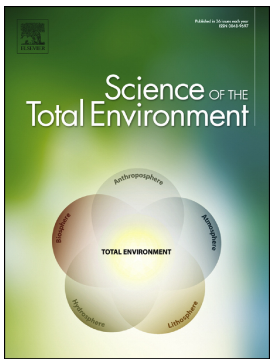
Journal Pre-proof



Multi-generational effects under single and pulse exposure scenarios in two monophyletic Daphnia species

G.S. Araujo, D.M.S. Abessa, A.M.V.M. Soares, S. Loureiro

PII: S0048-9697(19)34008-2

DOI: https://doi.org/10.1016/j.scitotenv.2019.134031

Reference: STOTEN 134031

To appear in: *Science of the Total Environment*

Received date: 17 October 2018

Revised date: 18 July 2019

Accepted date: 20 August 2019

Please cite this article as: G.S. Araujo, D.M.S. Abessa, A.M.V.M. Soares, et al., Multi-generational effects under single and pulse exposure scenarios in two monophyletic Daphnia species, *Science of the Total Environment* (2018), https://doi.org/10.1016/j.scitotenv.2019.134031

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2018 Published by Elsevier.

Journal Pre-proof

Multi-generational effects under single and pulse exposure scenarios in two monophyletic *Daphnia* species

Araujo, G.S.[1]; Abessa, D.M.S[2]; Soares; A.M.V.M[1]; Loureiro, S. [1]

[1] Department of Biology & CESAM, University of Aveiro, 3810-193, Portugal

[2] NEPEA, Campus do Litoral Paulista, Universidade Estadual Paulista Júlio de Mesquita Filho, Praça Infante Dom Henrique, s/n, CP 11330-900 São Vicente, SP, Brazil

* Corresponding author: Giuliana Seraphim de Araujo

Department of Biology & CESAM, University of Aveiro, 3810-193, Portugal

e-mail: giuliana@ua.pt

Journal Pre-proof

Abstract

Anthropogenic activities commonly relate to a set of diffuse and point contamination sources, from industrial, domestic or agricultural outputs, characterized by a chemical cocktail exposure and consequent disturbances of natural ecosystems. Different species may present different sensitivities to contaminants, even when phylogenetically close. This study used two monophyletic *Daphnia* species from tropical and temperate environments, *Daphnia similis* and *Daphnia magna* respectively, to evaluate the variation of their sensitivity to Pb (if any) and fitness during a multi-generational exposure and recovery. To accomplish that, standard acute immobilization tests were done on specific generations. Tests were carried out with exposures to 1) potassium dichromate ($K_2Cr_2O_7$) to evaluate organisms' sensitivity/fitness, 2) Pb, to monitor variation on Pb sensitivity and 3) the fungicide mancozeb, providing a pulse toxicity approach on generational Pb acclimated daphnids. Since growth is an important trait related to organisms' fitness, organisms' size measurements were also monitored. In addition, organisms were maintained under two different dietary regimes. Our results indicate no variation on daphnids sensitivity to $K_2Cr_2O_7$, except for *D. similis* from a recovery period under food restriction. However, a lower Pb sensitivity was seen for both species throughout generations. Both species also showed that under food restriction neonates' size were larger than those kept under regular food, while reproduction was considerably reduced. Food restriction also generated opposite outcomes on both species, such as *D. magna* epigenetic changes and *D. similis* phenotypic acclimation to Pb. Besides, *D. magna* pre-exposed to Pb presented lower sensitivity to mancozeb, while the contrary was shown by *D. similis*. This study indicates that daphnids are capable of acquiring a lower sensitivity to Pb across a long-term exposure, and that Pb pre-exposure can affect the sensitivity to other chemicals. Also, different patterns in multi-generational responses from monophyletic species (especially under oligotrophic media, common on natural habitats) acknowledges the use of representative or native species to assess the effect of contaminants, since monophyletic species can provide different toxicity outputs.

Key-words: multi-generation, daphnids, metal, fungicide, acute toxicity, body length

1. Introduction

The human population keeps on an exponential increase trend and so it does the demand for manufactured products, automobiles and food. This higher demand increases the use of a wide range of chemicals such as metals, hydrocarbons, pesticides and pharmaceuticals. Lead (Pb) is a nonessential metal (Jaishankar et al., 2014) with no known biological function which is capable of being very harmful to natural biota (Stokes et al., 1985). Nearly 95% of Pb emitted to the ecosystem is of anthropogenic source (Abrahim and Parker, 2002) including leaded gasoline, lead based paints, mining residues and others (Jartun et al., 2008). This metal is known to cause neurotoxicity (e.g.

(Reddy et al., 2003)), renal dysfunction and enzymatic inhibition (e.g. (Hernández-Flores, S. and Rico-Martínez, 2006)). It was recently added (June of 2018) to the Candidate List of substances of very high concern (SVHCs) by the European Chemicals Agency (ECHA). The World Health Organization (WHO) stablished the limit of Pb for drinking water as 10 µg/L (WHO, 2011), which is also followed by the European Union; for surface waters the European Union applies the Annual Average Value for the Environmental Quality Standards (AA-EQS) of Pb of 7.2 µg/L (European Parliment, 2008). The Brazilian federal legislation allows a 50 µg/L of Pb as the maximum concentration permitted (Brasil, 2005) for freshwater bodies.

The growth of human population also increases the demands for food, leading to the expansion, intensification and mechanization of agricultural practices and consequently raising the use of pesticides, insecticides and fungicides. Mancozeb is a worldwide used fungicide belonging to the dithiocarbamate group (Kubrak et al., 2012) which is applied on several crops like orchards and vineyards (Morgado et al., 2016), fruit plants, flowers and ornamental trees (Calviello et al., 2006). It can also develop neurotoxicity (e.g. (Axelstad et al., 2011)) and trigger negative effects on the reproductive, endocrine and immune systems of the exposed organisms (e.g. (Tsang, M. M. and Trombetta, 2007)). For human hazard assessment, according to FAO/WHO, the acceptable daily intake (ADI) for humans is 0.05 mg/Kg/day for mancozeb, while for the environmental hazard a No Observed Effect Concentration for *D. magna* (NOEC) for this compound was established as 0.0073 mg/L (European Commission, 2009). The predicted environmental concentration (PEC) of mancozeb proposed by the German authority for risk assessment of surface waters is 12.7 µg/L (Sabero Europe, 2017). However, such concentration can change in the case of crops and farmed vegetables, and thus they may achieve levels as high as 210.8 µg/L, for tomatoes farms for example (Environmental Protection Agency, 2005).

In general, contaminants are released into the environment and may reach aquatic ecosystems, negatively affecting the biota (Jartun et al., 2008). Organisms inhabiting natural aquatic systems may therefore be exposed to a complex mixture of chemicals and other stressors (Chen et al., 2015). Native biota can be useful to predict the ecological impacts of chemical substances, since their responses to stressors can produce a suitable and robust scenario of the pollution effects (Castillo et al., 2006). Although native species are the best choice in this case, some authors state that phylogenetically close related species produce redundant data regarding chemical toxicity (Hammond et al., 2012; Magalhães et al., 2014; Manusadžianas et al., 2003). Such species may occupy the same niche in different environments, such as water bodies at different latitudes (Ghilarov, 1967). In this sense, studies relying on similar sensitivities have used species from temperate regions to predict contamination on tropical environments (Flohr et al., 2012; Terra, N. R. and Gonçalves, 2013). However, different outcomes from similar species (Lyu et al., 2013; Magalhães et al., 2014; Regaldo et al., 2013; Völker et al., 2013) indicate the need of deeper assessments comparing close related species for their sensitivity to pollutants.

The genus *Daphnia* is a well-studied aquatic crustacean group with standard acute and chronic ecotoxicological tests well established for some species (e.g. *D. magna, D. similis, D. laevis*), commonly used in ecotoxicological studies (OECD, 2012, 2004). These standardized tests are based on short-term effects, and do not represent a suitable approach to deal with long-term ecological effects due to continuous contamination (Hammers-Wirtz, M. and Ratte, 2000; Moliner, 1992). Standard ecotoxicological tests may underestimate the influence of chemicals at the population level (e.g. no prenatal exposure). To overcome this issue, breeding organisms under chemical exposure for successive generations can be a more realistic approach (Tanaka, Y. and Nakanishi, 2002). Multi-generational studies can be more sensitive than single-generation experiments, being more predictive of chronic exposure (long-term) under field conditions (Chen et al., 2014), and therefore ecologically more relevant than single-generational studies (Tsui, M. T. K. and Wang, 2005).

Daphnids reproduce by parthenogenesis, proliferating genetically identical individuals (Adema, 1978); this is a key point on multi-generational tests because even though daphnids' reproduction is essentially asexual, these organisms are capable of generating epigenetic variability in offsprings in few generations (Frost et al., 2010). The physiological variation that occurs in a *Daphnia* mother can be transmitted to the offspring and affect their development, and may also induce epigenetic alterations (Ramírez, 2014). However, multi-generational tests are still not well widespread and more research regarding this approach is needed (Stoddard and Harper, 2007). Moreover, studies performed with few generations (e.g. 3 generations) may mislead to different sensitivity findings, because this time interval may be not enough to stabilize sensitivity variation fluctuation. Therefore, using a larger number of generations is necessary to achieve a more accurate result on organisms' sensitivity (increased stabilization of sensitivity variation) (Krylov, V. V. and Osipova, 2013; Silva et al., 2017). Studies performed with several generation exposures (six generations or more) are available in the literature (Jacobasch et al., 2014; Muyssen and Janssen, 2004; Silva et al., 2017). In addition, it has been demonstrated that a nine generation Pb exposure induced considerable Pb bioaccumulation and morphological alterations in *D. magna* and *D. similis* (Araujo et al., 2019). In this last-mentioned study, six generations were enough to stabilize Pb body burden in daphnids, being this dependent on the food regime.

Daphnids metal acquired sensitivity is widely believed to be physiological acclimation and not genetic adaptation (Stoddard and Harper, 2007) or epigenetic changes. Organisms' physiological acclimation to chemicals may alter their sensitivity by involving different physiological processes; it can be detected if a full recovery is achieved when chemical exposure is cessed. If recovery fails, organisms may be presenting epigenetic changes (transgenerational inheritance) (Schultz et al., 2016). Epigenetics include DNA methylation, histone tail modifications and microRNA expression (Stoccoro et al., 2013). It can lead to a transfer of new phenotypic characteristics with no gene sequence modification (Berger et al., 2009). Epigenetic in *Daphnia* has already been shown and Frost

et al., (2010) suggests that *Daphnia* may alter the genotypic expression of offspring directly through genetic changes or via epigenetic regulation.

With that in mind it is important to investigate the organisms' recovery by removing chemical exposure to better estimate if the observed sensitivity was due to acclimation or epigenetics. Being a more realistic approach, the multi-generational test can provide valuable information on the real threat of chemicals on natural environments (Fernández-González et al., 2011), which are unseen on classical chronic exposure tests (Brausch and Salice, 2011).

The aim of this study was therefore to compare the outcome of two monophyletic *Daphnia* species, the well-established temperate organism *D. magna* and the tropical species *D. similis* during a continuous generational sublethal exposure, using Pb as a relevant metal model. Since natural environments can be contaminated a non-simultaneous entry of contaminants, pulse exposures to the fungicide mancozeb on Pb pre-exposed organisms was also evaluated. Chemical exposures were performed under two different dietary regimes, in order to consider fluctuations on the availability of nutrients in the natural environments. To achieve such goals, a multi-generational test was performed on which *D. magna* and *D. similis* were submitted to different setups: a control, a sublethal Pb exposure and a recovery period, where Pb pre-exposed organisms were transferred to clean media for three generations, under two different dietary regimes for a total of nine generations. Therefore, the hypotheses of this study were to evaluate if 1) a long-term Pb exposure modifies the sensitivity of Daphnids throughout generations (continuous Pb exposure); 2) Organisms pre-exposed to Pb present changes on their sensitivity to other chemicals (e.g. mancozeb); 3) Recovery after Pb exposure ceases; 4) Food regime alters the sensitivity of organisms to contaminants; 5) *D. magna* and *D. similis* under similar exposure design would give different responses.

2. Methodology

The OECD 202 immobilization and OECD 211 reproduction test guidelines for *Daphnia* sp. testing recommends the use of broods N3 to N5 to perform assays (OECD, 2004; 2012). Therefore, the same brood (N3) was chosen to start all generational assays (all generations started at the N3 brood of the previous generation) and all toxicity tests to avoid any changes in organisms' sensitivity.

2.1. Chemical solutions and analysis

The $Pb(NO_3)_2$ (CAS No. 10099-74-8, 98.5% purity, VWR chemicals®) and mancozeb (CAS No.8018-01-7, 97.5% purity, Fluka®) stock solutions were prepared in mili-Q water and diluted (in ASTM media) for the preparation of the other concentrations.

For the chemical analysis (by ICP-OES; Horiba Jobin Yvon, Activa M) Pb samples were acidified with nitric acid. The limit of quantification (LOQ) for Pb (25 µg/L) were adequate regarding

samples concentrations. Chemical analysis was evaluated in triplicate and certified material in duplicate (to ensure chemical optimum recovery).

2.2. Culture maintenance

All organisms were kept in ASTM hard water medium (American Society for Testing Materials) (ASTM, 2002), under controlled photoperiod and temperature (16:8h light/dark; 20º ± 2ºC). Daphnids were fed with the microalgae *Raphidocellis subcapitata* ($3x10^5$ cells/mL) and enriched with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). ASTM medium and food was renewed every other day. Both species were maintained exactly under the same conditions to exclude the interference of other abiotic factors.

2.3. Multi-generation

For the multi-generation test, an F was used as the nomenclature to denominate the daphnids' generations (F0 to F9) and N to indicate the broods (Figure 1). The test began with 20 neonates (F0, from brood N3) with less than 24 hours old being randomly placed in one litter vials; three replicates were prepared for every treatment. Each species was exposed to a 2x2 experimental setup with four treatments in total: a negative control (clean ASTM media) and the Pb treatment (50 μg/L of Pb in ASTM), being the legislation maximum permitted by the Brazilian federal law for fresh water bodies (Brasil, 2005). Both setups were maintained under two different dietary regimes (during the whole 9-generation experiment), the usually used algae concentration in reproduction tests ($3x10^5$ cells/mL) and a food restriction regime ($1.5x10^5$ cells/mL). After the release of the F6 generation, the experiment was divided in another two sets: while one of the sets of F6 was kept in the same condition as before (Pb contaminated media), in the second set daphnids were moved to clean media (ASTM), named as recovery period, for 3 more generations (F6 to F8, till the F9 was released).

Journal Pre-proof

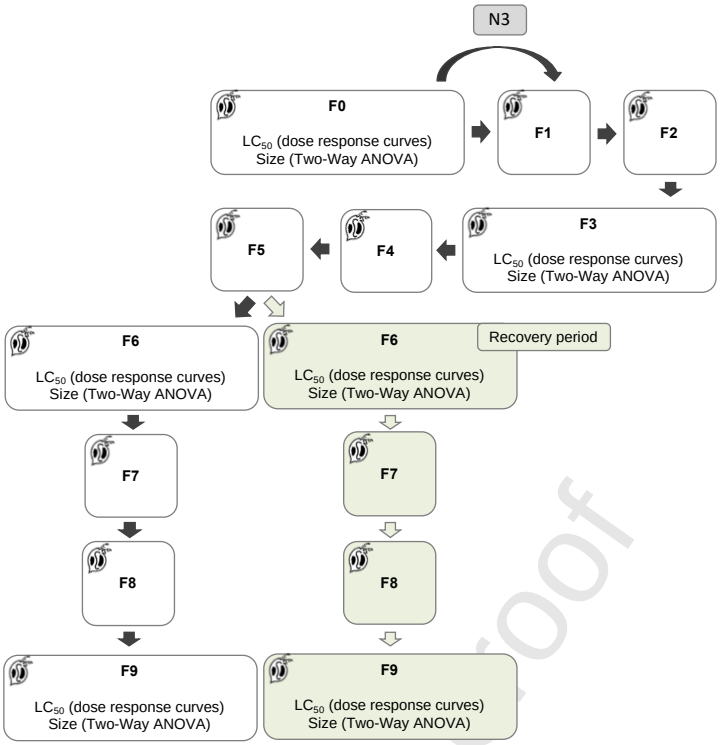


Figure 1: Experimental design of the multi-generation test. F refers to the generation and N to the brood used to start each generation. Toxicity tests ($K_2Cr_2O_7$, Pb and mancozeb) carried out (in all setups) are mentioned in appropriate boxes. Darker arrows indicate chemical exposure to Pb and lighter arrows indicates recovery period. This same design was made for the two different food regimes (usual and restricted) and the control (ASTM media). Figure adapted from Araujo et al., 2019.

