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Giuliana Seraphim de Araujo

Respostas de Daphnia magna e Daphnia similis expostas a químicos durante várias gerações

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro), do Doutor Denis Moledo de Souza Abessa (Professor Assistente Doutor da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Campus Experimental do Litoral Paulista) e do Doutor Amadeu Mortágua Velho da Maia Soares (Professor Catedrático do Departamento de Biologia da Universidade de Aveiro).

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palavras-chave Daphnia magna, Daphnia similis, chumbo, mancozebe, multigerações, exposições a pulso.

resumo A pressão antropogénica pode afetar negativamente o meio ambiente, através da introdução de vários tipos de stressores, desde químicos, físicos ou bióticos. Diferentes espécies podem apresentar distintas sensibilidades a estes stressores e por isso este estudo comparou a sensibilidade de duas espécies monofiléticas, a espécie tropical Daphnia similis e a espécie modelo de regiões temperadas Daphnia magna a uma exposição a chumbo (Pb), assim como à sua combinação com o fungicida mancozebe. Tendo em conta que os organismos aquáticos podem estar expostos a químicos por longos períodos, ou a pulsos de exposição, o desenho experimental utilizado foi baseado na exposição por várias gerações de forma a avaliar a aptidão dos neonatos, a sua sensibilidade e possível recuperação de efeitos. Os objetivos principais deste estudo foram: 1) Comparar a toxicidade de diferentes químicos a duas espécies de Daphnia, 2) Comparar os efeitos de uma exposição longa a Pb em ambas as espécies, 3) Estimar se a exposição prévia a Pb afetaria a sensibilidade a outros químicos (ex: exposição a pulsos), 4) Avaliar se a quantidade de alimento afeta a sensibilidade dos organismos, 5) Estimar se os organismos são capazes de recuperar após uma longa exposição. Neste âmbito, a espécie D. magna demonstrou uma menor sensibilidade a Pb em comparação com D. similis e, o mancozebe desencadeou resultados opostos nas duas espécies. A exposição a Pb por nove gerações indicou uma diminuição de sensibilidade para ambas as espécies. Contudo, a D. magna aclimatada a Pb exibiu uma diminuição de sensibilidade a mancozebe, enquanto a D. similis demonstrou o contrário. A exposição a Pb por gerações indicou um crescimento da taxa de aumento populacional (r) para ambas as espécies, enquanto um aumento da taxa de alimentação foi demonstrado para D. similis. Outros efeitos contrários entre as espécies causados pela longa exposição ao Pb são demonstrados pela taxa reprodutiva líquida (R_0), a qual não apresentou efeito em D. magna, porém, foi reduzida para D. similis. Enquanto isso, uma reprodução mais acelerada e diminuição da longevidade e da AChE foi exibida para D. magna, mas não para D. similis. As diferenças entre as espécies não foram demonstradas quanto a efeitos adversos do Pb. A longa exposição a Pb desencadeou malformações na carapaça, possíveis grânulos de Pb na parte dorsal dos neonatos, e uma coloração vermelha nas extremidades, podendo ser indicador do aumento de hemoglobina. Ao nível da reprodução, houve a produção de machos, efípias, variação da cor dos ovos (verdes e brancos) e ovos abortados. A acumulação do Pb ao longo do tempo em D. magna ocorreu de forma rápida na presença de uma quan tidade usual de alimento e um aumento gradual da acumulação quando em restrição alimentar, com uma recuperação bem-sucedida para ambos os regimes

resumo (Cont.)

alimentares. A recuperação pós-exposição a Pb demonstrou um padrão geral no qual a D. magna demonstrou uma falha na recuperação (talvez efeito epigenético) e D. similis demonstra geralmente recuperações para níveis semelhantes ao controlo bem-sucedidas (aclimatação fisiológica). O efeito do Pb na reprodução, respiração, indução de malformações e outros efeitos adversos sugerem que uma exposição crónica de Pb por diversas gerações pode ser prejudicial para ambas espécies. Os destaques principais deste estudo foram que: 1) Daphnia magna e Daphnia similis são capazes de diminuir a sensibilidade ao Pb através de exposições crónicas de longa duração, 2) Pré-exposição a Pb pode afetar a sensibilidade de organismos a outros químicos, 3) Aclimatação a Pb pode induzir crescimento populacional de organismos menos sensíveis, 4) Ambas as espécies diferem quanto a recuperação (variação epigenética ou aclimatação fisiológica), 5) Respostas distintas entre espécies ocorrem quando os organismos são submetidos a exposição única ou geracional (especialmente em ambientes oligotróficos, comum em habitats naturais). Estes resultados sublinham a importância de utilizar espécies adequadas ao ambiente em questão em ensaios ecotoxicológicos (e de longa duração), para uma melhor avaliação de risco ecológico em áreas contaminadas.

