1.2 a	0.320 ± 0.003 a
0.04 Li	9	10	11	0.16 ± 0.01 a,b	9 ± 0 a	5 ± 0 a	62 ± 4 b,c	43 ± 4 b	19 ± 2 b	0.32 ± 0.01 a,b
0.08 Li	8	20	6, 10	0.124 ± 0.008 c	11.5 ± 0.5 b	4 ± 0 b	20 ± 3 c	6 ± 2 b	15 ± 2 b	0.11 ± 0.03 c
0.02 Li + 0.04 MP	10	0	–	0.152 ± 0.008 b,c	9 ± 0 a	5 ± 0 a	90 ± 3 a	89 ± 3 a	0.9 ± 0.7 a	0.316 ± 0.004 a,b
0.04 Li + 0.09 MP	10	0	–	0.141 ± 0.005 b,c	9 ± 0 a	5 ± 0 a	61 ± 3 b,c	42 ± 2 b	19 ± 2 b	0.256 ± 0.002 b,c
0.08 Li + 0.19 MP	6	40	5–18	0.115 ± 0.007 c	12 ± 0 b	4 ± 0 b	21 ± 2 c	9 ± 2 b	11.3 ± 0.8 a,b	0.14 ± 0.02 c
Kruskal-Wallis				$H_6 = 58.668$ $p < 0.001$	$H_6 = 56.575$ $p < 0.001$	$H_6 = 62.000$ $p < 0.001$	$H_6 = 54.645$ $p < 0.001$	$H_6 = 55.173$ $p < 0.001$	$H_6 = 55.969$ $p < 0.001$	$H_6 = 49.901$ $p < 0.001$
NOEC Li (mg/L)				0.04	0.04	0.04	0.02	0.02	0.02	0.04
LOEC Li				0.08	0.08	0.08	0.04	0.04	0.04	0.08
NOEC Li + MP				< 0.02 + 0.04	0.04 + 0.09	0.04 + 0.09	0.02 + 0.04	0.02 + 0.04	0.02 + 0.04	0.02 + 0.04
LOEC Li + MP				0.02 + 0.04	0.08 + 0.19	0.08 + 0.19	0.04 + 0.09	0.04 + 0.09	0.04 + 0.09	0.04 + 0.09

Table 4
– Estimated effect concentrations of lithium alone (Li) and lithium-microplastic mixtures (Li-MP) causing 10% (EC_{10}), 20% (EC_{20}) and 50% (EC_{50}) of reduction on *Daphnia magna* living offspring number per female after 21 days of exposure with the respective 95% confidence limits (95% CL). MP – microplastics. In the mixtures, the EC_x are expressed in relation to the concentration of Li and in relation to the concentration of MP, as indicated in the column entitled "Substance".

Condition	Substance	21-d EC_{10}		21-d EC_{20}		21-d EC_{50}	
		EC_{10} (mg/L)	95% CL (mg/L)	EC_{20} (mg/L)	95% CL (mg/L)	EC_{50} (mg/L)	95% CL (mg/L)
Li alone	Li	0.023	0.021–0.025	0.028	0.027–0.029	0.039	0.038–0.040
Li-MP	Li	0.023	0.021–0.025	0.028	0.026–0.029	0.039	0.038–0.040
Li-MP	MP	0.047	0.043–0.052	0.059	0.055–0.063	0.086	0.084–0.089

4. Discussion

In general, the concentrations of Li in natural freshwaters are low, with documented ranges such as 0–0.091 mg/L in surface water and 0–0.097 mg/L in groundwater of Ireland (Kavanagh et al., 2017), and 0.09–1.54 μg/L in drinking water sources of Nigeria (Ewuzie et al. (2020). Nevertheless, concentrations of Li in the low ppm range were also reported, such as 1.7 mg/L in groundwater drinking sources of the United States of America (Lindsey et al., 2021), 2.98 mg/L in drinking waters of the Argentinean Andes (Steinmetz et al., 2021), and 2.21 mg/L in bottled natural mineral water of Portugal (Neves et al., 2020). In industrial and other types of effluents, the concentrations of Li can be considerably higher, such as 15 mg/L in a plant facility effluent (Kszos and Stewart, 2003). Much higher concentrations (e.g., > 4000 mg/L) have been documented in brines of salt flats, such as the Salar de Atacama (López Steinmetz and Salvi, 2021). The contamination of environmental freshwater with MP and other plastic particles variates considerably and includes ranges such as 0.04–9.97 MP/m^3 in the Rhine River, Swiss (Mani and Burkhardt-Holm, 2020), 58–1265 items/m^3 in the Antuã River, Portugal (Rodrigues et al., 2018), up to more than 466,000 items/Km^2 in the Laurentian Great Lakes, North America (Eriksen et al., 2013), mean 24 h counts of 1,146,418.36 items/m^3 in the Los Angeles River (Moore et al., 2011), and mean concentrations of 1.56 ± 1.64 mg/L in lakes and 5.51 ± 9.90 mg/L in wetlands of Texas, USA (Lasee et al., 2017). Moreover, in general, very small MP are not included in the counts. Therefore, the Li and MP concentrations tested are environmentally relevant at least for aquatic ecosystems in naturally Li enriched regions, near Li extraction areas, hotspots of MP pollution, near e-trash dumping sites, and in industrialized and densely populated areas with moderate and high Li and MP contamination or under their impact.