2.4. Acute immobilization tests

The acute immobilization tests were conducted for every three generations (F0, F3, F6 and F9) and followed the OECD guideline 202 (OECD, 2004). The respective N3 broods (aged between 6 and 24 hours) were exposed to the following chemicals' concentrations ranges: potassium dichromate ($K_2Cr_2O_7$) for 24 hours, at standard concentrations proposed by OECD (202) up to 2.4 mg/L and decreasing concentrations were achieved by a factor of two; Pb from 3 mg/L and decreasing concentrations achieved by a factor of two, and the fungicide mancozeb, from 1.2 mg/L and decreasing concentrations were achieved by a factor of 2 for 48 hours. The exposure to the reference substance ($K_2Cr_2O_7$) is recommended by OECD (OECD, 2004) in order to determine the organisms' fitness used in ecotoxicological assays, organisms should present a LC_{50}-24 h within the range 0.6 mg/L to 2.1 mg/L to be considered fit to use in such assays. Each concentration contained five replicates with five neonates each exposed to 50 mL of experimental solutions. Thereafter, mortality and immobility were recorded to derive the lethal concentration for 50% of the exposed organisms (LC_{50}).

2.5. Neonate's measurement

Every three generations (F0, F3, F6 and F9), 30 neonates' younger than 24h from specific broods (N1, N3 and N5) and from each treatment were measured under a stereomicroscope. Body length was measured from the top of the head to the base of the apical spine (excluding the spine).

2.6. Statistical analysis

The lethal concentrations (LC_{50}) were estimated using a nonlinear regression curve fit (dose response curves) and differences among the LC_{50} of each setup were assessed through a global fitting (extra sums of squares F-test), using always the one with the best adjustment (see with more details in Pestana et al. (2016)). These statistical procedures were performed in order to confirm if the LC_{50} of each setup (control, Pb exposure and recovery) would differ when the organisms were re-exposed to each chemical ($K_2Cr_2O_7$, Pb and mancozeb) after generational Pb pre-exposure. Such approach permitted the statistical evaluation of hypothesis 1, 2 and 3. Hypothesis 4 and 5 were evaluated qualitatively looking at the LC_{50}s' variations for each treatment of each species and food regimes.

After checking the data for normality (Kolmogorov–Smirnov), the differences between juvenile sizes using as factors the generations (F0, F3, F6 and F9) and setups (control, Pb exposure and recovery period) were evaluated through a Two-Way Analysis of Variance (ANOVA), followed by a post-hoc test (Bonferroni) when statistical differences were first highlighted (GraphPad Prism®). Statistical analyses were performed for each species individually.

3. Results

3.1. Chemical analyses

The results of Pb chemical analyses showed a retrieval of >79% recovery comparing nominal to measured concentrations and a 25 µg/L of Pb limit of quantification (LOQ). The ASTM media samples (control) presented values <LOD for both chemical evaluations (Table 1S). The analysis of the certified material achieved above 80% of recovery. Mancozeb chemical analysis are not possible to report due to some technical constrains. However, as effects are similar to others reported in the literature, we considered nominal concentrations for all calculations and discussion.

3.2 Acute toxicity tests

3.2.1. $K_2Cr_2O_7$

The exposure to the reference substance $K_2Cr_2O_7$ is shown in the Figure 2. For *D. magna*, comparing the LC_{50} under usual food regime, no statistical difference was shown between treatments, however, F3 (continued Pb exposure) and F9 (control and Pb treatments) were statistically less sensitive than F0 (originally from cultures) (Figure 2a). *D. magna* under food restriction presented the

smallest variations in the calculated LC_{50} between generations showing no statistical difference towards F0 (Figure 2b).

For *D. similis* (usual food), generations F6 (continued Pb exposure) and F9 (control and Pb treatments) presented a statistically significant lower sensitivity in comparison to F0 (originally from cultures) (Figure 2c). When under food restriction, both treatments (control and Pb) presented lower sensitivity to $K_2Cr_2O_7$ in comparison to F0.

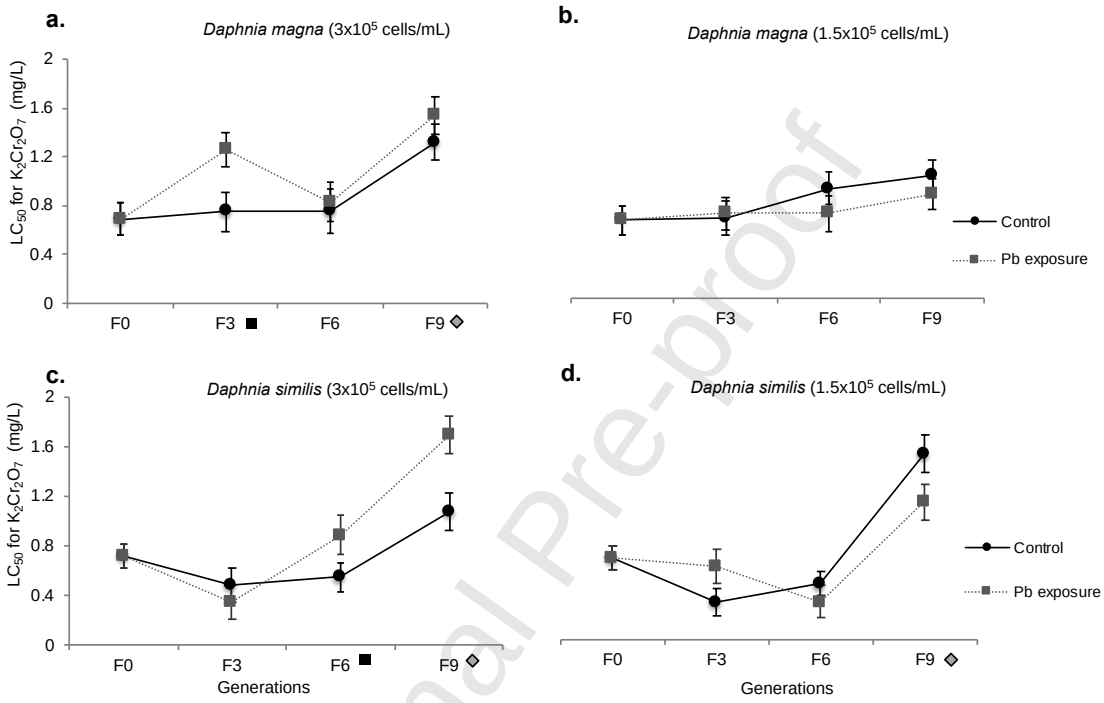


Figure 2: LC_{50} for a $K_2Cr_2O_7$ 24h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a 1) black square for those statistical different from F0, in Pb treatment and 2) a grey diamond when both control and Pb treatments presented difference in comparison to F0 (Bonferroni, $p<0.05$).

3.2.1.1. Recovery period

Regarding the potential recovery, *D. magna* showed no relevant variations in both dietary regimes (Figure 3a,b). However, organisms from recovery period under usual food regime on generation F9 presented lower sensitivity (to $K_2Cr_2O_7$) in comparison to F0.

Concerning recovering *D. similis* (usual food regime), no difference was shown among treatments or generations (Figure 3c). Under food restriction, F9 *D. similis* under recovery presented a contrary pattern regarding the sensitivity to $K_2Cr_2O_7$ when compared to both control and continued Pb exposure (Figure 3d). While the control and continued Pb exposure showed a trend to decrease in toxicity to $K_2Cr_2O_7$, the under recovery daphnids showed an increase in their sensitivity.

Journal Pre-proof

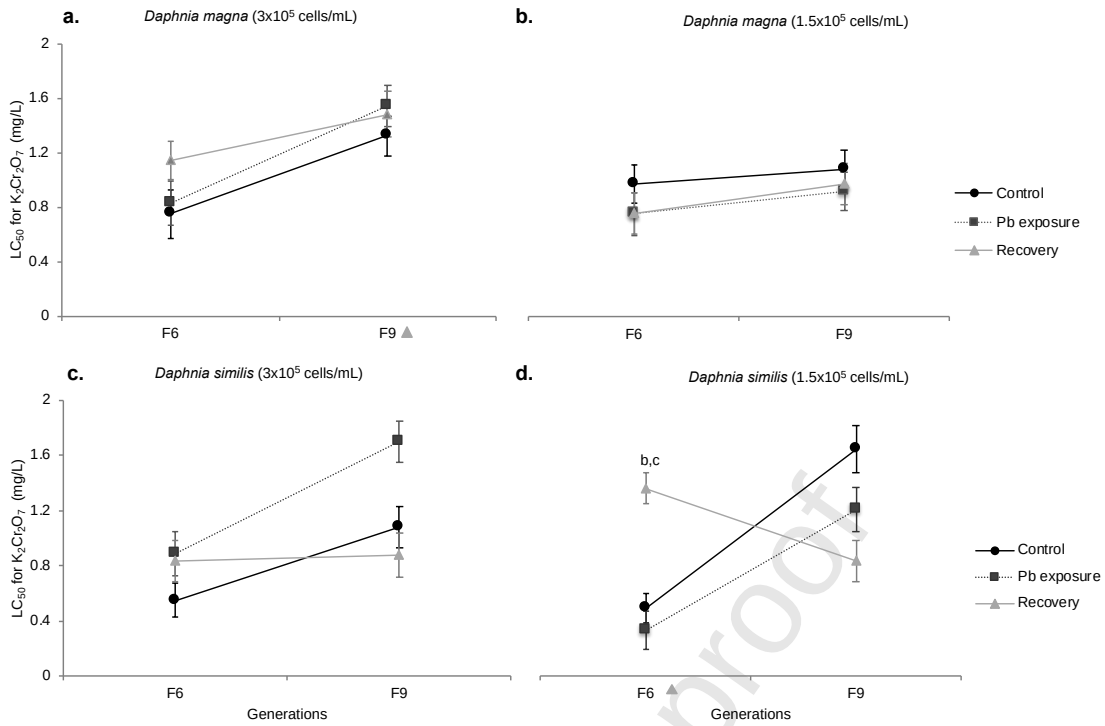


Figure 3: LC_{50} for a $K_2Cr_2O_7$ 24h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a grey triangle when recovery treatment presented difference in comparison to F0. Letters indicate statistical difference between treatments within the same generation, being (b) for recovery vs. control and (c) for Pb vs. recovery (Bonferroni, $p<0.05$). Data presented for control and Pb are the same as presented in figure 2, just for comparison.

3.2.2. Pb

For assessing the sensitivity to Pb, LC_{50} derived for the different generations exposed continuously to Pb showed a lower sensitivity for *D. magna* under the usual food treatment (Figure 4a). The same pattern is maintained for organisms under food restriction, in generations F3 (control and Pb treatments), F6 (continued Pb exposure) and F9 (control and Pb treatments) which presented lower Pb sensitivity in comparison to F0. *D. magna* Pb sensitivity showed a continuous decrease, going from LC_{50}= 0.43 mg/L (F0) up to 2.11 mg/L (F9) under usual food treatment and to 3 mg/L (F9) under food restriction, while the LC_{50} of control organisms varied from 0.43 mg/L to 0.89 mg/L under usual food and to 1.3 mg/L under food restriction (Figure 4b).

Regarding *D. similis* (under usual food), organisms from F9 presented a statistically lower sensitivity to Pb than organisms from F0 for both control and Pb exposure (Figure 4c). Control organisms from generation F6 showed a lower sensitivity in comparison to F0 organisms and to continued Pb exposure neonates. During generation F3, a lower Pb sensitivity was exhibited and it was not possible to calculate an LC_{50} for the tested concentrations range (with the highest being 0.75

mg/L), forcing the increase of Pb concentration range to the same as *D. magna* (being the highest 3 mg/L) for the following generations. *D. similis* under food restriction also indicated lower Pb sensitivity throughout generations (Figure 4d). The LC_{50} values obtained for *D. similis* on generations F6 and F9 under food restriction were statistically higher than for F0 (for control and Pb treatments). Pb exposed *D. similis* (excluding F6 from usual food treatment) diminished Pb sensitivity, rising its LC_{50} from 0.29 mg/L (F0) up to 0.94 mg/L (F9) under usual food treatment and to 1.76 mg/L (F9) under food restriction, while control organisms varied LC_{50} from 0.29 to 0.9 mg/L (F6) for usual food and to 1.16 mg/L (F9) under food restriction.

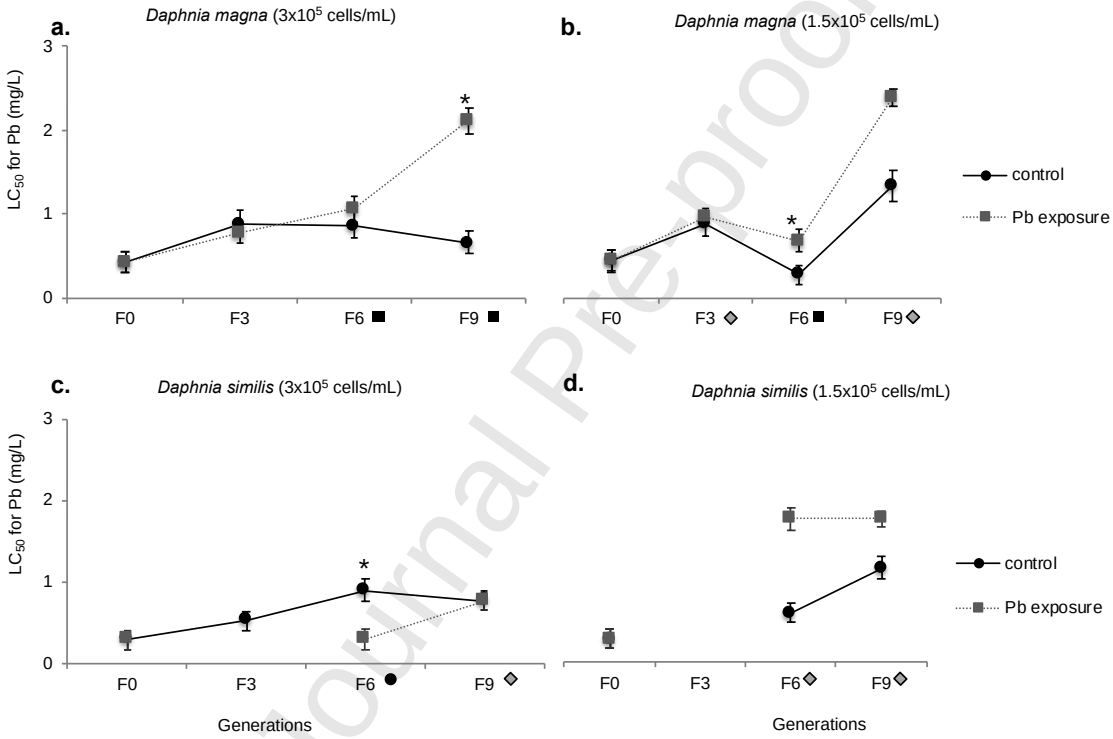


Figure 4: LC_{50} for a Pb 48h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a 1) black circle for those statistical different from F0 in the control treatment, 2) a black square for those statistical different from F0, in Pb treatment and 3) a gray diamond when both control and Pb treatments presented difference in comparison to F0 (Bonferroni, $p<0.05$). Asterisk (*) indicate statistical difference between treatments at each generation (Bonferroni, $p<0.05$). Data missing on *Daphnia similis* from generation F3 ($1.5x10^5$) was due to a subtle lower Pb sensitivity, preventing the calculation of the LC_{50}.