Keywords Daphnia magna, Daphnia similis, lead, mancozeb, multi-generation, pulse exposure

Abstract Anthropogenic pressure negatively affects natural environments through the introduction of various types of stressors, from chemical, physical or biotic. Different species may present different sensitivities to such stressors, therefore, this research evaluated the sensitivity of two monophyletic species of daphnids, the tropical Daphnia similis and the model temperate species Daphnia magna to a lead (Pb) exposure, as well as combined with the fungicide mancozeb. Moreover, since aquatic organisms may be exposed for long periods, or pulse exposures, the experimental design used was based on a multi-generation exposure to evaluate offspring sensitivity/fitness, sensitivity and possible recovery through generations. The main goals of this study were: 1) Compare the toxicity of chemical compounds to two daphnid species, representing different climate regimes 2) Compare the effects of Pb between species in a long-term exposure, for more realistic responses 3) Estimate if Pb exposure would affect the sensitivity to other chemicals (e.g. pulse exposure), 4) Evaluate if food quantity affect organisms' sensitivity to long-term exposures and, 5) Assess organisms' recovery after a generational Pb exposure. In this context, D. magna presented a lower Pb sensitivity than D. similis, and mancozeb triggered opposite outcomes for the two species tested. The nine generation Pb exposure indicated a diminished Pb sensitivity for both species. A lower mancozeb sensitivity for Pb exposed organisms occurred for D. magna, while D. similis showed an opposite response. The generational assay indicated an enhanced rate of population increase (r) for both species, however, enhanced feeding rate occurred only for D. similis. Other contrasting effects among species caused by a long-term Pb exposure was seen for the Net Reproductive Rate (R_0) which was not affected for D. magna and was diminished for D. similis. Meanwhile, early reproduction, reduced lifespan and AChE activity were shown for D. magna but not for D. similis. No disparity was shown between species considering adverse Pb effects. Generational Pb exposure led to carapace malformations, possible Pb granules in neonates' dorsal region, reddish extremities in neonates (possibly indicating increased haemoglobin content). Considering reproduction, negative effects occurred such as male production, ephippias (or dormant haploid egg), eggs color variation (green and white) and abortion. Pb accumulation in D. magna through generations presented a faster and saturated Pb accumulation under usual food and a gradual concentration increase under food restriction, with a successful recovery (both food regimes). Pb exposure presented a general pattern of D. magna failed

Abstract (Cont.)

retrieval (may be due to ephigenetics) and D. similis successful retrieval (phenotypic acclimation) to levels similar to control (successful) organisms. The effect of Pb on reproduction, respiration, induction of malformation, and other adverse effects suggests that a chronic generational exposure can be harmful to both Daphnia species. The most important highlights of this study were; 1) Daphnia magna and Daphnia similis are capable of diminishing Pb sensitivity across a long-term Pb exposure, 2) Pb pre-exposure can affect daphnids' sensitivity to other chemicals, 3) Pb exposure may increase the population of acclimated organisms in natural habitats, 4) Both species differ regarding recovery (epigenetics or physiological acclimation), 5) Different responses occur in single and generational exposures from monophyletic species (especially under oligotrophic media, typical of natural habitats). These results indicate the importance of accomplishing ecotoxicological assays with species adequate to the environment being evaluated (and of long-term), for a more realistic ecological risk assessment of contaminated areas.

Table of contents

Figures and Tables

Chapter 1

General Introduction

1. General Introduction

1.1. *Contaminants in aquatic environments*

Water is an essential resource for humans, economy and ecosystems (Vitousek et al., 1997; Vörösmarty et al., 2010). Human population has been increasing and consequently the contamination of aquatic ecosystems has intensified as well (Jiang et al., 2015). Growing population leads to increased demands of resources and energy for industries, agriculture, urbanization and other goods, which are often accompanied by damage of ecosystems and biodiversity (Vörösmarty et al., 2010). All this pressure can enhance the chemical levels in aquatic ecosystems, which frequently occurs by runoff or effluent discharge (Mosleh et al., 2005).

Metals are important contaminants worldwide and can be highly hazardous to organisms and the environment (Su et al., 2014). They can have natural or anthropogenic sources, such as industrial waste, atmospheric input, volcanic activity, mining activities, dredging, sewage sludge, aquaculture, ports and marinas, urban drainage, and metal-containing pesticides (Mager et al., 2011; Valavanidis & Vlachogianni, 2010; Zhang et al., 2011). Such chemicals may end up in rivers, which are the major metal contributors to marine ecosystems (Valavanidis & Vlachogianni, 2010). Once in water bodies, chemicals may be transported and spread, contaminating distant areas from the respective pollution sources (Tornero & Hanke, 2016). Old and recent studies on metal contamination are available in the literature (Dave, 1984; Völker et al., 2013; Zuo et al., 2018).