The decrease of MP concentrations in the test medium of Li-MP mixtures within 24 h was probably mainly due to the uptake of particles by *D. magna* because it ingests this type of MP and possibly also incorporates them by other ways (Guilhermino et al., 2021), bioconcentration of MP by *D. magna* as found in other studies (An et al., 2021; Hoffchröer et al., 2021), sedimentation of some MP particles in the bottom of the beakers (Luís et al., 2015; Pacheco et al., 2018; Chen et al., 2020), settlement of microalgae cells and cell debris with MP adsorbed to their surface, and formation of aggregates among MP particles and of these with microalgae cells and cell debris, and their further sedimentation (Lagarde et al., 2016; Prata et al., 2018), among other processes (Luís et al., 2015; Martins and Guilhermino, 2018; Chen et al., 2020).

4.1. Lithium decreased the population fitness of D. magna

The means obtained for the effect criteria determined in females of the control group (Table 3) are in the ranges documented in different clones of *D. magna* exposed to comparable experimental conditions (e.g., Vandenbrouck et al., 2011; Martins et al., 2013; Cardoso et al., 2020; An et al., 2021), confirming that the environmental conditions used were adequate for *D. magna*.

The lack of significant differences in all the effect criteria between females exposed to the lowest concentration of Li (0.02 mg/L) and those of the control group indicate that *D. magna* was able to overcome the chemical stress with slight effects only, such as a few dead juveniles. Exposure to 0.04 mg/L of Li caused reproductive toxicity as indicated by the reduced number of total offspring and living offspring, and the considerable number of dead juveniles. The sum of the living offspring number and dead offspring number equal to the total offspring number observed under exposure to 0.04 mg/L of Li suggests lethal effects induced directly on the juveniles developing in the brood chamber. Moreover, the lower total offspring number relatively to the control group suggests that maternal effects also occurred. The dead of one parental female after 11 days of exposure suggests mortality by cumulative effects. The rise of Li concentration to 0.08 mg/L increased the severity of the intoxication, as shown by the augment of the parental mortality, the decrease of the somatic growth and the

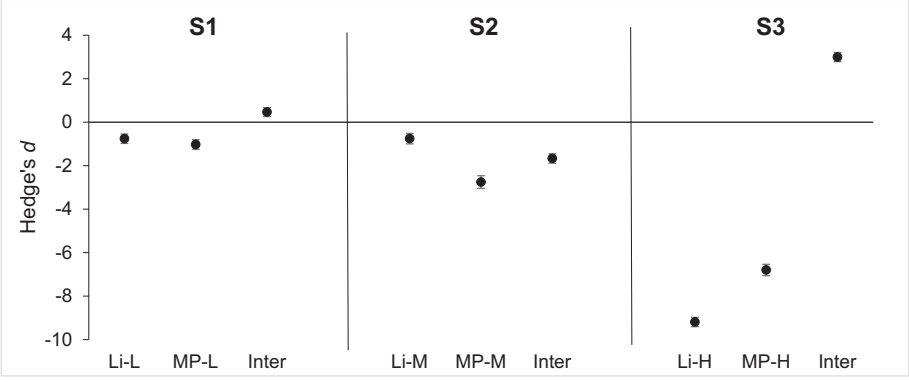

Fig. 1. – Independent and combined effects of lithium (Li) and microplastics (MP). The dots represent the individual and interactive effect sizes measured with Hedge's *d* and the corresponding 95% confidence interval (vertical bars). The following scenarios were considered: low concentrations of Li and MP (S1), medium concentrations of Li and MP (S2), and high concentrations of Li and MP (S3). Li-L – low concentration of Li (0.02 mg/L); Li-M – medium concentration of Li (0.04 mg/L); Li—H – high concentration of Li (0.08 mg/L); MP-L – low concentration of MP (0.04 mg/L); MP-M – medium concentration of MP (0.09 mg/L); MP-H – high concentration of MP (0.19 mg/L); inter – interaction, combined effects of the stressors (mixture).

increase of the time until the first brood release suggesting growth and development delay, and the reduction of the brood number, the total offspring number, the living offspring number and the dead offspring number indicating reproductive impairment. Such effects resulted in the decrease of the population growth rate by 67%. Therefore, in the range of Li concentrations tested, *D. magna* population fitness was significantly reduced, leading to the refusal of H_{01} and acceptance of H_{A1}. Based on the LOEC values, the most sensitive effect criteria were the total offspring number and the living offspring number that were significantly reduced at concentrations of Li equal or higher than 0.04 mg/L.