3.2.2.1. Recovery period

Recovering *D. magna* (usual food) presented enhanced sensitivity to Pb compared to control and continued Pb exposure organisms at generation F6. On generation F9, Pb exposed organisms

were significantly less sensitive than control and recovery period. Under food restriction, *D. magna* from recovery period diminished Pb sensitivity from F6 to F9. However, only on generation F6, recovering organisms presented statistical difference to control and continuous Pb exposure organisms. Neonates from recovery period showed a lower sensitivity to Pb (F9) in comparison to F0. Considering *D. similis*, recovering organisms presented a lower Pb sensitivity in comparison to continuous Pb exposure and similar sensitivity to control (F6). A different trend occurred under food restriction, with Pb continuous exposure maintaining the lower sensitivity to Pb, while the control and recovering organisms diminished their sensitivity from F6 to F9. Recovering organisms (F9) presented a lower sensitivity in comparison to F0.

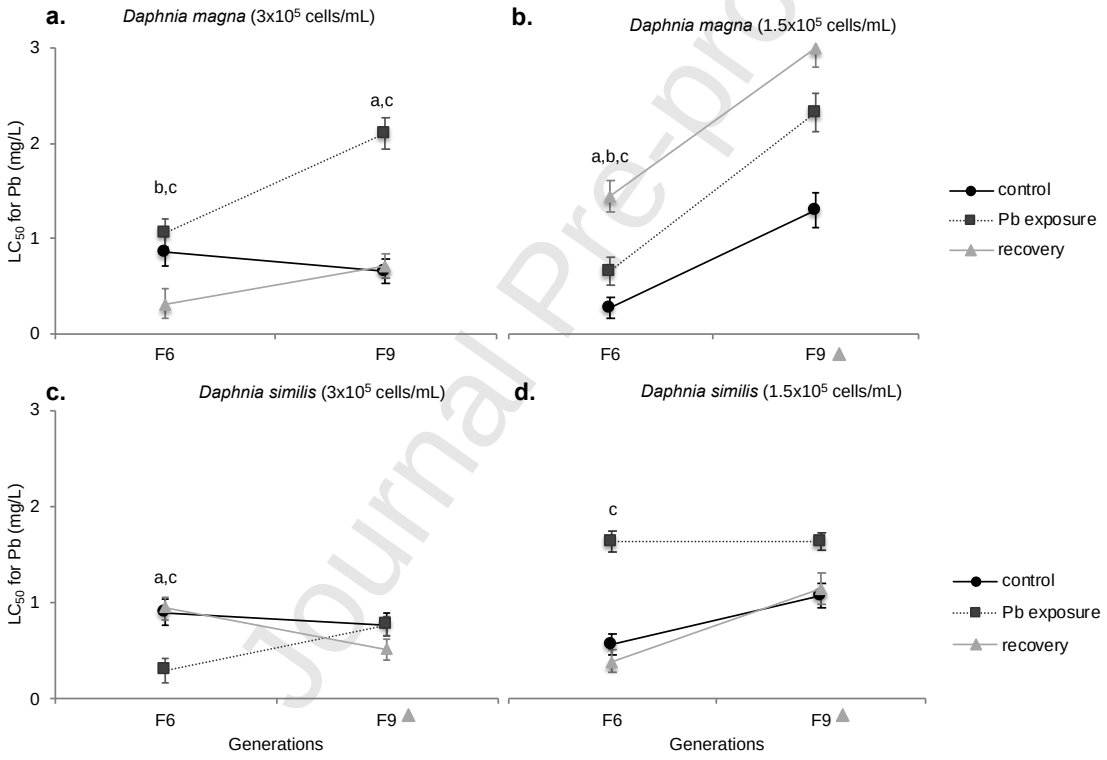


Figure 5: LC_{50} for a Pb 48h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a gray triangle for those statistical different from F0 in the recovery treatment (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control, (b) for recovery vs. control and (c) for Pb vs. recovery (Bonferroni, $p<0.05$). Data presented for control and Pb are the same as presented in figure 4, just for comparison.

3.2.3. Mancozeb

The acute toxicity test with mancozeb, in which organisms from all treatments were exposed to mancozeb for 48h, showed that F6 from control organisms and F9 from both control and Pb treatments presented statistical difference compared to F0 (Figure 6a). However, no statistical difference among treatments was achieved. Under food restriction, sensitivity to mancozeb exposure diminished regarding *D. magna* continuously exposed to Pb, being statistically different from control on generation F9 (Figure 6b). Generations F6 (control and Pb exposure) and F9 (continuous Pb exposure) presented lower mancozeb sensitivity in comparison to F0. *D. similis* under usual food regime presented similar outcomes as *D. magna*, except for a diminished control sensitivity on F6 (Figure 6c). However, under food restriction, organisms from control treatment showed lower sensitivity to mancozeb in comparison to continuous Pb exposure (F3 and F9) (Figure 6d). Regarding generations, F6 (continued Pb exposure) and F9 (control) exhibited lower mancozeb sensitivity in comparison to F0. Control organisms from generation F6 were not tested for mancozeb toxicity because of a lack of neonates' production due to food restriction, not generating enough neonates to start the test.

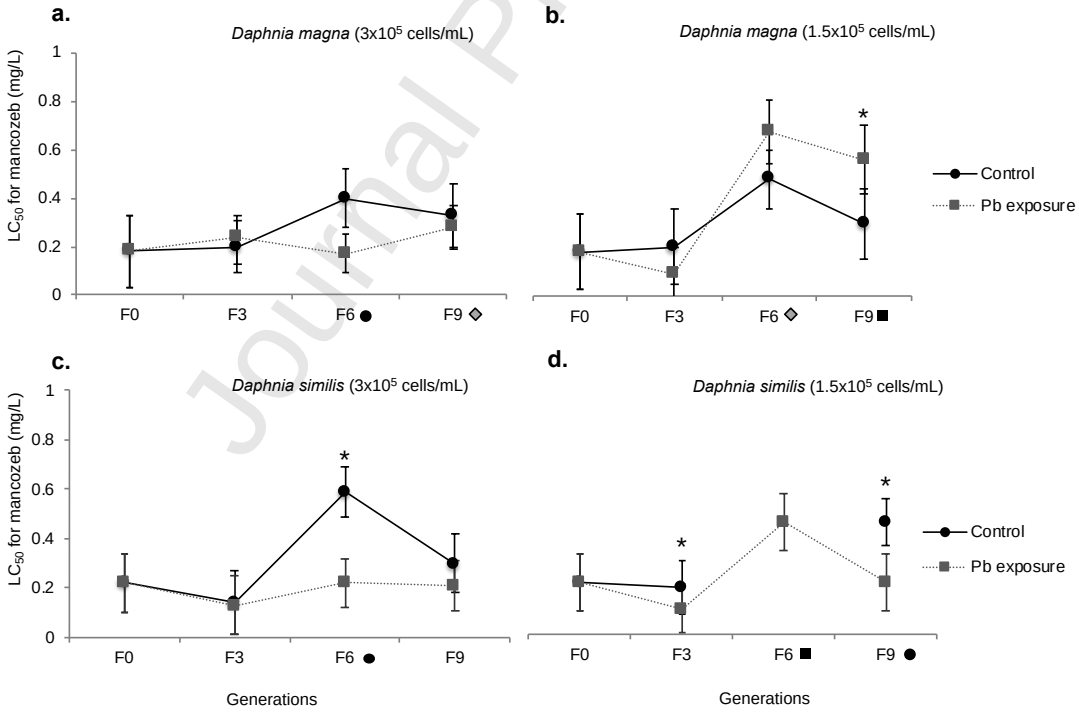


Figure 6: LC_{50} for a mancozeb 48h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a 1) black circle for those statistical different from F0 in the control treatment, 2) a black square for those statistical different from F0, in Pb treatment and 3) a gray diamond when both control and Pb treatments presented difference in comparison to F0 (Bonferroni, $p<0.05$). Asterisk (*) indicate statistical

difference between treatments at each generation (Bonferroni, $p<0.05$). The lack of data on control *D. similis* ($1.5x10^5$) from generation F6 was due to a lack of neonates' production due to food restriction.

3.2.3.1. Recovery period

During the recovery period, no difference was found between treatments on *D. magna* under usual food (Figure 7a). Organisms from recovery period presented a trend of diminished mancozeb sensitivity, but with no statistical difference (among treatments). However, comparing recovering organisms from F9 with generation F0, a statistical difference appears. *D. magna* under food restriction presented different results, with recovering organisms showing enhanced sensitivity to mancozeb in comparison to organisms under continuous Pb exposure on generation F6. On generation F9, organisms under continuous Pb exposure showed a lower sensitivity in comparison to other treatments (control and recovery period) (Figure 7b). Regarding *D. similis*, the control treatment (usual food) showed a lower sensitivity to mancozeb in generation F6, being less sensitive than the other treatments (Pb and recovery period) (Figure 7c). However, the sensitivity returned (in F9) to similar values as presented before. Different results are shown by organisms under food restriction, with the control treatment of generation F6 not producing enough neonates and consequently with no possibility to test further (Figure 7d). *D. similis* under continuous Pb exposure from generation F9 were significantly more sensitive to mancozeb than the respective control.

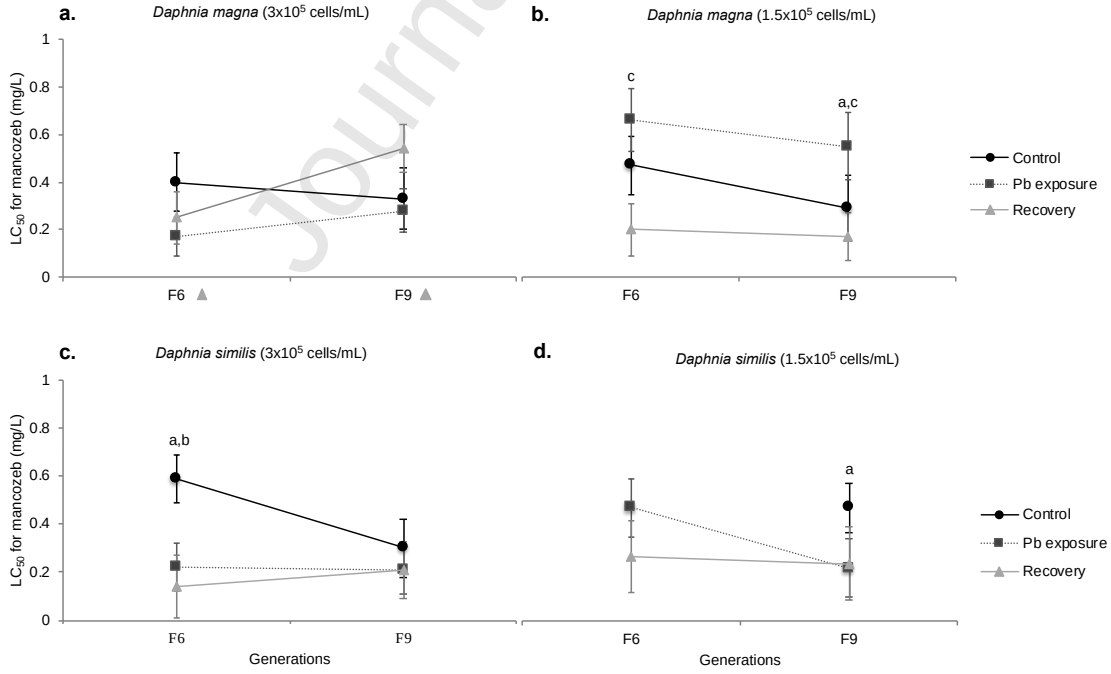


Figure 7: LC_{50} for a mancozeb 48h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-

exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a gray triangle for those statistical different from F0 in the recovery treatment (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control, (b) for recovery vs. control and (c) for Pb vs. recovery (Bonferroni, $p<0.05$). Data presented for control and Pb are the same as presented in figure 6, just for comparison.

3.3. Neonate's measurement

To determine if organisms' sensitivity was related to the fitness of neonates which may be translated into a bigger or smaller size, 30 neonates were measured in each treatment at first, third and fifth brood (N1, N3, and N5). Size measurement through generations showed a clear pattern for both species and food regimes, with juveniles from brood N1 being the smaller and N5 the largest, together with a size enhancement for food restricted neonates when compared to usual food regime (Figure 8). Two-way ANOVA statistical analysis is detailed in Tables 2S (*D. magna*) and 3S (*D. similis*).

Regarding N1 broods from *D. magna* (under usual food), statistical difference between control and continuous Pb exposure is shown in *D. magna* (usual food) in F3, with control organisms being bigger than those from Pb exposure (Figure 8a). In this brood generation F3 also presented a diminished size when compared to F0 for continued Pb exposure. For generation F6, N1 neonates exposed to Pb showed a smaller size in comparison to F0, as well as both treatments (control and Pb) from generation F9.

Daphnia magna (usual food) from brood N3 and generation F3 (both control and Pb treatments) exhibited a smaller size in comparison to F0. In brood N5, however, control organisms from generation F6 were statistically bigger than F0 and, in generation F9, statistically larger than Pb treatment.

Under food restriction, no statistical difference among treatments for brood N1 was depicted. However, continued Pb exposure neonates in generations F3 and F6 were smaller than F0. Regarding broods N3, Pb exposed neonates presented an enhanced size in comparison to those from F0 in generation F9; although it does not differ from control organisms, a trend of Pb exposed neonates increased size is seen from F0 to F9. This same trend of increased size through generations is also seen for Pb exposed neonates from brood N5, being bigger than control organisms from generation F9 (but smaller than control in F3). For all generations, N5 neonates from both treatments (control and Pb) were larger than those from F0 (Figure 8b).

Regarding *D. similis* (under usual food), N1 control neonates were bigger than those from continuous Pb exposure in generation F3 (Figure 8c). This same generation showed that N1 Pb exposed neonates presented a smaller size in comparison to F0, as well as both treatments (control and Pb) in generations F6 and F9.

Regarding broods N3, neonates' size showed no statistical difference between treatments (control and Pb) and only continuous Pb exposure neonates from generation F3 presented statistical bigger sizes than those from F0.

Journal Pre-proof

For broods N5, both treatments (control and Pb) differed at all generations. Continuous Pb exposure neonates were bigger than control in generation F3 and the opposite occurred on generations F6 and F9. A trend of increasing in size through generations was observed for control neonates, opposite from what was observed for Pb exposed neonates. N5 control and Pb treatments differed from F0 in generations F3 and F6, with Pb exposed neonates from F3 being bigger and control smaller than F0, the contrary occurring in F6.

Under a restricted diet, N1 control *D. similis* exhibits a statistically larger size than Pb treatment on generation F3, which were also smaller than neonates from F0. However, neonates' size increased among generations and, in generation F9, both treatments (control and Pb) presented enhanced size compared to F0. Regarding broods N3, a decrease in size occurred from F0 to F3 and a statistical difference was detected. However, neonates from both treatments increased in size and end up being larger than neonates from F0 in generation F9. During generation F6, only control neonates indicate statistical difference in comparison to F0. N5 control *D. similis* under food restriction could not been measured during generation F6 due to reproduction impairment (mentioned above). Although, F3 N5 neonates showed enhanced size for continuous Pb exposure (compared to control), the opposite occurring in F9. Neonates from N5 control in generation F6 were not measured do to reproductive impairment of the treatment (mentioned above).