Despite of metal pollution, pressure for agricultural production has increased due to enhanced need for food, biofuel and fibers (Popp et al., 2013). To meet this high demand, the use of pesticides and fertilizers has been highly required. However, such enhanced chemical use can have damaging consequences (Maltby et al., 2009; Ochoa-Acuña et al., 2009; Popp et al., 2013). Once used, pesticides can be transported by runoff, percolate in the soil or volatilize into the atmosphere, and consequently affect non-target organisms (Novelli et al., 2012). Aquatic biota may be exposed to contaminants either by food pathway, dermal contact or respiratory surface (Newman et al., 2003).

In aquatic ecosystems several chemicals are prone to be simultaneously present, negatively affecting the biota (Jartun et al., 2008; Pavlaki et al., 2014), which may be exposed to a complex mixture of chemicals (Chen et al., 2015). However, the majority of toxicity studies are still conducted with single chemical exposure (Barata et al., 2006).It is already known that a complex mixture of chemicals in an environment can have unpredictable consequences (Mattsson et al., 2009). Chemical emission can occur in several ways, such as in pulse exposures (short episodes) or in a chronic way with constant

uninterrupted exposure, in a constant concentration or presenting broad oscillations (Leeuwen, 2007).

1.1.1. *Metals in aquatic environments*

Metal toxicity can harm the environment and organisms (Valavanidis & Vlachogianni, 2010). The harmfulness depends on the dose, exposure route, composition, as well as some organisms' factors, such as species, age, gender, nutritional status (Tchounwou et al., 2012). In terms of physiology, metals are mainly divided in two groups, essential and non-essential metals. Essential metals are retained in organisms' bodies at low concentrations through organic molecules linkage, playing an important role in metabolic activities but being toxic at high concentrations; whereas non-essential metals do not play any role regarding biological function and can be toxic even in trace amounts (Tchounwou et al., 2012; Valavanidis & Vlachogianni, 2010; Yilmaz et al., 2010).

Lead (Pb) is a non-essential metal highly toxic to organisms and the environment (Valavanidis & Vlachogianni, 2010), presenting a bluish-gray color. It occurs naturally in the Earth's crust but it is generally combined with other elements, forming lead compounds. Pb has natural and anthropogenic sources, such as erosion, volcanic activity, industries, urban effluents, vehicular exhaustion, bullets, batteries, fossil fuels, construction and mining process, being the anthropogenic sources greater than natural inputs (Cheng & Hu, 2010; Grosell et al., 2006; Valavanidis & Vlachogianni, 2010). Pb is a soft, malleable, corrosion resistant metal, which awaken the industry interest (Flora et al., 2012; Jan et al., 2015). Due to its non-degradable nature, it is persistent in the environment and its presence is of great concern; highly toxic metals are part of the Commission Regulation of the European Union for hazardous metals and Pb is one of the few metals that takes part in this list (EC, 2008). Pb is worldwide disperse through anthropogenic actions and its concentration has increased during and after the industrial age (Cheng & Hu, 2010; Valavanidis & Vlachogianni, 2010).

Lead can trigger mortality, affect growth, reproduction, neurological impairment, metabolism, respiration, renal dysfunction, anemia (decrease haemoglobin) and enzymatic inhibition (Ha & Choi, 2009; Hernández-Flores & Rico-Martínez, 2006; Mansour et al., 2015; Yilmaz et al., 2010) in exposed organisms. Pb effects are shown in the literature in aquatic organisms such as fish (Gašpić et al., 2002; Rogers et al., 2003; Yilmaz et al., 2010), mollusks (Grosell & Brix, 2009; Labrot et al., 1996), crustaceans (Komjarova and Blust, 2008; Regaldo et al., 2013; Shuhaimi-Othman et al., 2011) and algae (Koukal et al., 2007; Lukavský et al., 2003; Slaveykova & Wilkinson, 2002).

The neurotoxic influence of Pb is due to the substitution of calcium ions, inhibiting the regulator function that calcium accomplishes (Sharma et al., 2014). Since Pb has a high affinity for thiol (-SH) groups, it has the ability to affect enzymatic activities composed of sulfhydryl groups, leaving cells more susceptible to oxidative damage (Jan et al., 2015; Sharma et al., 2014). Apart from targeting -SH groups, Pb can also influence on substrate binding of chemicals, leading to activity loss (e.g. zinc) (Piast et al., 2005).

The concentration of Pb has been rising and studies show concentrations above limits worldwide. The World Health Organization (WHO) stablished the Pb limit for drinking water as 10 µg/L (WHO, 2011). The European Union (since 2013), Canada (Federal-Provincial-Territorial Committee on Drinking Water (CDW), (Health Canada, 2016)) and Australia ((National Health and Medical Research Council (NHMRC), 2016)) follow this recommendation and the Environmental Protection Agency (EPA) in the United States recommends a Pb limits of 15 µg/L. However, the United Kingdom and Scotland tap water levels reached concentrations above 50 µg/L (WHO, 2011). Besides elevated levels in drinking water, high Pb concentrations are also found in food items. According to Sanches-Filho et al. (2017), Brazilian fish presented Pb concentrations above limits. Concentrations exceeding legal standards were also observed in Nigerian rivers (Imasuen & Egai, 2013), soil in New Orleans, USA (Karrari et al., 2012), and vegetable crops grown in contaminated soils in Brazil (Lima et al., 2009). With that in mind it is clear that Pb contamination control is not being achieved, and, since no Pb level is necessary to organisms, no harmless levels is found (Flora et al., 2012).