The reduction of the somatic growth, delay of the first reproduction, and decrease of the brood number and of the total offspring number observed in *D. magna* exposed to 0.08 mg/L of Li suggest deficit of energy resulting in the need of allocating it from growth and reproduction to face chemical stress. Under exposure to stressors, *D. magna* often responds by increasing the food intake to get more energy; when this is not enough, energy reserves are likely used and, after their depletion, the energy is allocated from growth and reproduction to activate defences against stress, repair increased damages, promote chemical elimination, and other processes (Vandenbrouck et al., 2011; Sengupta et al., 2016; Guilhermino et al., 2021). Under long-term exposure to Li, energy may be a critical issue because this metal alters the metabolism possibly leading to a decreased energy production, and disrupts ionic regulation (Nagato et al., 2013; Tkatcheva et al., 2015; Viana et al., 2020), what may interfere with water filtration, food intake, respiration, swimming and several other functions. Moreover, along the exposure period, *D. magna* likely bioconcentrated Li from the water and bioaccumulated it from food, as documented in other animals exposed to Li (Aral and Vecchio-Sadus, 2008; Bolan et al., 2021; Viana et al., 2020), potentially increasing the energy shortage and other toxic effects. In *D. magna* 21-day exposed to 0.25 mg/L of lithium cobalt oxide nanomaterials (LCO) used in Li batteries, down-regulation of genes important to metabolism, metal detoxification, metabolism, and cell maintenance was found (Bozich et al., 2017). After 48 h of exposure to 1 mg/L of LCO, *D. magna* showed changes in metabolic pathways compatible with response to energy depletion (Niemuth et al., 2021). In other aquatic animals, feeding alterations under Li exposure have been documented, such as Li-contaminated food aversion learning in crayfish (Arzuffi et al., 2000), and preference for more energetic food rather than the usual one (Rodríguez et al., 2021). These findings support the hypothesis of energy deficit in *D. magna* long-term exposed to Li. In addition to adverse effects on somatic growth and reproduction, energy depletion may have also contributed to the parental and juvenile mortality observed at the medium and highest concentrations of Li.

Li is a neuroactive substance, a property that is the basis of its therapeutic use. *D. magna* has a complex central nervous system and signalling pathways with many similarities to other invertebrates (e.g., *Daphnia pulex, Drosophila*) and vertebrates, including humans (e.g., Kim et al., 2017; Bedrossiantz et al., 2020, 2021; Niemuth et al., 2021). Serotonergic, cholinergic, dopaminergic, glutamatergic and GABAergic systems are involved in the regulation of basic physiological functions and of sensorial-based individual and population responses to the presence of food, light, stressors and other environmental changes (Weiss et al., 2015; Bedrossiantz et al., 2020; Issa et al., 2020; Bedrossiantz et al., 2021). Neurotoxic environmental contaminants can disrupt *D. magna* signalling resulting in changes of the swimming activity, phototaxis and feeding behaviour, and other adaptative responses to stressors (Bedrossiantz et al., 2020, 2021; Fuertes and Barata, 2021), which can lead to adverse effects at individual and population levels (e.g., Campos et al., 2012, 2016). A well know effect of Li is to increase the serotonin levels in the brain. In *D. magna*, serotonin is an important regulator of metabolism, growth and reproduction (Hansen et al., 2008; Campos et al., 2012, 2016). As shown in studies with *D. magna* exposed to serotonin and serotonin reuptake inhibitors, alterations in the levels of this neurotransmitter may lead to life-history strategy changes (Hansen et al., 2008; Campos et al., 2012, 2016). Exposure to relative high concentrations of serotonin reuptake inhibitors (e.g., 0.05, 0.125 mg/L), which increase serotonin levels, and adequate food decreases *D. magna* growth and delays reproduction leading to reduced population growth rate (Hansen et al., 2008). Li can also inhibit the activity of acetylcholinesterase and other cholinesterase enzymes (Oliveira et al., 2011; Viana et al., 2020; Costa et al., 2021), interfere with Na^+/K^+ ATPase activity through the competition with Na^+ (Kszos and Stewart, 2003; Tkatcheva et al., 2015), and is suspect of interfering with the synthesis and/or use of neurotransmitters, such as dopamine (Nagato et al., 2013). Such neurotoxic effects, can change the behaviour, growth, reproduction and life-history strategy leading to adverse effects at population level, as found in *D. magna* exposed to other neurotoxicants (e.g., Guilhermino et al., 1999; Issa et al., 2020; Bedrossiantz et al., 2020, 2021). Therefore, neurotoxicity likely also contributed to the effects observed in *D. magna* long-term exposed to concentrations of Li equal or higher than 0.04 mg/L.

In *D. magna* exposed for 24 h to LiOH (12.58 mg/L), up-regulation of genes encoding for cuticle-related proteins that are needed for moulting and that also likely confer protection against chemical stress were found in *D. magna* exposed for 24 h to LiOH (12.58 mg/L), as well as probable interference in several signalling pathways (Kim et al., 2017). Under short-term (48 h) exposure to Li (1.15 mg/L), *D. magna* showed decreased levels of several amino acids possibly negatively affecting moulting, development and growth (Nagato et al., 2013). Compromised redox homeostasis, oxidative stress, lipid peroxidation, metabolic and endocrine alterations, among other adverse effects have been also found in other aquatic animals exposed to Li (Tkatcheva et al., 2015; Liu et al., 2018; Viana et al., 2020; Costa et al., 2021; Jing et al., 2021; Pinto-Vidal et al., 2021; Vidal et al., 2021). If such effects, or at least some of them, occurred in *D. magna* long-term exposed to Li, they may have also contributed to the reduced individual and population fitness observed.