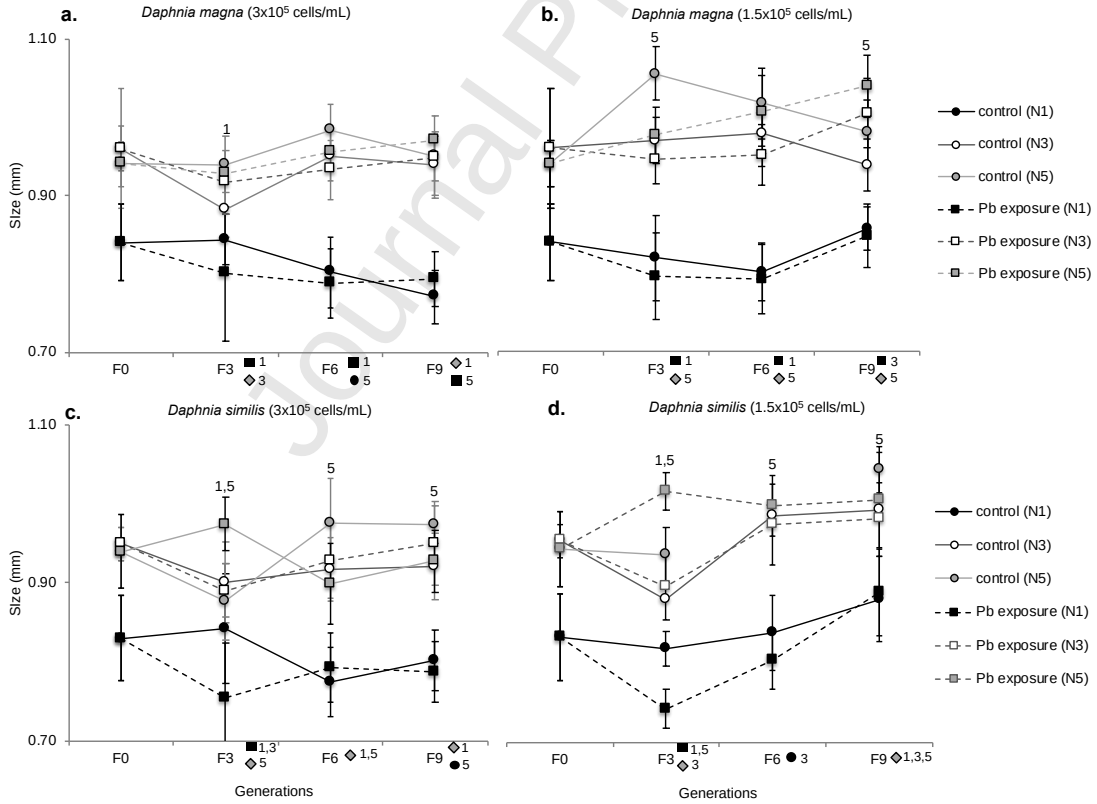


Figure 8: Body length of neonates from broods N1 (dark), N3 (white) and N5 (grey) of *Daphnia magna* and *Daphnia similis* in control and Pb continuous exposure through 10 generations. Generations are marked with a 1) black circle for those statistical different from F0 in the control treatment, 2) a black square for those statistical different from F0 in Pb treatment and 3) a gray diamond when both treatments presented difference in comparison to F0 (Bonferroni, $p<0.05$). Numbers (1,3

and 5) indicate statistical difference between treatments from each brood, being 1 for N1, 3 for N3 and 5 for N5 (Bonferroni, $p<0.05$).

3.3.1. Recovery period

Looking at the recovery period, a similar pattern for neonates' size occurred, with N1 being smaller than N5. *D. magna* (usual food) recovering N1 did not differ from any other treatment (control and Pb) at all generations evaluated, however, neonates were statistically smaller than F0 in generations F6 and F9. Neonates from brood N3 in F9 reduced drastically their size from F6 to F9, being smaller than control, continuous Pb exposure and F0 neonates. However, broods N5 presented an enhanced size compared to control in generation F9 and compared to F0 in generations F6 and F9.

When food was restricted, *D. magna* from recovery period (brood N1) showed size enlargement, being bigger than control and continuous Pb exposure at both F6 and F9. These neonates increase their size from F6 to F9, being bigger than F0 in generation F9. The same trend that happened under usual food also happened for food restriction, and a drastic size decrease from F6 to F9 of neonates from brood N3 was observed. In this case, recovering neonates (N3) were bigger than Pb exposed and generation F0 on generation F6. However, such neonates were smaller than control and Pb treatments on generation F9. Neonates N5 under recovery in generation F9 had a similar size than those in control and smaller than those from continuous Pb exposure, continuing to be bigger than F0 (F6 and F9).

Neonates from *D. similis* under usual food presented no difference among treatments for any generation in N1 broods, however, with a smaller size when compared to F0. Neonates from broods N3 showed no statistical difference whatsoever, nor among treatments, nor among generations. Broods from N5 under recovery appeared to be the largest individuals, differing from continued Pb exposure ones at F6 and both treatments (control and Pb) in F9, being bigger than F0 at both generations (F6 and F9).

When food was scarce, *D. similis* N1 recovering neonates were bigger than continuous Pb exposure ones in generation F6. Regarding generations, brood N1 from generation F9 presented enhanced size when compared to F0. N3 recovering neonates showed smaller sizes concerning both treatments (control and Pb) in generation F6 and a larger dimension in F9 when compared to F0. A size increase trend can be seen from F6 to F9 for such organisms. For recovering individuals from brood N5, no statistical difference to any other treatment was shown during generation F6, however, recovering organisms were smaller than control in generation F9. These same organisms also differed from F0, being bigger than F0 neonates.

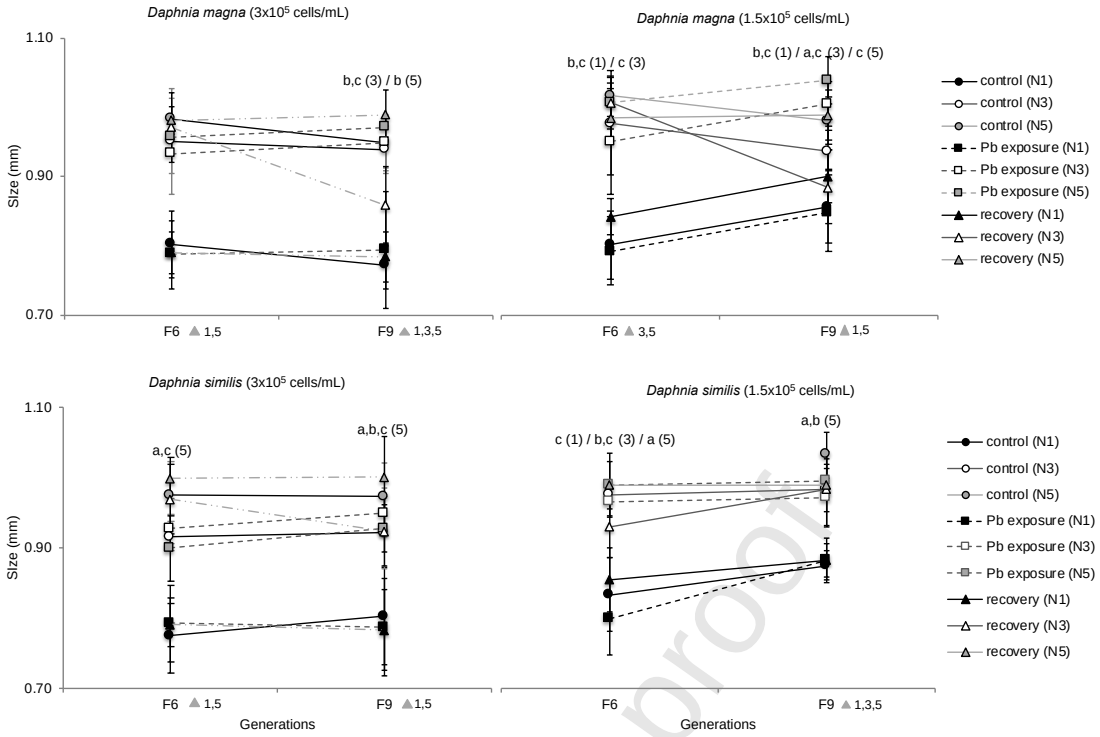


Figure 9: Body length of recovering neonates from broods N1 (dark), N3 (white) and N5 (grey) of *Daphnia magna* and *Daphnia similis* in control and Pb continuous exposure through 10 generations. Generations marked with a gray triangle indicate statistical difference for recovering organisms in comparison to F0 (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control, (b) for recovery vs. control and (c) for Pb vs. recovery (Bonferroni, $p<0.05$). Number (1, 3 and 5) indicates which brood showed statistical difference, being 1 for N1, 3 for N3 and 5 for N5.

4. Discussion

During the multi-generational exposure to Pb, no significant effect on daphnids sensitivity to $K_2Cr_2O_7$ was observed, except for *D. similis* under recovery and food restriction. Both species diminished significantly their sensitivity to Pb, from F0 to F9 in both food regimes. Neonates' size was increased during food restriction. However, food restriction triggered different outcomes for both species that may highlight potential epigenetic changes during recovery period for *D. magna* and diminished sensitivity to mancozeb when pre-exposed to Pb, contrarily to *D. similis*.

Control variability over generations

D. magna control under a usual food regime ($3x10^5$ cell/mL) presents a trend of diminished sensitivity to $K_2Cr_2O_7$ exposure from F0 to F9. This sensitivity fluctuation may be considered a natural fluctuation occurring in organisms derived from laboratory cultures (and within the range proposed by OECD (2004) as previously discussed above). Other studies also indicate natural fluctuations on *D. magna* sensitivity to chemicals (Silva et al., 2017; Stoddard & Harper, 2007). *D.*

similis shows a similar lower sensitivity pattern for both food regimes, however, it was more pronounced under food restriction. Heugens (Heugens et al., 2001) stated that neonates at high food concentration can be more sensitive than those from restricted nutrition. Daphnids from poor fed mothers produce fewer but larger neonates, which may be acutely less sensitive to chemicals (Pieters and Liess, 2006). Neonates size measured in this study validate these results. Neonates' size measured from restricted food daphnids are bigger than under usual food and a trend of increase size for control organisms is seen also at generations F6 and F9.

The control under usual food regime *D. magna* showed no difference towards F0, making the lower Pb sensitivity developed by the continuous Pb exposure even more explicit. Control organisms with restricted nutrients present a sensitivity variation, indicating lower sensitivity of generation F9 in comparison to F0, a pattern also shown by *D. similis* (for both food regimes). These data indicate that food restriction can alter daphnids sensitivity. Under food restriction, both cladocerans species diminished Pb sensitivity, which is probably linked with the trade-off that can occur under low-food supply, leading to a smaller quantity but higher quality neonates born from poor-fed mothers (Enserink et al., 1995). Sustaining this data, *D. similis* control neonates from generation F3 showed no mortality under the highest Pb concentration tested as discussed in the results.

In general, both species under both food regimes kept under control condition throughout generations showed lower sensitivity when tested for mancozeb toxicity at generation F6. However, such low sensitivity changed (enhanced) in generation F9, being probably a natural sensitivity fluctuation. Such results corroborate with neonates' size, in which control organisms generally presented increased size on brood N5 through generations and may explain the lower sensitivity pattern shown. Pavlaki et al. (2014) data also corroborate with these findings, with enhanced somatic growth shown by *D. magna* under poor-food media. *D. similis* under food restriction were not evaluated at F6 due to reproduction impairment and presented a lower mancozeb sensitivity at generation F9, corroborating studies that suggests that poor-nutritional females give birth to less but larger (and low sensitive) neonates (Enserink et al., 1995). Other studies confirm low or no reproduction of daphnids under food restriction (Enserink et al., 1995; Pavlaki et al., 2014; Pereira & Gonçalves, 2008; Ward, & Robinson, 2005).

Multi-generation Pb exposure

A lower sensitivity to Pb is shown for both daphnids species through the multi-generation test (higher LC_{50} for Pb). Neonates size measurement corroborates this hypothesis, with brood N5 showing an increased neonate size (differing from F0) in both species and food regimes (excluding *D. similis* usual food). *D. magna* has shown acclimation (LC_{50} of 40 to 149 μg/L) under chronic Hg (3.8 μg/L) exposure for two generations (Tsui, M. T. K. and Wang, 2005). Dietrich et al. (2010) indicated a lower sensitivity development over six generations of *D. magna* exposed to four pharmaceuticals

(carbamazepine (CBZ), diclofenac (DIC), 17a-ethinylestradiol (EE2) and metoprolol (MET)). Other authors also found diminished sensitivity to metal, such as the physiological acclimation exhibited by *D. magna* pre-exposed to Cu (0.01 mg/L) for 20 days (LeBlanc, 1982) and increased LC_{50} (0.19 to 0.3 mg/L of Cu^{2+}) shown on five generations acclimation (Bossuyt et al., 2005). *D. longispina* under a historically contaminated habitat exhibited a LC_{50} variation of 0.095 mg/L on reference sites to 0.36 mg/L on Cu impacted areas (Agra et al., 2011). *D. magna* LC_{50} range varied from 0.26 to 0.49 mg/L under Cd exposure to two generations (Bodar et al., 1990). As stated before, this change in sensitivity could be acquired through physiological acclimation or epigenetic changes.

Contrarily to the lower Pb sensitivity acquired by organisms under continuous Pb exposure, no sensitivity variation to $K_2Cr_2O_7$ of organisms pre-exposed to Pb was shown. *D. magna* and *D. similis* showed differences between generations exhibiting a diminished sensitivity to $K_2Cr_2O_7$, however, this sensitivity occurred for all treatments in a similar order of magnitude (including the control group) meaning that all the organisms were less sensitive and that neither the Pb pre-exposure nor the dietary regime had affected this endpoint, thus such change was probably a natural fluctuation. Absence of sensitivity variation to xenobiotics was also demonstrated for *D. magna* pre-exposed to 0.01 mg/L of Cu when sequentially exposed to Pb (LC_{50} = 0.12 to the pre-exposed and 0.15 mg/L to the non-exposed animals) or Zn (LC_{50} = 0.20 to pre-exposed and 0.24 mg/L to the non-exposed) (LeBlanc, 1982).

Considering that chemicals are not present alone in the ecosystem, and non-static environmental conditions are also present, pulse exposures often occur in the environment and therefore the fungicide mancozeb exposure was tested on Pb pre-exposed daphnids as a potential example. The acute exposure (48h) to mancozeb (on Pb pre-exposed organisms) shows that *D. magna* under continuous Pb exposure revealed no difference among treatments under usual food regime. Studies support our results, showing weak antagonist or no effect on organisms pre-exposed to other chemicals at low concentrations such as the lack of sensitivity variance (shown before in this study) of *D. magna* acclimated to Cu when exposed to Pb or Zn (LeBlanc, 1982) and; the lack of Zn uptake disparities found on *D. magna* pre-exposed to Cd (<0.06 mg/L) (Guan, R & Wang, 2004). *D. magna* under food restriction showed different outcomes, with organisms pre-exposed to Pb decreasing sensitivity to mancozeb exposure along generations. There are studies showing lower sensitivity to a chemical after a long-exposure to another, such as an induced Cd lower sensitivity was acquired after Zn pre-exposure on *D. magna* (Barata et al., 2002). This lower sensitivity of continuous Pb exposure neonates is corroborated by the size shown for such organisms, which increased (for broods N3 and N5) among generations, reaching a larger size at generation F9. Regarding usual food regime, similar results shown by *D. magna* are also shown by *D. similis*, which indicated no significant difference between treatments. This outcome, as it happened for *D. magna*, also varied under food restriction. In an opposite way, organisms under continuous Pb exposure were statistically more sensitive than control. The toxicity of combinations concerning metals and pesticides has already been shown and

studies corroborates with our results of metal pre-exposure increasing the fungicide's toxicity. Increased toxicity was reported to acute mixtures of Cd and the fungicide carbendazim to *D. magna* (Ferreira et al., 2008), acquiring higher toxicity when Cd is dominant; also in mixtures of Cd and an organophosphorous insecticide (diazinon) to the mayfly (*Ephoron virgo*) (Van Der Geest et al., 2000) and in mixtures of metals (As, Cd and Cu) and organophosphorous and carbamate insecticides (diclorvos, dalathion and carbofuran) to the microcrustacean *Tigriopus brevicornis* (Forget et al., 1999).