1.1.2. *Fungicides in aquatic environments*

Pesticides are intended to control harmful organisms and diseases, facilitating agricultural production. According to the United States Environmental Protection Agency (US EPA) and the European Commission (EC), the term covers all substances used to control pests, such as insecticides, herbicides, fungicides and others. The enhanced use of pesticides has a partial positive impact on economy due to enhanced food production. However, it does negatively impact the health of humans and ecosystem (Aktar et al., 2009). The group of pesticides mostly applied worldwide are fungicides, aiming to control parasitic fungi and its spores (inhibit germination) on seeds and leaves of various vegetables (e.g. potato, cucumber), crops, fruits (e.g. banana, tomato and mango) and ornamental plants (Calumpang et al., 1993; Cuco et al., 2017; Gullino et al., 2010; Maroni et al., 2000). After sprayed, such chemicals can reach aquatic ecosystems through run-off, reaching non-target organisms and harming the ecosystem (Aktar et al., 2009; Jansen et al., 2015).

According to the Fungicide Resistance Action Committee, fungicides are grouped by its mode of action, such as from the group dithiocarbamate, having a multi-site-action mode of action (FRAC, 2018). Mancozeb is a fungicide registered since 1948 (US), belonging to the ethylene-bis-dithio-carbamate (EBDC) group (Durkin, 2015). This fungicide acts on fungi´s Krebs cycle, presenting the mode of action of Multi-Site Action (Cecconi et al., 2007), having not only one target but a range of impacts (different effects), which reduce the development of resistance to such chemicals (AWRI, 2010). In other organisms (non-target), it can affect the reproductive, endocrine (thyroid disruption), immune and nervous system as well as react with haemoglobin (Axelstad et al., 2011; Corsini et al., 2005; Maroni et al., 2000; Tsang & Trombetta, 2007).

Dithiocarbamate chelates can bind to Cd, Cu, Zn and Pb, which are then transformed into lipophilic compounds and redistributed into the brain (US EPA, 2001). Moreover, the active toxicants of such fungicides may react with –SH groups of specific enzymes (Gullino et al., 2010). EBDCs are absorbed by mucus membranes, respiratory and gastro-intestinal tracts, skin, and are metabolized by hepatic enzymes (Houeto et al., 1995). Mancozeb composition presents Zinc (Zn^{2+}) and Manganese (Mg^{2+}) ions (Kubrak et al., 2012) and it is toxic to a large range of non-target organisms such as fish (Kubrak et al., 2012), rats (Axelstad et al., 2011; Calviello et al., 2006; Castro et al., 1999), annelids (Silva et al., 2010) and crustaceans (Leeuwen, 1986; Terra & Gonçalves, 2013), being highly toxic to aquatic organisms (US EPA, 2005).

According to FAO/WHO, the acceptable daily intake (ADI) for humans of mancozeb is 0.05 mg/Kg/day (EC, 2009). The No Observed Effect Concentration for *D. magna* (NOEC) for mancozeb is 0.0073 mg/L (EC, 2009). The application rate (frequency) of mancozeb depend on the crop being cultivated, varying from a frequency of 3 days (tomatoes) to 28 days (apple three) (SAPEC, 2011). With that in mind and with the increased concentration of such chemical in the environment, studies should be accomplished to better determine the environmental concentration and harmfulness caused by mancozeb exposure on terrestrial and aquatic non-target organisms (Calviello et al., 2006).

1.2. *Daphnia as test organism*

The genus *Daphnia* includes more than 100 known species and generally have a length of less than 0.5 to more than 6 mm (Ebert, 2005). *Daphnia magna* and *Daphnia similis* are monophiletic species (Figure 1.1) (Lehman et al., 1995), differing in some morphologic characteristics. *D. magna* body length achieve from 4 to 6 mm, having a darker brownish colour (dependent on food and ambient eutrophisation), denticles in the post-

abdomen, variable caldal spine length and a dorsal edge of the post-abdomen that separates the anal spines into two series (Amoros, 1984). In its turn, *D. similis* have a body length from 2 to 4 mm, having a rounded head above the compound eye, sinuate post-abdomen (without identation), less melanine in ocellus, antennullar mouth not fully developed, lighter body coloration (also dependent on food and ambient eutrophisation) and long caldal spine (Amoros, 1984; Hebert and Finston, 1997).