Independently of the mechanisms involved, the results of the present study showed that 21-day exposure to Li concentrations equal or higher than 0.04 mg/L caused adverse effects in *D. magna*. In a previous study with this species, 21-day exposure to Li (provided as LiOH), at temperature of 20 °C, decrease of survival (by 100%), significant reduction of reproduction (by 97.8%) at 2.5 mg/L of Li, and no significant effects at 0.5 and 1 mg/L were found (Bozich et al., 2017). Differences of sensitivity between the populations of *D. magna* tested, and in several of the experimental conditions used (e.g., source of Li, food, test medium, number of animals, among others) may have contributed to the discrepancy in the concentrations that caused significant effects on reproduction between the two studies.

At appropriate levels, the presence of Na in the water provides protection against Li at least in some species, as shown by the increase in the EC_{50}s of Li on the reproduction of the freshwater zooplankton species *Ceriodaphnia dubia* from 0.72 mg/L in diluted mineral water (~2.8 mg/L of Na) to >4 mg/L in stream water (~ 17.4 mg/L of Na), at water temperature of 25 °C in both cases (Kszos et al., 2003). In the test medium used in the present study, the Na concentration was ~52.5 mg/L, and calcium, potassium and magnesium ions were also present (ASTM components), which may also provide some protection against Li. These findings indicate that despite the protection that environmental waters rich in Na and other ions may provide, the individual and population fitness of *D. magna* can be considerably reduced under long-term exposure to Li.

The 21-day EC_{50} on reproduction (0.039 mg/L) and LOECs (0.04 and 0.08 mg/L) of Li determined in the present study are lower than the concentrations of Li that caused significant adverse effects in other animal species after prolonged exposure, such as: EC_{50}s on reproduction from 0.72 mg/L to more than 4 mg/L in *C. dubia* exposed for 7 days at water temperature of 25 °C; 2.5 mg/L in tadpoles (*Lithobates catesbeianus*) exposed for 21 days at 22 ± 1 °C (Pinto-Vidal et al., 2021); 25 mg/L in the zebrafish (*Danio rerio*) exposed for two weeks at water temperature of 29 ± 1 °C (Liu et al., 2018); 20 mg/L in carps after 30 days of exposure at 23 ± 0.5 °C (Jing et al., 2021); and 0.250 mg/L in marine mussels (*Mytilus galloprovincialis*) after 28-day exposure at 18 ± 1 °C (Viana et al., 2020). Although differences in exposure conditions and in the effect criteria used do not allow a rigorous comparison, these findings suggest that *D. magna* may be more sensitive to long-term Li exposure than several other aquatic

species, therefore being a good model to study the toxic effects resulting from long-term exposure to this metal.

4.2. Li-MP mixtures reduced the population fitness and toxicological interactions occurred

Concentrations of Li-MP mixtures equal or higher than 0.04 Li + 0.09 MP mg/L significantly decreased the fitness of *D. magna*, leading to rejection of H_{02} and acceptance of H_{A2}.

Based on the LOEC values, the most sensitive effect criterion to Li-MP mixtures was the somatic growth that was significantly reduced by the lowest concentration of Li-MP mixtures (0.02 Li + 0.04 MP mg/L). This was an early sign of Li-MP mixture toxicity because none of the other effect criteria were significantly affected, and Li and MP when tested separately only reduced the somatic growth at 0.08 mg/L (Table 3) and 0.09 mg/L (Guilhermino et al., 2021), respectively. Under exposure to Li-MP mixtures, in addition to Li, *D. magna* must have uptake MP as found under single exposure to the same type of plastic particles (Guilhermino et al., 2021). Moreover, during the exposure period and additionally to Li, *D. magna* likely bioconcentrated MP from the water, as documented in this species exposed to other plastic particles (Hoffchröer et al., 2021), and probably also accumulated MP through the ingestion of MP-contaminated food because plastic particles bind to microalgae cells (Lagarde et al., 2016), including the tested MP (Prata et al., 2018). At least three hypotheses, not mutually exclusive, can be raised to explain why the somatic growth was specifically affected by the lowest concentration of Li-MP mixtures. First, a change of *D. magna* life-history strategy towards earlier and increased reproduction occurred. This switch is common in *D. magna* under stress and was documented under long-term exposure to serotonin reuptake inhibitors (Campos et al., 2012), which increase serotonin levels, an effect that Li may have caused as previously discussed. However, our data do not support this hypothesis because the release of the first brood was not anticipated and the total number of juveniles did not increase under exposure to the lowest concentration of Li-MP mixtures. These findings suggest that more likely, the additional energy required to respond to stress induced by exposure to Li-MP mixtures was first allocated from the somatic growth in an attempt of assuring reproduction (second hypothesis). The third hypothesis are specific effects induced by the mixture and/or its components separately on internal targets (e.g., basic aminoacids, enzymes, neurotransmitters) and/or mechanisms (e.g., signalling pathways involved in their synthesis and/or use) leading to development and growth impairment, as suggested by alterations at molecular level in *D. magna* exposed to Li (Nagato et al., 2013; Kim et al., 2017) and MP (Trotter et al., 2021). Testing the second and third hypotheses requires further investigation at lower levels of biological organization that was not in the scope of the present study.