The enhanced sensitivity could be due to a lack of energy (food restriction), which may reduce the ability to detoxify (Heugens et al., 2001). Another hypothesis that can be raised is the possible full consumption of the food provided in a restricted regime, resulting in a higher ingestion rate of Pb which can be fully absorbed on the algae surface (Heugens et al., 2006). Both stressors (Pb exposure and food restriction) may also jointly impair organisms' detoxification physiology, making organisms unable to cope with another stressor (mancozeb exposure). All these described hypotheses can also be occurring combined. If pre-exposure to a certain contaminant alters (enhances or diminishes) the toxicity of other substance it can represent a problem in natural environments contaminated by multiple contaminants (Ward & Robinson, 2005) or under pulse exposure to contaminated habitats (Barata et al., 2004). We highlight that the results showed above indicate, again, opposite responses regarding *D. magna* and *D. similis*.

Daphnids recovery after chemical exposure

Organisms from recovery period are offspring from Pb exposed mothers that were moved to clean water for further three generations. Under the usual food regime, both *Daphnia* species did not vary from control outcomes regarding Pb sensitivity, although, *D. similis* from F9 presents a lower sensitivity to Pb compared to F0. Such outcomes vary when organisms are submitted to restricted nutrients and, *D. magna* demonstrates a Pb sensitivity higher than control and Pb treatments. Since these neonates were not exposed to Pb and their lower sensitivity was derived from their progenitors under former Pb exposure, this result may indicate a probable epigenetic change. *D. similis*, however, presented a different outcome in which organisms under recuperation did not differ from control, indicating that their progenitors were physiologically acclimated to Pb and when such exposure ended the neonates' sensitivity returned to non-exposed daphnids sensitive levels. If the acclimated population is as fit as the control in the absence of Pb, its existence is as likely as the control population in a non-contaminated environment. However, the acclimated population may be more fit than the control during episodes of Pb exposure (Ward & Robinson, 2005). Consequently, the data acquired by this study shows that this is an essential information to risk assessment studies as the exposed organisms can remain less sensitive to specific contaminants (Lopes et al., 2006). Vandegehuchte et al. (2009a) indicated DNA methylation in *D. magna*, suggesting that potentially

epigenetic effects may occur in this species. Moreover, Vandegehuchte et al. (2009b) indicated that different generational levels of Zn exposure can entail different levels of DNA methylation (epigenetic phenomena). Physiological acclimation is also shown by other authors such as the re-established sensitivity on only one generation under recovery of *D. magna* exposed for 12 generations to 0.03 mg/L of Cu (LeBlanc, 1982) and the re-established sensitivity of *D. magna* under 14 generations of Cu exposure (0.0005 to 0.1 mg/L) when Cu exposure no longer exists (Bossuyt, B. T. A. and Janssen, 2004).

Similar outcomes exhibited for Pb sensitivity was also shown for $K_2Cr_2O_7$ concerning usual food regime. Recovering organisms (both species) presented similar sensitivity as control individuals. This outcome does not change for recovering *D. magna* under food restriction, which presents similar outcome as control and Pb treatments. However, *D. similis* from recovery period under food restriction presented diminished sensitivity to $K_2Cr_2O_7$ (at generation F6) than both other treatments (control and Pb). Nonetheless, such sensitivity enhances from F6 to F9, exhibiting the lowest LC_{50} of generation F9. The diminished recovering organisms sensitivity could be probably because during food impairment organisms increase the offspring's energy input and produce less but larger neonates, which can be less sensitive to chemicals (Enserink et al., 1990; Gliwicz & Guisande, 1992; Pieters and Liess, 2006). However, if that was the case, the control treatment with food restriction would also been less sensitive. Since neither the control or Pb treatments under restricted food conditions had diminished their respective sensitivities, the lower sensitivity acquired by recovering organisms was probably due to the synthesis of metallothioneins during former Pb exposure (Ferreira et al., 2008) and therefore, organisms pre-exposed to Pb were able to better cope to a late $K_2Cr_2O_7$ exposure. The synthesis of metallothioneins costs energy, thus bigger neonates with higher energy reserves can better manage these production than smaller offspring (Enserink et al., 1990). Together with the metallothioneins, Pb pre-exposure under food restriction could have diminished the sensitivity of *D. similis* due to molecular, biochemical or physiological mechanisms (together with the enhanced neonates size due to food restriction), such as enhanced anti-oxidant systems to inhibit reactive oxygen species (ROS) production, since ROS are known to be enhanced by aquatic pollution (Maria et al., 2009). The different responses of *D. magna* and *D. similis* regarding sensitivity under food restriction is a critical outcome specially concerning natural habitats where food impairment may be a common condition, as in most freshwater lakes in northern Europe, North America and Canada (Brown and Yan, 2015). Other studies have shown different results relating close Cladocera species such as a lower sensitivity of *D. magna* to AgNP in comparison to *D. pulex* and *D. galeata* (Völker et al., 2013), and the lower *D. magna* sensitivity to the organic chemical 3,4-dichloroaniline (DCA) in comparison to *Ceriodaphnia quadrangula* (Klüttgen et al., 1996).

D. magna under usual food regime are the only organisms that show statistical sensitivity difference among generations regarding mancozeb exposure, with recovering organisms from generations F6 and F9 being less sensitive than generation F0. These organisms show a trend of lower

mancozeb sensitivity, indicating that neonates from mothers formerly exposed to Pb could be diminishing their sensitivity to mancozeb. Literature shows that daphnids adapted to specific chemicals can be less sensitive to other compounds, such as the cadmium-adapted (0.061 mg/L) *D. magna* population that was less sensitive to Pb (although more sensitive to phenol) (Ward & Robinson, 2005), and the *D. magna* under physiological acclimatization to Zn (<0.4 mg/L) diminished sensitivity to Cd (Barata et al., 2002). Here again, being formerly exposed to Pb, *D. magna* probably enhanced its metallothioneins production (Ferreira et al., 2008), which diminished the sensitivity to mancozeb, since it is an organometallic fungicide (constituted by manganese (Mn) and zinc (Zn)), therefore, this type of fungicide can also increase metallothioneins production (Mosleh et al., 2005). When food is limited, *D. magna* respond in a different way, being similar to control and more sensitive (to mancozeb) than Pb treatment. However, *D. magna* under food restriction and *D. similis* at both food regimes exhibits no LC_{50} variation from F6 to F9. And, after three generations in clean media (F9) no differences regarding control and recovery period for such organisms are presented. Full recovery of organisms and similar to control is also shown in other studies (cited before) (Bossuyt, B. T. A. and Janssen, 2004; LeBlanc, 1982).

Daphnia magna vs. Daphnia similis

Regarding the ecological relevance of choosing appropriate species for ecotoxicological studies regarding multiple chemicals and long-term exposure, some suggestions can be made: 1) A standard protocol for multi-generation tests is in order; 2) Both species became less sensitive to Pb under a long-term exposure, being indicative of a development of acclimated populations; 3) Despite being less sensitive to Pb, the acclimation to a chemical may lead to enhanced sensitivity to others, therefore, the dissemination of multiple chemical exposure tests is also crucial; 4) The recovery period is crucial to evaluate the type of chemical acclimation and the future of exposed populations, keeping in mind that monophyletic species may differ; 5) Food quantity has an important role regarding organisms sensitivity and may differently influence on the observed responses to chemicals, even considering phylogenetically close species and; 6) Some adaptations regarding native and more reliable species in standard tests should be carried, together with a range of species instead of only one to better comprehend risk assessment managements. Further, epigenetic modifications (phenotypic characters transgenerational transferred) on *Daphnia* should be done to elucidate if results shown were inherited from exposed mothers.

Acknowledgment

The study was supported by project RePulse— Responses of *Daphnia magna* Exposed to Chemical Pulses and Mixtures Throughout Generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010) and through CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), from FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Giuliana Araujo received a Ph.D. grants from the Brazilian National

Council for Scientific and Technological Development (CNPq, 201788/2014-4) and support from the PhD program Biology and Ecology of Global Change from the Department of Biology, University of Aveiro, Portugal. The authors are grateful to Abel Ferreira for all the laboratorial support. The authors declare that they have no conflict of interest.

Bibliography

Abrahim, G., Parker, R., 2002. Heavy-metal contaminants in Tamaki Estuary: impact of city development and growth, Auckland, New Zealand. Environ. Geol. 42, 883–890. https://doi.org/10.1007/s00254-002-0593-0

Adema, D.M.M., 1978. Daphnia magna as a test animal in acute and chronic toxicity tests. Hidrobiologia 59, 125–134.

Agra, A.R., Soares, A.M.V.M., Barata, C., 2011. Life-history consequences of adaptation to pollution. "Daphnia longispina clones historically exposed to copper." Ecotoxicology 20, 552–562. https://doi.org/10.1007/s10646-011-0621-5

Araujo, G.S., Pavlaki, M.D., Soares, A.M.V.M., Abessa, D.M.S., Loureiro, S., 2019. Bioaccumulation and morphological traits in a multi-generation test with two Daphnia species exposed to lead. Chemosphere 219, 636–644. https://doi.org/10.1016/j.chemosphere.2018.12.049

ASTM, 2002. Methods for Acute Toxicity Tests With Fish, Macroinvertebrates and Amphibians. Ecol. Res. Ser. EPA-600/3-75-009 96, 61. https://doi.org/10.1520/E0729-96R14.2

Axelstad, M., Boberg, J., Nellemann, C., Kiersgaard, M., Jacobsen, P.R., Christiansen, S., Hougaard, K.S., Hass, U., 2011. Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams but no behavioral effects in the offspring. Toxicol. Sci. 120, 439–446. https://doi.org/10.1093/toxsci/kfr006

Baird, D.J., Barber, I., Bradley, M., Calow, P., Soares, A.M.V.M., 1989. The Daphnia bioassay : a critique. Hydrobiology 403–406.

Barata, C., Markich, S.J., Baird, D.J., Taylor, G., Soares, A.M.V.M., 2002. Genetic variability in sublethal tolerance to mixtures of cadmium and zinc in clones of Daphnia magna Straus. Aquat. Toxicol. 60, 85–99. https://doi.org/10.1016/S0166-445X(01)00275-2

Barata, C., Solayan, A., Porte, C., 2004. Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to Daphnia magna. Aquat. Toxicol. 66, 125–139. https://doi.org/10.1016/j.aquatox.2003.07.004

Berger, S.L., Kouzarides, T., Shiekhattar, R., Shilatifard, A., 2009. An operational definition of epigenetics. Genes Dev. 23, 781–783. https://doi.org/10.1101/gad.1787609.Copyright

Bodar, C.W.M., Sluis, I. V, Montfort, J.C.P., Voogt, P.A., Zandee, D.I., 1990. Cadmium resistance in Daphnia magna. Aquat. Toxicol. 16, 33–40.

Bossuyt, B. T. A. and Janssen, C.R., 2004. Influence of multigeneration acclimation to copper on tolerance, energy reserves, and homeostasis of Daphnia magna straus. Environ. Toxicol. Chem. 23, 2029–2037. https://doi.org/10.1897/03-377

Bossuyt, B.T.A., Escobar, Y.R., Janssen, C.R., 2005. Multigeneration acclimation of Daphnia magna Straus to different bioavailable copper concentrations. Ecotoxicol. Environ. Saf. 61, 327–336. https://doi.org/10.1016/j.ecoenv.2005.03.004

Brasil, 2005. RESOLUÇÃO CONAMA N^{o} 357, DE 17 DE MARÇO DE 2005.

Brausch, J.M., Salice, C.J., 2011. Effects of an environmentally realistic pesticide mixture on Daphnia magna exposed for two generations. Arch. Environ. Contam. Toxicol. 61, 272–279. https://doi.org/10.1007/s00244-010-9617-z

Brown, A.H., Yan, N.D., 2015. Food quantity affects the sensitivity of Daphnia to road salt. Environ. Sci. Technol. 49, 4673–4680. https://doi.org/10.1021/es5061534

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F.I., Palozza,

P., 2006. DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: Involvement of the oxidative mechanism. Toxicol. Appl. Pharmacol. 211, 87–96. https://doi.org/10.1016/j.taap.2005.06.001

Castillo, L.E., Martínez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., Solis, E., 2006. Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Environ. 367, 418–432. https://doi.org/10.1016/j.scitotenv.2006.02.052

Chen, F., Yao, Q., Zhou, X., 2015. The influence of suspended solids on the combined toxicity of galaxolide and lead to Daphnia magna. Bull. Environ. Contam. Toxicol. 95, 73–79. https://doi.org/10.1007/s00128-015-1543-3

Chen, Y., Huang, J., Xing, L., Liu, H., Giesy, J.P., Yu, H., Zhang, X., 2014. Effects of multigenerational exposures of D. magna to environmentally relevant concentrations of pentachlorophenol. Environ. Sci. Pollut. Res. 21, 234–243. https://doi.org/10.1007/s11356-013-1692-z

Dietrich, S., Ploessl, F., Bracher, F., Laforsch, C., 2010. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in Daphnia magna - A multigenerational study. Chemosphere 79, 60–66. https://doi.org/10.1016/j.chemosphere.2009.12.069

Enserink, E.L., Kerkhofs, M.J.J., Baltus, C.A.M., Koeman, J.H., 1995. Influence of food quantity and lead exposure on maturation in Daphnia magna; Evidence for a trade-off mechanism. Funct. Ecol. 9, 175–185. https://doi.org/10.2307/2390562

Enserink, L., Luttmer, W., Maas-diepeveen, H., 1990. Reproductive strategy of Daphnia magna affects the sensitivity of its progeny in acute toxicity tests. Aquat. Toxicol. 17, 15–26. https://doi.org/10.1016/0166-445X(90)90009-E

Environmental Protection Agency, U., 2005. Reregistration Eligibility Decision for Chlorpyrifos. United States Environ. Prot. Agency 259. https://doi.org/EPA738-R-98-010

European Commission, 2009. Review report for the active substance mancozeb. Heal. Consum. Prot. Dir. 1–63.