D. magna is widespread in freshwater systems of temperate regions (Tsui and Wang, 2007). Usually present in rock pools, but also found in ditches, shallow ponds, and small eutrophic reservoirs (Hebert, 1978). *D. similis* however, prefer tropical regions (Rodgher et al., 2010) and is present in eutrophic and shallow ponds (Ramírez, 2014). Both species may be present in habitats likely to dry up (Ebert, 2005).

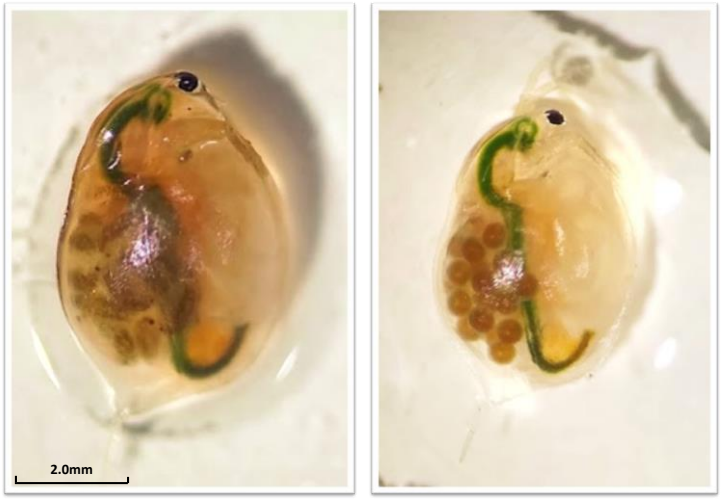

Figure 1.1: Adult female **a.** *Daphnia magna* and **b.** *Daphnia similis*

The contamination of aquatic bodies is a rising concern and needs to be mitigated. Aquatic organisms can assist the identification of contaminated areas and the chemical effects on the environment through ecotoxicological assays (Alves & Rietzler, 2015). Organisms survival, growth and reproduction are classic laboratory endpoints to determine chemicals toxicity (Van der Oost et al., 2003). *Daphnia* is a model species worldwide used to chemical exposure monitoring (Lampert, 2011). This species has been used to evaluate the toxicity of a wide range of chemicals such as metals (Dave, 1984; Griffiths, 1980; Kim et al., 2017; Massarin et al., 2010) and pesticides (Barata et al., 2004; Ochoa-Acuña et al., 2009; Zalizniak and Nugegoda, 2006). Studies comparing the sensitivity of both *D. magna* and *D. similis* has already been done (Beatrici et al., 2006; Buratini et al., 2004; Freitas and Rocha, 2011; Jardim et al., 2008; Tavares et al., 2014) and sensitivity of both species vary

according to the chemical being tested, the hardness of the media, among others (Yim et al., 2006).

Daphnia is an environmental sensitive planktonic crustacea of the cladocera order. These animals reproduce mainly by parthenogenesis (asexual) under favourable conditions, and due to this characteristic, population is mostly composed by females (Barata et al., 2000; Doke et al., 2017). Its body is enclosed by a carapace, in which haemolymph flows (Ebert, 2005). Daphnids are filter-feeders, filtrating the surrounding water and particles as food source; however, despite of food particles, they may filtrate contaminated particles as well, and play a role in the chemical flow within food chains. As zooplankton organisms which are in the bottom of the trophic chain, assessing their contamination is crucial, due to biomagnification and implications on energy and biomass transference along the trophic chain (Weltens et al., 2000).

Standard protocols with *Daphnia* (OECD, 2012, 2004) aim to determine acute (immobilization) and chronic (reproduction) assays, as well as studies suggesting the evaluation of other endpoints such as feeding, oxygen consumption and biomarkers (Allen et al., 1995; Guilhermino et al., 1996; Pestana et al., 2013). *Daphnia magna* is the model temperate species, worldwide used in ecotoxicological studies. However, studies demonstrating divergence regarding daphnid species sensitivity and responses to chemicals could be an alert to misapplied legislation in tropical areas, where the legislation is historically based on temperate organisms (Jardim et al., 2008; Pereira & Gonçalves, 2008; Printes & Callaghan, 2006).

These standard (short-term) protocols may not realistically mimic environmental contamination, since organisms may be exposed to contaminants (maybe more than one) for generations in natural ecosystems (Brausch & Salice, 2011). Due to the parthenogenetic characteristic of daphnids and consequently genetically identical offspring, such organisms are ideal to long-term exposure evaluation. Short-term experiments use neonates from unexposed mothers, disregarding oogenesis and embryogenesis exposure as well as ignoring the toxicant transfer from (exposed) mothers to neonates (Sánchez et al., 2000). Multi-generation exposures take into account the maternal effects (transgenerational transfer) transmitted to offspring, which is an additional route for adult females' detoxification (Tsui and Wang, 2007). Therefore, chronic exposure over only one generation of *Daphnia* (21-day tests) might lead to underestimate contaminants risk (Massarin et al., 2010). Corroborating with such statement, different outcomes between single and multi-generational exposure was shown by Tsui and Wang (2005). Therefore, Muyssen & Janssen (2004) recommend the use of multi-generational approach to evaluate long-term

chemical exposure, due to its more realistic methodology and better understanding organisms' behavior through a large amount of time. And, to achieve more realistic outcomes, Bossuyt et al. (2005) also recommends exposing organisms for a sufficient period of time, indicating at least a three-generation exposure.