The 21-day EC_{10}, EC_{20} and EC_{50} of Li and Li-MP mixtures (based on the concentration of Li) on living offspring were equal, indicating similar reproductive toxicity. The ratio between the 21-day EC_{50} of the MP alone on *D. magna* living offspring (0.146 mg/L, Guilhermino et al., 2021) and the corresponding value of Li-MP mixtures based on the concentration of MP (0.0864 μg/L, Table 4), indicates that the Li-MP mixtures were ~ 1.7 fold more toxic than the MP tested separately. Moreover, the highest concentration of Li-MP mixtures induced higher mortality (40%) than the same concentration of Li alone (20%), and the somatic growth and the population growth rate were significantly reduced at lower concentrations of Li when in mixture with MP than under exposure to Li and MP separately. Overall, these results point to toxicological interactions between Li and MP in *D. magna* long-term exposed to Li-MP mixtures, which were confirmed through the analysis of the combined effects of the chemicals on *D. magna* population growth rate Hedge's *d*. These findings lead to rejection of H_{03} and acceptance of H_{A3}. They are in line with the results from a study with filter feeding bivalves (*C. fluminea*) exposed for 96 h to Li and nano-sized MPs, individually and in mixture, also pointing to toxicological interactions between the two contaminants (Costa et al., 2021). Toxicological interactions between different types of MP and other metals, such as copper (Santos et al., 2020; Eom et al., 2021), chromium (Luís et al., 2015), mercury (Barboza et al., 2018b, 2018c; Oliveira et al., 2018), among others (e.g., Eder et al., 2021), were also documented in aquatic species.

In addition to adverse effects on *D. magna* somatic growth, reproduction and population growth rate, the studies available in the literature allow the identification of other common effects of Li and several types of MP in animals. They include possible energy depletion (An et al., 2021; Guilhermino et al., 2021; Nagato et al., 2013; Viana et al., 2020), feeding changes (Kszos et al., 2003; Oliveira et al., 2018; Rodríguez et al., 2021), cholinesterase inhibition (Oliveira et al., 2011; Santos et al., 2020; Viana et al., 2020; Costa et al., 2021), oxidative stress and lipid peroxidation (Barboza et al., 2018b; Liu et al., 2018), changes in the activity of biotransformation and energy-related enzymes (Barboza et al., 2018c; Viana et al., 2020), immunological alterations (Sadler et al., 2019; Pinto-Vidal et al., 2021), among others. Therefore, Li and some MP probably have mechanisms of toxicity and molecular targets in common, as also suggested by the comparison of changes at molecular level observed in *D. magna* exposed separately to Li (Nagato et al., 2013; Kim et al., 2017; Niemuth et al., 2021) and MP (Imholf et al., 2017; Sadler et al., 2019; Trotter et al., 2021). If so, their long-term combined action in such mechanisms and targets likely was an important contributor to the toxicological interactions found, a hypothesis deserving additional research. Nevertheless, other factors may have also contributed as further discussed in the next section.

In the wild, *D. magna* populations likely will have more diversity in the sensitivity to Li and Li-MP mixtures among individuals than the tested model populations, potentially decreasing the adverse impact of the contaminants at the population level. Nevertheless, long-term exposure to environmental contaminants and other stressors may lead to intra-population diversity loss (Guilhermino et al., 2021). The environmental conditions used were among the most adequate for *D. magna* growth and reproduction, and the only stressors were Li or Li-MP mixtures. In the wild, the UV radiation levels are in general much higher, water temperature and oxygen levels variate, food abundance and quality change, and other stressors may be present as well. Moreover, the most part of the MP found in the wild have irregular shape and such particles generally induce more severe effects than regular shaped MP (Ogonowski et al., 2016; An et al., 2021). Also, some effects of chemicals on *D. magna*, including MP, last for generations (Martins and Guilhermino, 2018; Schür et al., 2020), and the exposure period can be much longer. Furthermore, because distinct species often have distinct sensitivity to environmental contaminants, such as Li (Zhao-Xia et al., 2013) and MP (Jaikumar et al., 2019), the balances in interspecies relationship may be disrupted with potential negative impacts on biodiversity and ecosystem functioning.