European Parliment, 2008. DIRECTIVE 2008/105/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC,. Off. J. Eur. Union 84–97. https://doi.org/http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32008L0105

Fernández-González, M.A., González-Barrientos, J., Carter, M.J., Ramos-Jiliberto, R., 2011. Parent-to-offspring transfer of sublethal effects of copper exposure: Metabolic rate and life-history traits of Daphnia. Rev. Chil. Hist. Nat. 84, 195–201. https://doi.org/10.4067/S0716-078X2011000200005

Ferreira, A.L.G., Loureiro, S., Soares, A.M.V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on Daphnia magna. Aquat. Toxicol. 89, 28–39. https://doi.org/10.1016/j.aquatox.2008.05.012

Flohr, L., Castilhos Júnior, A.B. De, Matias, W.G., 2012. Acute and Chronic Toxicity of Soluble Fractions of Industrial Solid Wastes on Daphnia magna and Vibrio fischeri. Sci. World J. 2012, 1–10. https://doi.org/10.1100/2012/643904

Forget, J., Pavillon, J.F., Beliaeff, B., Bocquene, G., Bocquené, G., 1999. Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of Tigriopus brevicornis (Copepoda, Harpacticoida. Environ. Toxicol. Chem. 18, 912–918. https://doi.org/10.1897/1551-5028(1999)018<0912:jaopcp>2.3.co;2

Frost, P.C., Ebert, D., Larson, J.H., Marcus, M.A., Wagner, N.D., Zalewski, A., 2010. Transgenerational effects of poor elemental food quality on Daphnia magna. Oecologia 162, 865–872. https://doi.org/10.1007/s00442-009-1517-4

Ghilarov, A.A.M., 1967. The zooplankton of arctic rock pools. Zooplankt. Arct. Rock Pools 18, 82–95.

Gliwicz, Z M & Guisande, C., 1992. Family planning in Daphnia: resistance to starvation in offspring born to mothers grown at different food levels. Oecologia 91, 463–467.

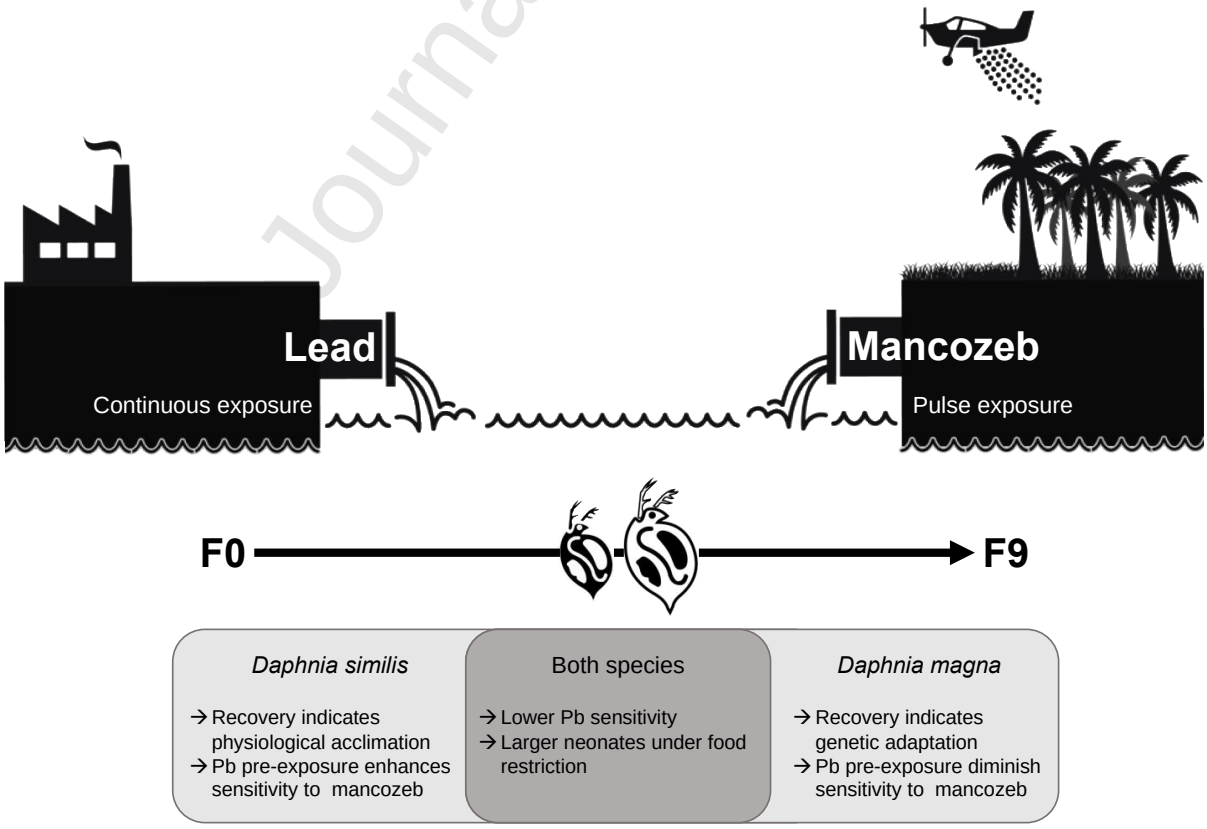
Guan, R & Wang, W., 2004. Cd and Zn Uptake Kinetics in Daphnia magna in Relation to Cd

Exposure History. Environ. Sci. Technol. 38, 6051–6058.
Hammers-Wirtz, M. and Ratte, H.T., 2000. Offspring Fitness in Daphnia: Is the Daphnia Reproduction Test Appropriate for Extrapolating Effects on the Population Level? Environ. Toxicol. Chem. 19, 1856. https://doi.org/10.1897/1551-5028(2000)019<1856:OFIDIT>2.3.CO;2
Hammond, J.I., Jones, D.K., Stephens, P.R., Relyea, R.A., 2012. Phylogeny meets ecotoxicology : evolutionary patterns of sensitivity to a common insecticide 5, 593–606. https://doi.org/10.1111/j.1752-4571.2011.00237.x
Hernández-Flores, S. and Rico-Martínez, R., 2006. Study of the effects of Pb and Hg toxicity using a chronic toxicity reproductive 5-day test with the freshwater rotiferLecane quadridentata. Environ. Toxicol. 21, 533–540. https://doi.org/10.1002/tox.20218
Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2001. A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment. Crit. Rev. Toxicol. 31, 247–284. https://doi.org/http://dx.doi.org/10.1080/20014091111695
Heugens, E.H.W., Tokkie, L.T.B., Kraak, M.H.S., Hendriks, A.J., Van Straalen, N.M., Admiraal, W., 2006. Population growth of Daphnia magna under multiple stress conditions: joint effects of temperature, food, and cadmium. Environ. Toxicol. Chem. 25, 1399–1407. https://doi.org/10.1897/05-294R.1
Jacobasch, C., Völker, C., Giebner, S., Völker, J., Alsenz, H., Potouridis, T., Heidenreich, H., Kayser, G., Oehlmann, J., Oetken, M., 2014. Long-term effects of nanoscaled titanium dioxide on the cladoceran Daphnia magna over six generations. Environ. Pollut. 186, 180–186. https://doi.org/10.1016/j.envpol.2013.12.008
Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B., Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. Interdiscip. Toxicol. 7, 60–72. https://doi.org/10.2478/intox-2014-0009
Jartun, M., Ottesen, R.T., Steinnes, E., Volden, T., 2008. Runoff of particle bound pollutants from urban impervious surfaces studied by analysis of sediments from stormwater traps. Sci. Total Environ. 396, 147–63. https://doi.org/10.1016/j.scitotenv.2008.02.002
Klüttgen, B., Kuntz, N., Ratte, H.T., 1996. Combined effects of 3,4-Dichloroaniune and food concentration on life-table data of two related cladocerans, Daphnia magna and Ceriodaphnia quadrangula. Chemosphere 32, 2015–2028.
Krylov, V. V. and Osipova, E.A., 2013. The response of Daphnia magna Straus to the long-term action of low-frequency magnetic fields. Ecotoxicol. Environ. Saf. 96, 213–219. https://doi.org/10.1016/j.ecoenv.2013.06.012
Kubrak, O.I., Atamaniuk, T.M., Husak, V. V., Drohomyretska, I.Z., Storey, J.M., Storey, K.B., Lushchak, V.I., 2012. Oxidative stress responses in blood and gills of Carassius auratus exposed to the mancozeb-containing carbamate fungicide Tattoo. Ecotoxicol. Environ. Saf. 85, 37–43. https://doi.org/10.1016/j.ecoenv.2012.08.021
LeBlanc, G., 1982. Laboratory investigation into the development of resistance of Daphnia magna(strauus) to environmental pollutants. Environ. Pollut. Ser. A, Ecol. … 27, 309–322. https://doi.org/10.1016/0143-1471(82)90159-3
Lopes, I., Baird, D.J., Ribeiro, R., 2006. Genetic adaptation to metal stress by natural populations of Daphnia longispina 63, 275–285. https://doi.org/10.1016/j.ecoenv.2004.12.015
Lyu, K., Wang, Q., Chen, R., Lu, Q., Yang, Z., 2013. Inter-specific differences in survival and reproduction of cladocerans to nitrite gradient and the ecological implications. Biochem. Syst. Ecol. 48, 151–156. https://doi.org/10.1016/j.bse.2012.12.002
Magalhães, D.P., Marques, M.R. da C., Baptista, D.F., Buss, D.F., 2014. Selecting a sensitive battery of bioassays to detect toxic effects of metals in effluents. Ecotoxicol. Environ. Saf. 110, 73–81. https://doi.org/10.1016/j.ecoenv.2014.08.019
Manusadžianas, L., Balkelyte, L., Sadauskas, K., Blinova, I., Põllumaa, L., Kahru, A., 2003. Ecotoxicological study of Lithuanian and Estonian wastewaters: Selection of the biotests, and correspondence between toxicity and chemical-based indices. Aquat. Toxicol. 63, 27–41. https://doi.org/10.1016/S0166-445X(02)00132-7
Maria, V.L., Santos, M. a, Bebianno, M.J., 2009. Contaminant effects in shore crabs (Carcinus maenas) from Ria Formosa Lagoon. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 150,

196–208. https://doi.org/10.1016/j.cbpc.2009.04.013
Moliner, E.A., 1992. Journal of Environmental Science and Health , Part B : Pesticides , Food Contaminants , and Agricultural Wastes Relative sensitivity of daphnia magna and brachionus calyciflorus to five pesticides 511–522.
Morgado, R.G., Gomes, P.A.D., Ferreira, N.G.C., Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod Porcellionides pruinosus. Chemosphere 144, 1845–1853. https://doi.org/10.1016/j.chemosphere.2015.10.034
Mosleh, Y.Y., Paris-Palacions, S., Couderchet, M., Biagianti-risbourg, S., Vernet, G., 2005. Metallothionein induction, antioxidative responses, glycogen and growth changes in Tubifex tubifex (Oligochaete) exposed to the fungicide, fenhexamid. Environ. Pollut. 135, 73–82. https://doi.org/10.1021/es203157f
Muyssen, B. T. A. and Janssen, C.R., 2004. Multi-generation cadmium acclimation and tolerance in Daphnia magna Straus. Environ. Pollut. 130, 309–316. https://doi.org/10.1016/j.envpol.2004.01.003
OECD, 2012. Guideline 211: Daphnia magna reproduction test. OECD Guidel. Test. Chem. Section 2, 23 . https://doi.org/10.1787/9789264070127-en
OECD, 2004. Guideline 202: Daphnia sp., Acute Immobilisation Test. OECD Guidel. Test. Chem. 1–12.
Pavlaki, M.D., Ferreira, A.L.G., Soares, A.M.V.M., Loureiro, S., 2014. Changes of chemical chronic toxicity to Daphnia magna under different food regimes. Ecotoxicol. Environ. Saf. 109, 48–55. https://doi.org/10.1016/j.ecoenv.2014.07.039
Pereira, J L & Gonçalves, F., 2008. Daphnia fitness over a food gradient: is body size the single trait predicting exploitative ability ? Ann. Limnol. - Int. J. Lim 44, 169–179.
Pestana, J.L.T., Novais, S.C., Norouzitallab, P., Vandegehuchte, M.B., Bossier, P., De Schamphelaere, K.A.C., 2016. Non-lethal heat shock increases tolerance to metal exposure in brine shrimp. Environ. Res. 151, 663–670. https://doi.org/10.1016/j.envres.2016.08.037
Pieters, B.J., Liess, M., 2006. Maternal nutritional state determines the sensitivity of Daphnia magna offspring to short-term Fenvalerate exposure. Aquat. Toxicol. 76, 268–277. https://doi.org/10.1016/j.aquatox.2005.09.013
Ramírez, D.C.S., 2014. Tolerance of a metal adapted natural Daphnia magna population to new stressors. Master´s thesis Univ. Gent, Belgium.
Reddy, G.R., Basha, M.R., Devi, C.B., Suresh, A., Baker, J.L., Shafeek, A., Heinz, J., Chetty, C.S., 2003. Lead induced effects on acetylcholinesterase activity in cerebellum and hippocampus of developing rat. Int. J. Dev. Neurosci. 21, 347–352. https://doi.org/10.1016/S0736-5748(03)00071-6
Regaldo, L., Reno, U., Gervasio, S., Troiani, H., Gagneten, A.M., 2013. Effects of metals on Daphnia magna and cladocerans representatives of the Argentinean Fluvial Littoral. J. Environ. Biol. 35, 689–697.
Sabero Europe, B.V., 2017. Registration Report, Part A: Risk Management.
Schultz, C.L., Wamucho, A., Tsyusko, O.V., Unrine, J.M., Crossley, A., Svendsen, C., Spurgeon, D.J., 2016. Multigenerational exposure to silver ions and silver nanoparticles reveals heightened sensitivity and epigenetic memory in Caenorhabditis elegans. Proc. R. Soc. B Biol. Sci. 283, 20152911. https://doi.org/10.1098/rspb.2015.2911
Silva, A.R.., Cardoso, D.N., Cruz, A., Pestana, J.L.T., Mendo, S., Soares, A.M.V.M., Loureiro, S., 2017. Multigenerational effects of carbendazim in Daphnia magna. Environ. Toxicol. Chem. 36, 383–394. https://doi.org/10.1002/etc.3541
Stoccoro, A., Karlsson, H.L., Coppedè, F., Migliore, L., 2013. Epigenetic effects of nano-sized materials. Toxicology 313, 3–14. https://doi.org/10.1016/j.tox.2012.12.002
Stoddard, J L & Harper, R., 2007. Effects of multi-generational exposure of Daphnia magna to copper. Inst. Environ. Toxicol. Chem. 1–26.
Stokes, P.M., Bailey, R.C., Groulx, G.R., 1985. Effects of Acidification on Metal Availability to Aquatic Biota , with Special Reference to Filamentous Algae. Environ. Health Perspect. 63, 79–87.
Tanaka, Y. and Nakanishi, J., 2002. Chronic effects of p-nonylphenol on survival and reproduction of

Daphnia galeata: Multigenerational life table experiment. Environ. Toxicol. 17, 487–492. https://doi.org/10.1002/tox.10083
Terra, N. R. and Gonçalves, S.P., 2013. Daphnia magna Straus, 1820 response to sediment samples from a contaminated river (Rio Grande do Sul, Brazil). Acta Limnol. Bras. 25, 19–33.
Tsang, M. M. and Trombetta, L.D., 2007. The protective role of chelators and antioxidants on mancozeb-induced toxicity in rat hippocampal astrocytes. Toxicol. Ind. Health 23, 459–470. https://doi.org/10.1177/0748233708089039
Tsui, M. T. K. and Wang, W., 2005. Multigenerational acclimation of Daphnia magna to mercury: relationships between biokinetics and toxicity. Environ. Toxicol. Chem. 24, 2927–2933. https://doi.org/10.1897/05-085r.1
Van Der Geest, H.G., Greve, G.D., Boivin, M.E., Kraak, M.H.S., Van Gestel, C.A.M., 2000. Mixture toxicity of copper and diazinon to larvae of the mayfly (Ephoron virgo) judging additivity at different effect levels. Environ. Toxicol. Chem. 19, 2900–2905. https://doi.org/10.1897/1551-5028(2000)019<2900:MTOCAD>2.0.CO;2
Vandegehuchte, M.B., Kyndt, T., Vanholme, B., Haegeman, A., Gheysen, G., Janssen, C.R., 2009a. Occurrence of DNA methylation in Daphnia magna and influence of multigeneration Cd exposure. Environ. Int. 35, 700–706. https://doi.org/10.1016/j.envint.2009.01.002
Vandegehuchte, M.B., Lemière, F., Janssen, C.R., 2009b. Quantitative DNA-methylation in Daphnia magna and effects of multigeneration Zn exposure. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 150, 343–348. https://doi.org/10.1016/j.cbpc.2009.05.014
Völker, C., Boedicker, C., Daubenthaler, J., Oetken, M., Oehlmann, J., 2013. Comparative Toxicity Assessment of Nanosilver on Three Daphnia Species in Acute, Chronic and Multi-Generation Experiments. PLoS One 8. https://doi.org/10.1371/journal.pone.0075026
Ward, T J & Robinson, W.E., 2005. Evolution of cadmium resistance in Daphnia magna. Environ. Toxicol. Chem. 24, 2341–2349.
WHO, 2011. Lead in drinking-water. Guidel. Drink. Qual. 9. https://doi.org/10.1155/2013/959637

Graphical abstract



Journal Pre-proof

Highlights

- *Daphnia magna* and *Daphnia similis* mancozeb sensitivity differs when pre-exposed to Pb.
- Adaptation of both *Daphnia magna* and *Daphnia similis* to low concentrations of Pb under two different food regimes.
- Adverse outcomes regarding recovery period with *Daphnia magna* relying possibly on genetic adaptation and *Daphnia similis* on physiological acclimation.