The majority of the multi-generational studies focus on the development of organisms changing (or not) chemical sensitivity, however, studies to determine chemical uptake and biokinetics are also essential. Organisms recovery can be evaluated in order to assess the persistence of chemical sensitivity and altered biokinetics (Tsui and Wang, 2007). A population recovery after a chemical exposure is seen as a contaminant risk reduction, however, recovery from metal exposure can increase organisms' sensitivity (even for a period of time) (Tsui and Wang, 2005). The ability of aquatic organisms to physiologically acclimate to chemical exposure can be viewed as an advantage for the survival of the organism (Sánchez et al., 2004). Organisms' physiological acclimation to chemicals may alter its sensitivity by involving different physiological processes and it can be detected if organisms achieve a full recovery when chemical exposure is excluded. If recovery fail, organisms may be presenting epigenetic changes (transgenerational enheritance) (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. The ecological structure may even be more susceptible to fluctuation of metal exposure then to a constant exposure (Tsui and Wang, 2005). Therefore, it is ecologically relevant to estimate the recuperation of long-term exposed organisms regarding risk assessment evaluation. Besides this, in literature different definitions or nomenclatures are used to define the same process. In this thesis, this (physiological acclimation and epigenetic changes) was the adopt definitions and nomenclatures.

Usually, organisms in the natural environment are exposed to more than one chemical, and maybe even to a complex mixture of chemicals. Toxicity studies concerning individual contaminants may underestimate the effects of a mixture toxicity, where chemicals may indicate additive (same as independent chemical toxicity, but experiencing the effect of all chemicals) or worse, synergistic effects (more than additive) (Cooper et al., 2009). Chemical mixture may occur in a continuous form or by pulse exposure. Agricultural chemicals (such as pesticides) are episodically applied, generating short-term pulse

exposures (Liess et al., 1999). Aquatic organisms are likely to suffer with pulse exposures, which may affect in a even more pronounced way then continuous exposures (Andersen et al., 2006). Mixture toxicity is not commonly used in risk assessment studies, however, it will definitely be required for future regulatory risk assessment studies (Nys et al., 2015).

1.3. *Conceptual framework and aims of the thesis*

With the background presented by this introduction, it is realistic to infer that both Pb and mancozeb may be found and affect the environment. Despite of the negative effects already shown (Altindag et al., 2008; Corsini et al., 2005; Forbes & Calow, 1999), they are still being largely used and discharged in aquatic ecosystems. Therefore, the aim of this study was to evaluate a generational Pb exposure and compare the effects between two *Daphnia* species. Considering that chemicals are not present alone in the ecosystem, pulse exposures to the fungicide mancozeb on Pb pre-exposed daphnids were also evaluated. Moreover, food quantity in the environment may be scarce and can also influence organism's sensitivity, therefore, food quantity variability (usual and restricted) was also evaluated during the multi-generation approach. To assess the persistence of chemical effects, recuperation on long-term chronically Pb exposed organisms (as recovery period of three generations) was also accomplished (Figure 1.2). The toxicity of Pb and mancozeb to daphnids was already shown in single exposures (Castillo et al., 2006; Enserink et al., 1995; Offem & Ayotunde, 2008). Due to non-static environmental conditions, pulse exposures of other chemicals are possible and often usual, therefore, a generational approach of continuous Pb exposure along with single pulse mancozeb exposure on Pb pre-exposed daphnids has been done.

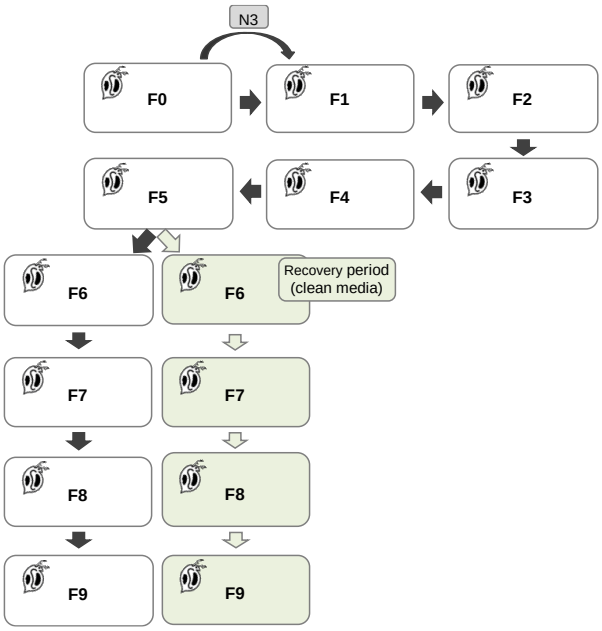

Figure 1.2: Experimental design of the multi-generation test. Dark arrows indicate Pb exposure and light arrows indicate recovery period for Pb pre-acclimated daphnids (F6 to F9). The same design was followed for control organisms. F refers to generation and N to broods.