4.3. Li-MP interaction was antagonistic at low and high concentrations, and synergistic at medium concentrations

The type of interaction between Li and MP, assessed through the population growth rate, changed with the concentration of Li-MP mixtures. At the lowest concentration of Li-MP mixtures, it was slightly antagonistic, almost additive. Adsorption of several metal ions to different types of MP was documented in distinct aqueous media (e.g., Holmes et al., 2012; Yuan et al., 2020; Tuccori et al., 2019). Small MP particles have a considerable adsorption capability due to their high surface/volume ratio (Yuan et al., 2020), and those tested had 1–5 μm. The concentrations of MP in test medium decreased within 24 h (Table 2) mainly due to *D. magna* uptake and bioconcentration from the water, and the sedimentation of MP particles and aggregates, as previously discussed. Moreover, adsorption of Li to microalgae cell surface and bioaccumulation of this metal by *C. vulgaris* was documented (Kaštánek et al., 2018). Therefore, it is hypothesised that adsorption of Li to MP and to microalgae cell surface, and uptake of the metal by *C. vulgaris* occurred in our experimental conditions, and that the settlement of some MP particles, microalgae cells, and aggregates on the bottom of the beakers reduced the concentration of Li and Li-contaminated food available in the water column of Li-MP mixtures relatively to the treatments containing the same concentrations of Li alone.

As *D. magna* feeds preferably by filtering the water column, respiration occurs through water filtration in gills, and the body surface is in contact with the surrounding water, the decrease of Li and contaminated food in the water column likely reduced the uptake of Li by animals exposed to Li-MP mixtures in relation to the treatments containing Li alone. The decrease of MP concentrations in test medium within 24 h (Table 2) supports this hypothesis, but the lack of significant differences in the actual concentrations of Li between treatments with and without MP apparently does not. However, because the test medium was renewed at each 24 h and fresh food was added daily, the potential difference of the Li concentration in the water column at each day due to the sedimentation of MP particles, microalgae cells and aggregates may have been too small to be detected because only the total amount of Li was determined. Nevertheless, it may have contributed to the antagonism because *D. magna* was exposed for 21 days, and likely bioconcentrated Li from the water and bioaccumulated it from Li-contaminated food along the exposure period, as documented in other animals exposed to this metal, including filter feeders (Aral and Vecchio-Sadus, 2008; Viana et al., 2020; Bolan et al., 2021). The concentrations of the chemicals in test medium were low, therefore the magnitude of these processes should have been relatively reduced, what can explain the slight antagonistic interaction under exposure to the lowest concentration of Li-MP mixtures. Reduction of Li availability in the water column due to interactions among Li, MP and microalgae cells, and settlement of part of them on the bottom of the beakers, and bioconcentration/bioaccumulation of Li and MP by *D. magna* likely occurred in all the exposure scenarios. However, their magnitude and relative contribution to the toxicological interaction probably variated with the concentration of Li and MP, and the influence of other factors.

Under exposure to the medium concentration of Li-MP mixtures, the toxicological interaction between Li and MP changed to synergism. It is hypothesised that four main factors contributed to this change, all them potentially increasing the concentrations of Li and MP inside the body of *D. magna*, their interaction with internal targets, and their bioconcentration/bioaccumulation along time, leading to higher toxicity. First, the concentrations of Li and MP in test medium augmented with the increase of Li-MP mixture concentration, likely leading to higher uptake and internalization of Li and MP by *D. magna*. Second, the rise of chemical concentrations in test medium probably increased the adsorption of Li to MP, as found in experiments with other metals and MP (Yuan et al., 2020), as well as the adsorption of Li to microalgae surface and its bioconcentration by microalgae cells, resulting in higher uptake of Li-contaminated MP and food and increased likelihood of internalization of MP and food components containing the metal. Third, inside *D. magna* body, desorption of some Li from MP may have also occurred, as suggested to other metals and MP (Yuan et al., 2020), particularly in the gut, where release of Li from contaminated microalgae cells may had also happened, increasing the absorption of free Li. Moreover, in the gut, some MP may have been fragmented as documented in krill exposed to MP (Dawson et al., 2018), facilitating the desorption of Li from MP, and the absorption of Li and smaller MP particles through the gut walls. Four, more MP in test medium may have stimulated the feeding behaviour of *D. magna* at least during the first part of the exposure period when the intoxication was not yet very severe. Feeding stimulation may have been caused by the presence of the particles themselves, because part of the ingested particles were MP and not real food (Chen et al., 2020) and more food needed to be ingested to get the same amount of energy, and/or due to increased chemical stress requiring additional energy to deal with it, triggering higher feeding behaviour. Increased water filtration in *D. magna* exposed to MP was documented (Chen et al., 2020), and it was also found in *C. fluminea* exposed for 96 h to a mixture of Li and nano-sized MP, with no significant effects of the chemicals on the filtering rate in single exposures (Costa et al., 2021). Moreover, in our experimental conditions, the test medium was changed every day, contributing to maintain more time the MP particles and microalgae cells in the water column potentially stimulating *D. magna* feeding, despite the settlement of part of them as previously discussed. If the feeding behaviour was stimulated, this would also require more energy to support increased swimming activity, water filtration, respiration and metabolism. Along time, higher uptake, internalization and bioconcentration/bioaccumulation of Li and MP likely overcome the beneficial effects resulting from the sedimentation of some MP, microalgae cells, and aggregates, part of which likely contaminated with Li, resulting in synergistic interaction and increased toxicity.