Journal Pre-proof

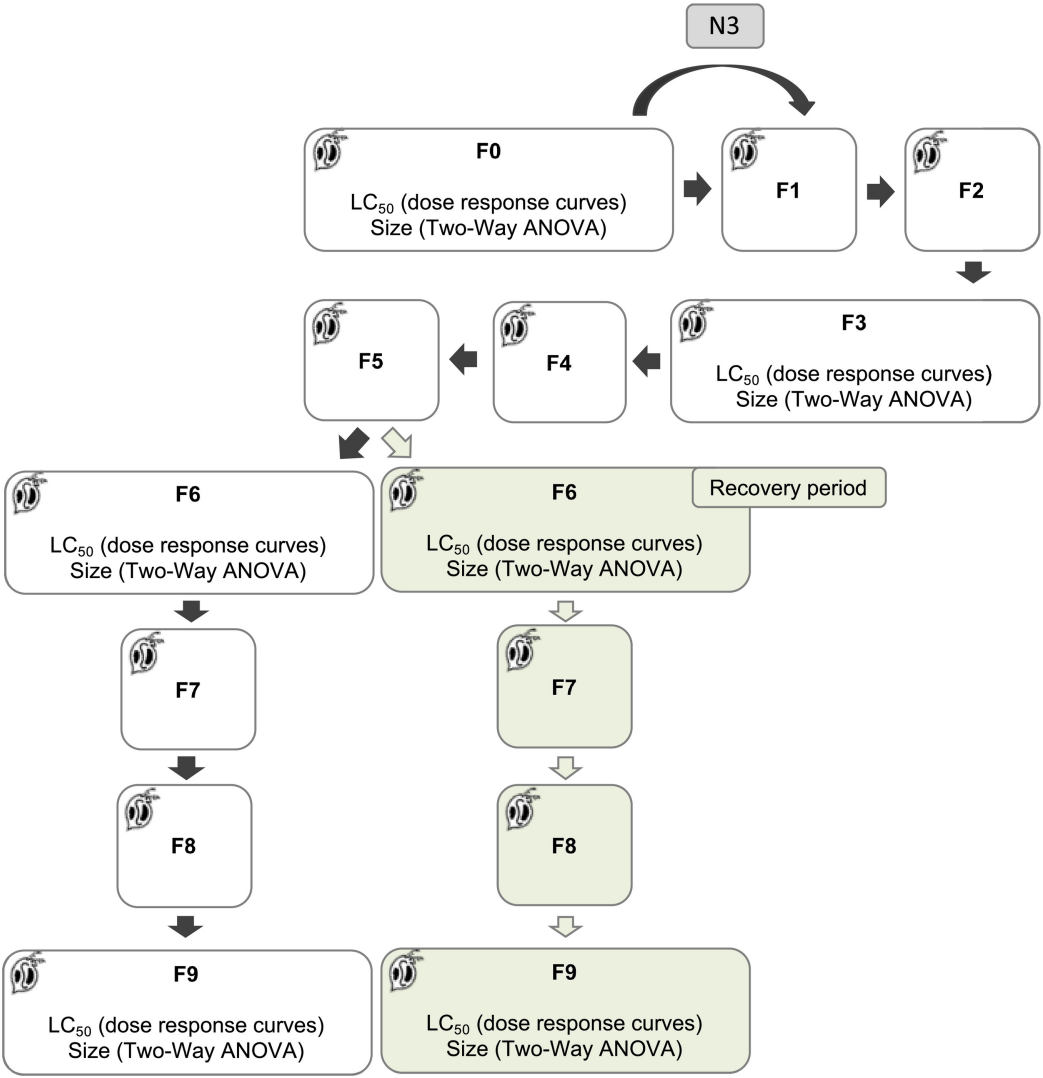

N3
F0
LC50 (dose response curves)
Size (Two-Way ANOVA)
F1
F2
F3
LC50 (dose response curves)
Size (Two-Way ANOVA)
F4
F5
F6
LC50 (dose response curves)
Size (Two-Way ANOVA)
F6
Recovery period
LC50 (dose response curves)
Size (Two-Way ANOVA)
F7
F8
F9
LC50 (dose response curves)
Size (Two-Way ANOVA)
F7
F8
F9
LC50 (dose response curves)
Size (Two-Way ANOVA)

Figure 1

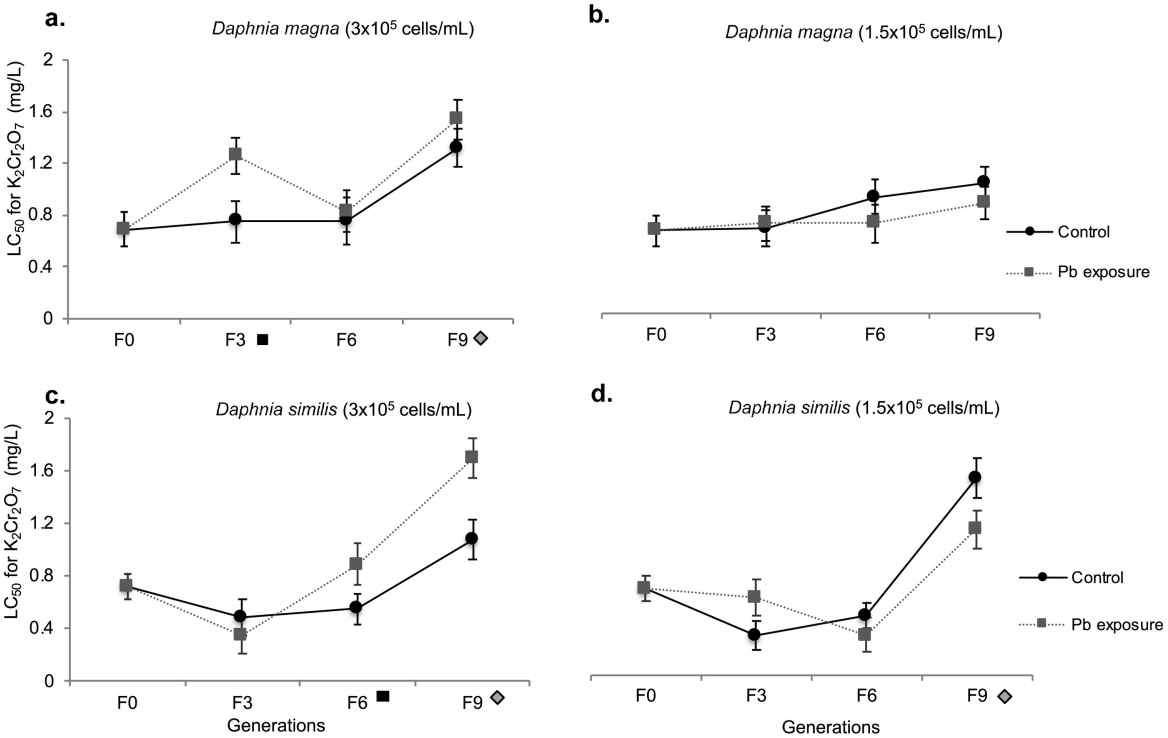

a.
Daphnia magna (3x10^5 cells/mL)
LC50 for K2Cr2O7 (mg/L)
2
1.6
1.2
0.8
0.4
0
F0
F3
F6
F9
b.
Daphnia magna (1.5x10^5 cells/mL)
Control
Pb exposure
F0
F3
F6
F9
c.
Daphnia similis (3x10^5 cells/mL)
LC50 for K2Cr2O7 (mg/L)
2
1.6
1.2
0.8
0.4
0
F0
F3
F6
F9
Generations
d.
Daphnia similis (1.5x10^5 cells/mL)
Control
Pb exposure
F0
F3
F6
F9
Generations

Figure 2

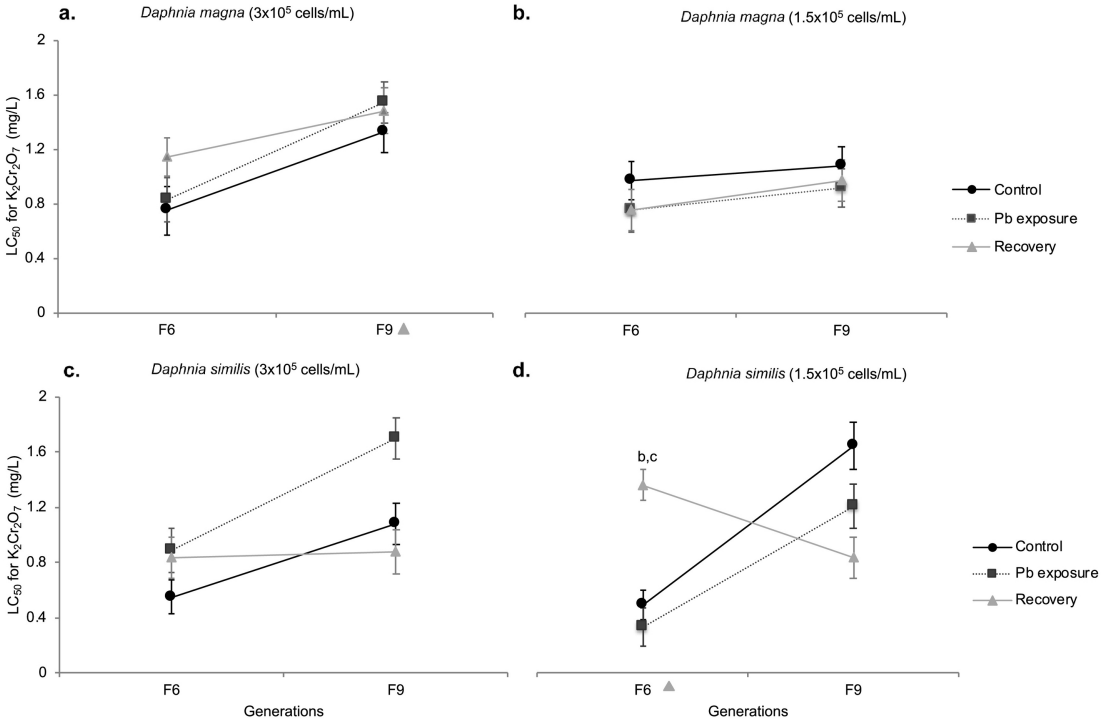

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
LC50 for K2Cr2O7 (mg/L)
2
1.6
1.2
0.8
0.4
0
F6
F9
b,c
Generations
Control
Pb exposure
Recovery

Figure 3

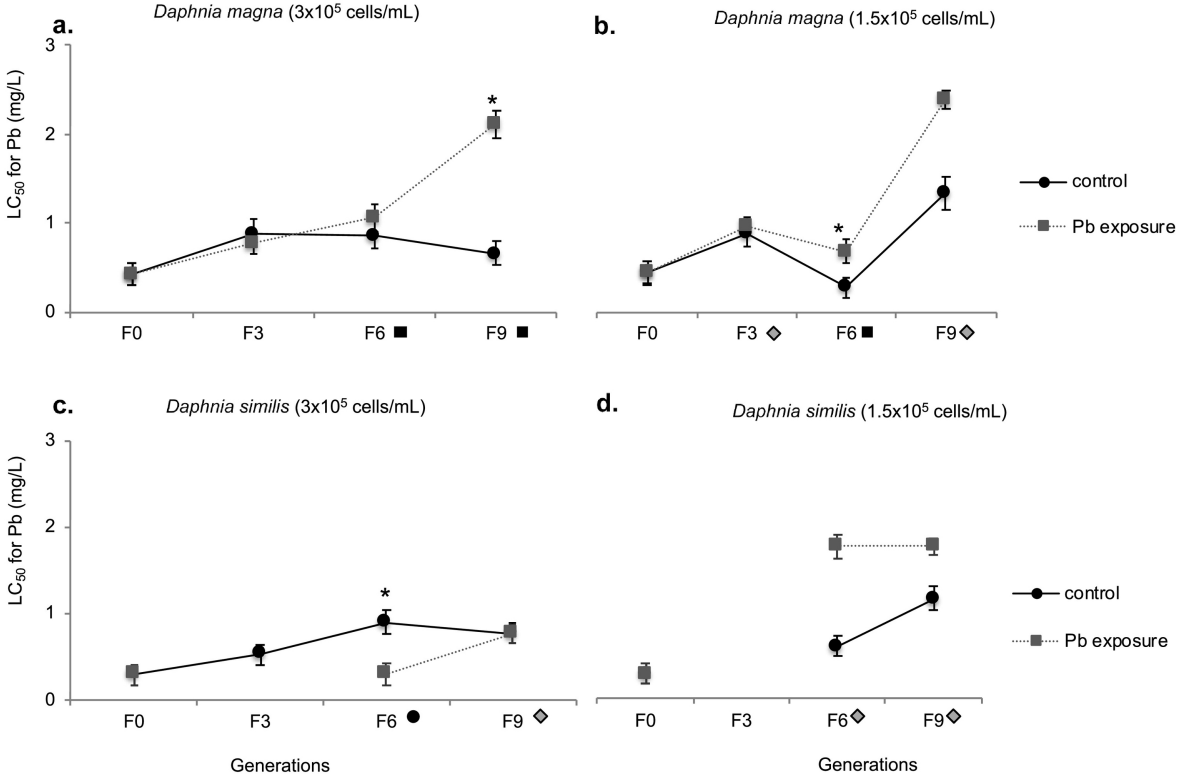

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
LC50 for Pb (mg/L)
0
1
2
3
F0
F3
F6
F9
*
control
Pb exposure
Generations