To achieve what was proposed, this thesis had several specific goals and was structured in seven chapters.

Chapter 1 includes the general introduction, providing general information of the chemicals exposure and the organisms used in this study, and effects;

Chapter 2 cover the effects of both Pb and mancozeb in single exposures, comparing the sensitivity of both chemicals to both species (*D. magna* and *D. similis*). In this chapter, standard toxicity tests (immobilization and reproduction) along with feeding inhibition, oxygen consumption and enzymatic activities (acetylcholinesterase "AChE") were performed to evaluate the effects of both chemicals.

In Chapter 3, the acute effects of a long-term Pb exposure are shown, aiming to compare the outcomes of both species. In this chapter, other outcomes were also evaluated, such as pulse mancozeb exposures on Pb exposed organisms, food variation and recovery. Standard immobilization tests were performed ($K_2Cr_2O_7$, Pb and mancozeb) to estimate variations on organisms' sensitivity. Since size influences on chemical sensitivity, neonates' size was also measured.

Chapter 4 provides the chronic effects of a long-term Pb exposure, being the first study (to our knowledge) to cover chronic endpoints after a generational Pb exposure on daphnids. We also aimed to compare the endpoints of both species along with organisms'

recuperation and food quantity variation. To achieve this goal, reproduction and feeding inhibition tests on Pb exposed (and recovering) daphnids was performed.

Chapter 5 provides *D. magna* and *D. similis* population effects after a generational Pb exposure (along with food variation and recovery evaluation). To reach this proposal, Net Reproductive Rate (R_0), Hatching delay, Lifespan and Biochemical Activity (AChE) were evaluated.

Chapter 6 brings the evaluation of bioaccumulation of Pb throughout a long-term exposure in both *Daphnia* species, along with the description of several morphological and physiological observable traits. These adverse effects included the production of male and ephippias (or haploid dormant eggs), reddish extremities in neonates, mal-formations, neonates and eggs color variation, granules on neonates' bodies.

Lastly, **Chapter 7** brings a general discussion and the main conclusions drawn from the results acquired in this study, along with suggestions for future research.

Chapter 2

Toxicity of Lead and Mancozeb differs in two monophyletic *Daphnia* species

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Toxicity of Lead and Mancozeb differs in two monophyletic *Daphnia* species

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Abstract

Lead and mancozeb are two important chemicals used for different human purposes and activities worldwide. Hazard assessment in different areas of the world is carried out with different but phylogenetically similar species, adapted to different climatic conditions, in order to increase relevance. This study evaluated the sensitivity of two monophyletic species, the tropical species *Daphnia similis* and the temperate species *Daphnia magna,* to the two chemicals lead and mancozeb. Standard acute (lethal) and chronic ecotoxicological tests (reproduction and growth) as well as other sublethal measurements such as the intrinsic rate of population increase (*r*), feeding inhibition and O_2 consumption were recorded along with the analysis of the AChE activity to determine the neurotoxicity of both contaminants. Albeit their similar evolutionary status, *D. magna* presented a lower sensitivity to Pb in comparison to *D. similis*. Despite the differences in sensitivity, both species presented similar patterns of response under Pb exposure, with diminished reproductive outputs, feeding impairment, reduced O_2 consumption and no effect on AChE activity. Mancozeb decreased the reproductive parameters and feeding rate, and increased the AChE activity in both species, but triggered opposite effects in the O_2 consumption. While *D. magna* increased O_2 consumption under mancozeb exposure, no effects were observed for *D. similis*. Thus, species may present different responses and sensitivities to different pollutants, regardless of their phylogeny. Therefore, the use of ecotoxicological assays with native species is crucial for a better ecological risk assessment in contaminated areas.

Key-words: daphnids, metal, fungicide, acute toxicity, chronic toxicity, acetylcholinesterase

1. Introduction

The growth of human population is directly or indirectly drastically affecting ecosystems, with hazardous chemicals such as metals, hydrocarbons, pesticides and pharmaceuticals being released abundantly in aquatic ecosystems. Lead is a non-essential metal which can accumulate in natural habitats naturally or through anthropogenic sources (mining, fossil fuels, industrial effluents) and be toxic to aquatic organisms (Tchounwou et al., 2014). The European Chemicals Agency (ECHA) recently added Pb to the Candidate List of substances of very high concern (SVHCs) on June of 2018. The growth of human population also increases the demands for food, increasing the pressure on agricultural systems and consequently intensifying the use of pesticides and fungicides. Mancozeb is a fungicide commonly used worldwide which belongs to the dithiocarbamate group and is frequently used by farmers (London et al., 1997) on several field crops like orchards and vineyards (Morgado et al., 2016), fruits, ornamental plants and vegetables (Maroni et al., 2000).