At the highest concentration of Li-MP mixtures, the interaction between Li and MP changed to antagonism. The rise of Li and MP concentrations in test medium likely further increased the adsorption of Li to MP, as well as the amount of microalgae cells contaminated with Li. Therefore, although the MP decay in the test medium did not increase (Table 2), a higher percentage of Li-contaminated MP, microalgae cells and aggregates may have been removed from the water column at the highest concentration of Li-MP mixtures than at the medium one. If so, this would have reduced the uptake of Li from the water column and contaminated food by *D. magna*, as well as the bioconcentration/bioaccumulation of Li in relation to the treatment containing the same concentration of Li alone, contributing to change the interaction to antagonism. Additionally, a well know effect of MP in animals is feeding inhibition due to false food satiation caused by the presence of the particles in the gut, among other possible causes (e.g., Luís et al., 2015; Ogonowski et al., 2016). At relatively high concentrations, several types of MP, including regular shaped particles such as those tested here and irregular ones, were found to decrease *D. magna* water filtration and food uptake, as well as the population fitness (e.g., Ogonowski et al., 2016; Colomer et al., 2019; Serra et al., 2020; An et al., 2021). For example, significant reduction of *D. magna* food intake after 72 h of exposure to 2.25×10^5 MP items/mL of 1–5 μm plastic spheres was found (Ogonowski et al., 2016). The number of MP particles in the highest concentration of the Li-MP mixtures was lower (34,884 MP/mL) but the exposure time was much longer, and the test medium was changed every day. For the same type of MP and *D. magna* population, similar experimental conditions, and availability of adequate food, the effects depend on the MP concentration, MP/food ratio, MP elimination rate, exposure time, among other factors (e.g., Ogonowski et al., 2016; Colomer et al., 2019; Serra et al., 2020; An et al., 2021). In general, relatively low MP concentrations and MP/food ratios have no effect or slightly change (decrease or increase) *D. magna* filtering rate and feeding intake, and most of them are rapidly eliminated from the gut, whereas relatively high MP concentrations and MP/food ratios can considerably reduce the water filtration and feeding intake, and increase the retention time of the MP in the gut (Ogonowski et al., 2016; Colomer et al., 2019; Serra et al., 2020). Moreover, the filtration capability of *D. magna* decreases with the augment of the time of exposure to MP and the ratio MP/food concentration (Colomer et al., 2019). In the present study, *D. magna* was exposed for 21-days and the MP/food ratio was higher under exposure to the highest concentration of Li-MP mixtures than at lowest concentrations because the amount of food provided daily was the same for all the exposure scenarios. In filter feeder bivalves (*C. fluminea*) exposed for 8 days to the type of MP used in the present study (0.13 mg/L), mercury (Hg, 0.030 mg/L) or to a mixture of MP-Hg (same concentrations), the filtration rate was significantly decreased in all the treatments, the reduction under exposure to the mixture was lower than the sum of the reduction in bivalves exposed to the substances individually, suggesting antagonism, and the bioconcentration factor of Hg was much lower in bivalves exposed to the mixture (25) than in those exposed to Hg alone (55). Therefore, it can be hypothesised that in *D. magna* exposed to the highest concentration of Li-MP mixtures, which had 0.19 mg/L of MP, water filtration inhibition and feeding reduction induced by MP occurred, decreasing the uptake of Li, and the bioconcentration/bioaccumulation of Li relatively to the exposure to the same concentration of Li alone, contributing to the antagonism found. Moreover, the tested MP were very small and this type of particles tend to aggregate inside the digestive system of animals when ingested at high concentrations, a process that may increase the retention time of the particles in the gut and cause adverse effects, such as gut obstruction, lesions in the gut walls, and the release of additives and contaminants adsorbed to MP, such as metals (Ogonowski et al., 2016; Jabeen et al., 2018; Yuan et al., 2020). However, the formation of aggregates in the digestive system may also reduce the internalization of MP and the

chemicals that they might have because aggregates may not be able to cross the gut walls due to their size (Ogonowski et al., 2016; Jabeen et al., 2018). Moreover, the desorption of chemicals from the MP and microalgae cells stuck in the aggregates may be more difficult than when the particles and cells are isolated. Aggregates and MP may also decrease the absorption of other contaminants present freely in the gut, as activated charcoal does in the treatment of intoxications by chemicals able to bind to this material. Eventually, the MP and aggregates not translocated across the gut walls are eliminated (Ogonowski et al., 2016; Jabeen et al., 2018), as well as the contaminants and contaminated food that they might contain. Therefore, the potential contribution of these processes to the antagonism found at the highest concentration of Li-MP mixtures should not be excluded. Antagonism between copper, which likely has similarities in the mode of action with Li (Nagato et al., 2013), and other MP was also found in *Daphnia carinata* (Thi et al., 2021) and suspected in *Danio rerio* (Santos et al., 2020).

In addition, to the factors before discussed, MP can also disrupt the patterns of *D. magna* swimming activity by other ways, especially the vertical migration in the water column leading to modifications in feeding, energetic costs, and stress levels of the animals (Magester et al., 2021). Moreover, at high concentrations, MP can be retained in gills and cause local damage, what can negatively impact respiration and many other functions, and can also adsorb to *D. magna* body surface, including appendices, making swimming and other functions more difficult (Eltemsah and Bøhn, 2019; Guilhermino et al., 2021). Therefore, these potential effects may have also contributed to the changes on the type of Li-MP interaction with the increase of Li-MP mixture concentrations.