Figure 4

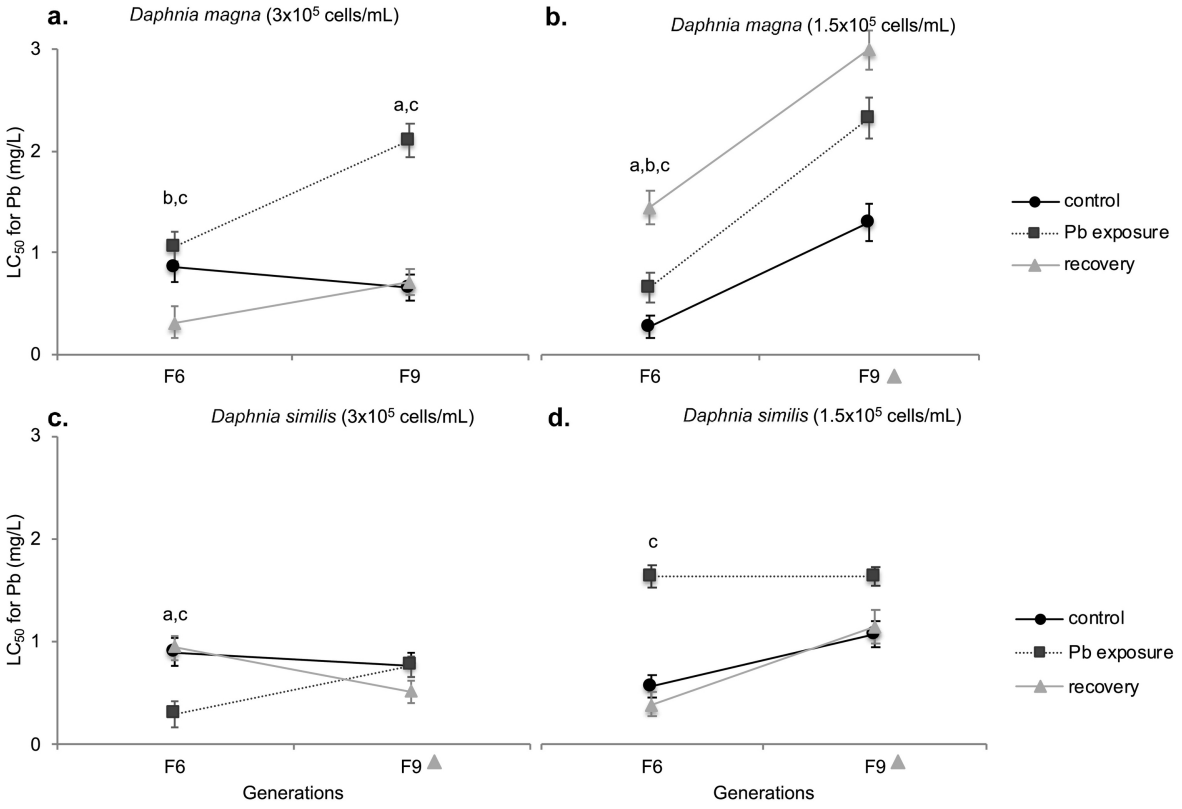

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
LC50 for Pb (mg/L)
0
1
2
3
F6
F9
Generations
a,c
b,c
a,b,c
c
control
Pb exposure
recovery

Figure 5

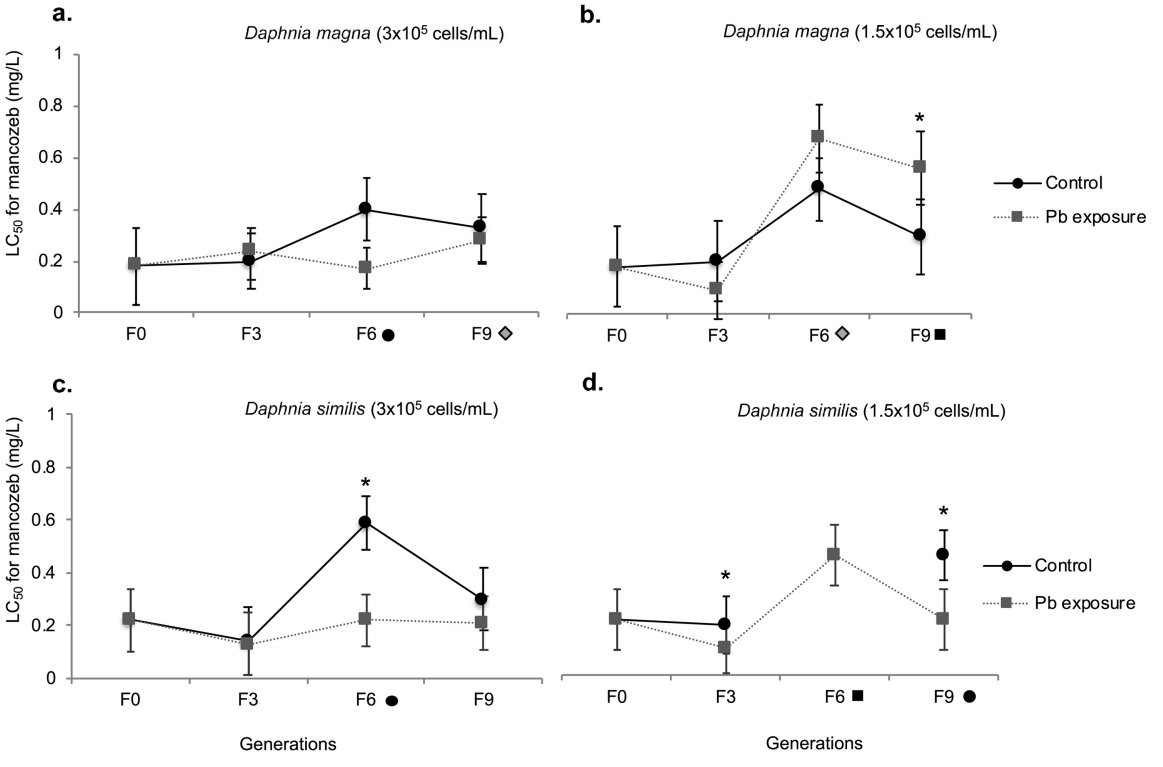

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
LC50 for mancozeb (mg/L)
0
0.2
0.4
0.6
0.8
1
F0
F3
F6
F9
*
Control
Pb exposure
Generations

Figure 6

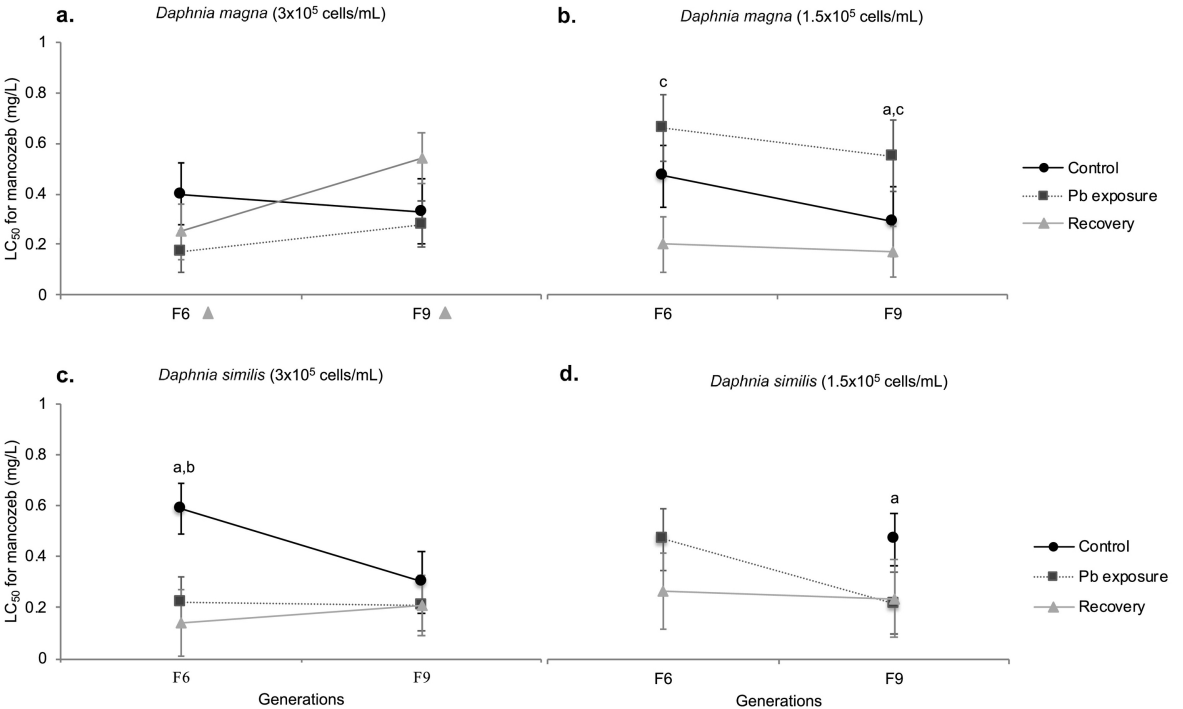

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
LC50 for mancozeb (mg/L)
1
0.8
0.6
0.4
0.2
0
F6
F9
c
a,c
a,b
a
Control
Pb exposure
Recovery
Generations

Figure 7

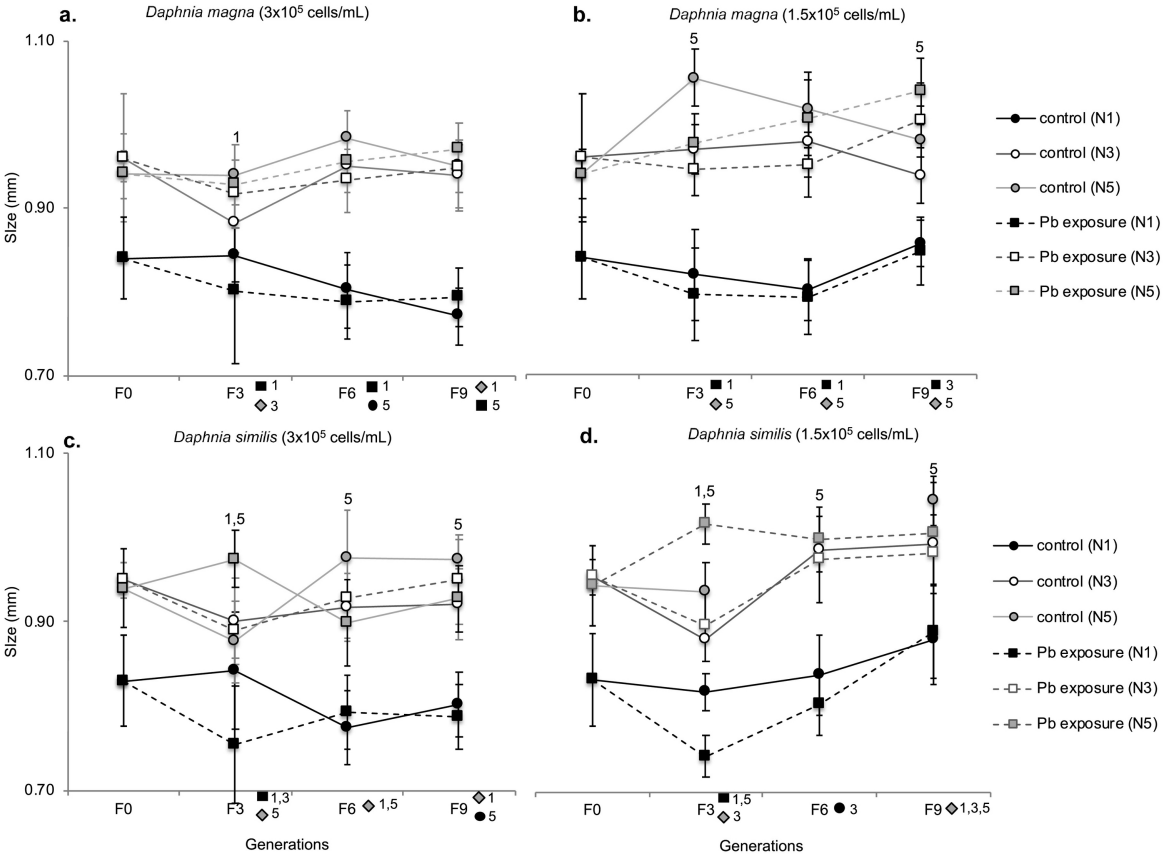

a.
Daphnia magna (3x10^5 cells/mL)
b.
Daphnia magna (1.5x10^5 cells/mL)
c.
Daphnia similis (3x10^5 cells/mL)
d.
Daphnia similis (1.5x10^5 cells/mL)
Size (mm)
1.10
0.90
0.70
F0
F3
F6
F9
Generations
control (N1)
control (N3)
control (N5)
Pb exposure (N1)
Pb exposure (N3)
Pb exposure (N5)

Figure 8

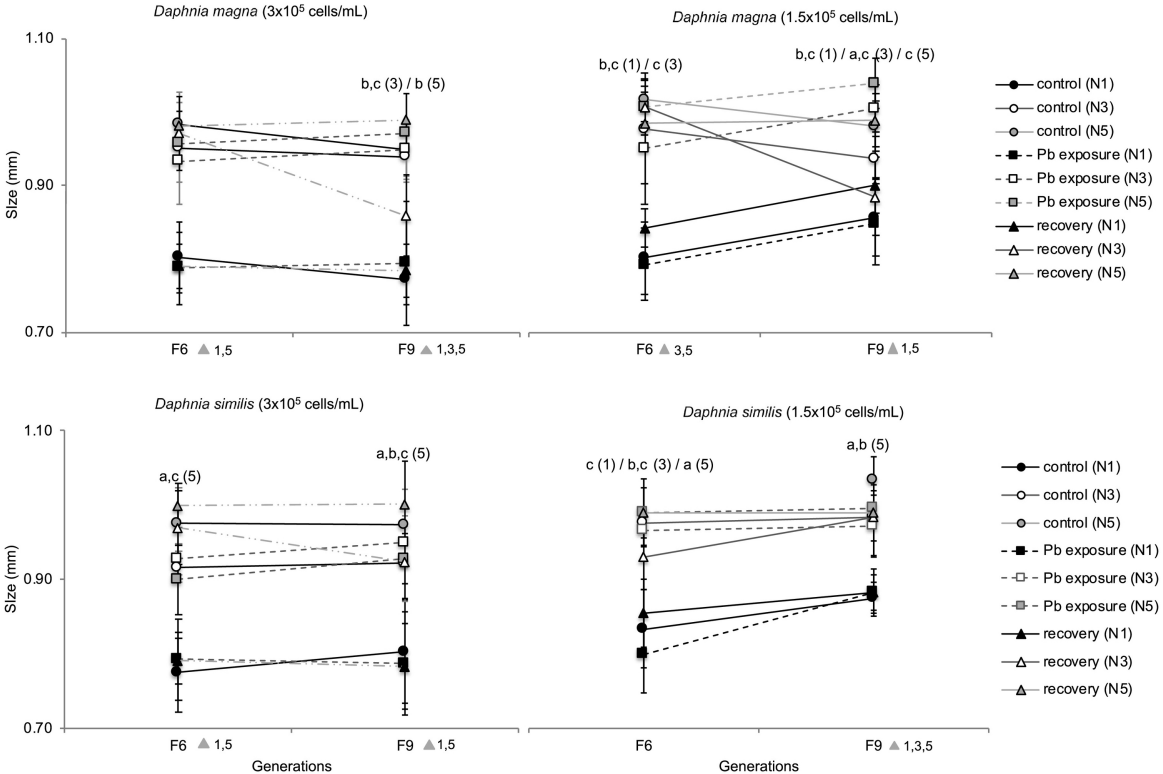

Daphnia magna (3x10^5 cells/mL)
Daphnia magna (1.5x10^5 cells/mL)
Daphnia similis (3x10^5 cells/mL)
Daphnia similis (1.5x10^5 cells/mL)
Size (mm)
1.10
0.90
0.70
b,c (3) / b (5)
b,c (1) / c (3)
b,c (1) / a,c (3) / c (5)
a,c (5)
a,b,c (5)
c (1) / b,c (3) / a (5)
a,b (5)
F6 ▲ 1,5
F9 ▲ 1,3,5
F6 ▲ 3,5
F9 ▲ 1,5
F6
F9 ▲ 1,3,5
Generations
control (N1)
control (N3)
control (N5)
Pb exposure (N1)
Pb exposure (N3)
Pb exposure (N5)
recovery (N1)
recovery (N3)
recovery (N5)

Figure 9