Overall, the findings of this study indicate that the type of interaction between Li and MP on *D. magna* population growth rate depends on the concentration of Li-MP mixtures. Different types of interaction at distinct concentrations of mixture components were also documented in *D. magna* exposed to mixtures of MP and other contaminants, such as some metals (Yuan et al., 2020), gold nanoparticles (Pacheco et al., 2018) and ammonium (Serra et al., 2020), as well as to mixtures not containing MP (Silva et al., 2022). They were also found in other animals exposed to mixtures, including aquatic (e.g., Sanches et al., 2018) and terrestrial species (e.g., Wang et al., 2021a).

5. Conclusions and implications to 'One Health'

The results of the present study showed that 21-day exposure to environmental relevant concentrations of Li and Li-MP mixtures significantly reduced the population fitness of *D. magna* (up to 67% and 58%, respectively), stressing their potential negative impacts on wild zooplankton populations.

A single lineage of *D. magna* was tested in laboratory conditions, which are different from many real scenarios. In the wild, *D. magna* populations generally have more diversity and strategies that may reduce the adverse effects of Li and MP. Nevertheless, at least in some real scenarios, the long-term effects of Li and Li-MP mixtures on *D. magna* and other zooplankton populations may be more severe than those observed here due to higher levels of UV radiation, presence of other stressors, longer exposure, and other factors. Zooplankton communities play ecological functions that are fundamental to reach the United Nations Sustainable Development Goals. Therefore, the long-term population effects of Li and Li-MP mixtures to the model species *D. magna* found in the present study are very concerning, especially considering the current dependency of Li and MP that our society has, as well as its expected increase in the next future.

Based on the population growth rate, toxicological interactions between Li and MP in *D. magna* were found. The interaction changed with the concentration of Li-MP mixtures, being slight antagonism at the lowest concentration, synergism at the medium concentration, and antagonism at the highest concentration. The existing knowledge on the combined effects of stressors is still limited and more studies are urgently needed to improve the basis for environmental and human risk assessment, considering more realistic scenarios, a most important step to increase 'One Health' safety.

Today, increased long-term human exposure to Li through drinking water occurs mainly in regions naturally enriched with this metal and in Li extraction areas. It may also happen in other situations, such as high consumption of bottled natural mineral waters rich in Li, and through the consumption of contaminated drinking water in areas with Li-related industries and near e-trash sites or under their impact. The therapeutic use of Li is expected to further rise as human population growths, socio-economic development allows more people to have access to health care, and new therapeutic applications emerge. Contaminated food of animal and vegetal origins, and air may also be important routes of exposure to Li and MP. Therefore, increased long-term human exposure to Li likely will be a common situation in many regions across the globe in the coming years.

Although relatively low concentrations of Li have beneficial effects on human health, increased exposure induces toxicity. There are evidences linking Li exposure through drinking water and adverse effects in humans, such as impairment of maternal calcium homeostasis, especially vitamin D, during pregnancy (Harari et al., 2016). Studies in animals have been also documenting several types of Li adverse effects, including the present one showing that long-term exposure to environmental relevant concentrations of Li, alone and combined with MP, can reduce the somatic growth, reproduction and the population fitness of animals. Moreover, Li over-exploration has been causing depletion of precious natural resources, such as freshwater of quality, increasing the risks to wildlife and human health, raising social injustices in their use, and leading to conflicts (Jerez et al., 2021) with potential consequences at global scale. This type of conflicts will tend to be more severe and expand to other regions as pollution, global warming and human population increase. Therefore, more research, technology and solutions to stop the increasing trends of Li, MP and other types of pollution are urgently needed to protect 'One Health'.

CRediT authorship contribution statement

A. Martins: Methodology – planning of the bioassay; Investigation – carried out the bioassay and determined the concentrations of microplastics in test medium; Data collection – biological and microplastic concentration data; Writing - Review & Editing.

D. Dias da Silva: Methodology – planning of lithium analyses; Investigation – determination of lithium concentrations in test medium; Data collection – lithium concentrations in test medium; Writing – method to determine lithium concentrations, contribution improve the first draft of the manuscript; Review & Editing.

R. Silva: Methodology – planning of lithium analyses; Investigation – determination of lithium concentrations in test medium; Data collection – lithium concentrations in test medium; Writing - Review & Editing.

F. Carvalho: Methodology – planning of lithium analyses; Investigation – determination of lithium concentrations in test medium; Data collection – lithium concentrations in test medium; Resources; Supervision; Funding acquisition; Writing – method to determine lithium concentrations, contribution to improve the first draft of the manuscript; Review & Editing.

L. Guilhermino: Conceptualization; Methodology – Planning of the bioassay and microplastic determinations; Investigation – Data analysis; Supervision; Writing – Original draft preparation, Review & Editing; Data curation; Visualization – Figures and Tables; Resources; Project administration; Funding acquisition.

Declaration of competing interest

None.

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