A significant percentage of chemicals may end up in the aquatic environment through runoff (Soares et al., 2012). Although the prediction of chemical pressure is usually regarded as more efficient and relevant if native aquatic organisms are used to assess the impact of xenobiotics, the idea to extrapolate ecotoxicity results from closely related species for conservation purposes seems practical (Bernardo et al., 2007). Some authors demonstrate that phylogenetically close related species often share similar levels of sensitivity and are likely to produce redundant ecotoxicity information (Hammond et al., 2012; Manusadžianas et al., 2003). However, this similarity does not constitute a definitive situation and species belonging to the same phylogenetic group can respond distinctly when exposed to the same substances at similar conditions (Magalhães et al., 2014; Summers & Clough, 2001). Test results are often extrapolated to similar organisms, and in cases of different sensitivities it may lead to over or under estimation of hazard and risk. Thus, knowing how similar organisms react to contamination becomes important to seek for a greater reliability on the use of derived ecotoxicological data.

Daphnia is a genus of the Cladocera order which has species living in different climatic scenarios, it is easy to cultivate in the laboratory, presents a short life cycle, highly sensitive to contaminants and included in standardized protocols for toxicity testing (Adema, 1978). Although *Daphnia* species are considered phylogenetically very close, there are some evidences of sensitivity differences among them when exposed to the same chemical compound (Klüttgen et al., 1996; Lyu et al., 2013; Völker et al., 2013). Therefore, comparing the effects induced by chemicals used worldwide to two monophyletic *Daphnia* species,

from temperate and tropical areas, may provide an important insight on the chemical hazard assessment in different climate scenarios.

With this in mind, the goal of this study was to compare the sensitivity of two monophyletic *Daphnia* species (*Daphnia magna* and *Daphnia similis*) from different climatic areas (temperate and tropical, respectively) to two different contaminants, the metal lead (Pb) and the fungicide mancozeb. To achieve this goal, standard laboratory toxicity assays (immobilization and reproduction) along with other sublethal enzymatic (AChE) and physiological endpoints (Feeding Rate and Respiration) were performed using both species at the same environmental conditions.

2. Methods

2.1. Culture maintenance

Both *Daphnia* species were maintained in ASTM hard water (American Society for Testing Materials) (ASTM, 2002) enriched with organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). Cultures were maintained under a 16:8h light/dark photoperiod at 20º±2ºC and daphnids fed with *Raphidocellis subcapitata* ($3x10^5$cells/mL). Maintaining both species at the same conditions is an asset to derive only genetically driven responses to both chemicals and disregard effects due to different abiotic exposures (e.g. temperature). ASTM medium and food was renewed every other day. New cultures and all the following assays were initiated with less than 24h old neonates from third to fifth brood.

2.2. Chemical solutions and analysis

Stock solution for $Pb(NO_3)_2$ (CAS No. 10099-74-8, 98.5% purity, VWR chemicals®) and mancozeb (CAS No.8018-01-7, 97.5% purity, Fluka®) were prepared in mili-Q water, and then used for preparing the different concentrations in ASTM medium.

Prior to analysis Pb samples were acidified with nitric acid and analysis performed by ICP-OES (Horiba Jobin Yvon, Activa M). The limit of quantification (LOQ) for Pb was 25 µg/L. Samples were evaluated in triplicate. Duplicate samples of certified material were used, to ensure chemical optimum recovery during procedures.

2.3. Acute Toxicity

2.3.1. Acute Immobilization test

Acute tests were based on OECD guideline 202 (OECD, 2004). Neonates from both species aged between 6 and 24 hours were exposed to a range of concentrations of Pb and mancozeb for 48 hours. At the end of the exposure, immobilization was recorded. For each concentration, five replicates with five neonates each exposed to 50mL of experimental solutions were used. Concentrations applied are presented as supplementary material (Table 2S.1).

2.4. Chronic Toxicity

2.4.1. Reproduction and growth

The 21-day reproduction tests were based on procedures described in the OECD guideline 211 (OECD, 2012). Neonates from both species aging between 6 and 24 hours were exposed to a range of chemical concentrations for 21 days (see Table 2S.1). Ten individual replicates per concentration and the control were used and organisms were exposed in glass vials containing 50mL of experimental solution. Food and medium were renewed every 48 hours. Replicates were checked daily and neonates counted in each brood; the parental *Daphnia* length was measured at the end of the test.

2.4.2. Feeding Rate

Feeding trials were established according to McWilliam and Baird (2002). Neonates from both species with less than 24 hours were separated from the main cultures until they reach the 4^{th} instar; then they were exposed to both chemicals for 24 hours (Table 2S.1). The test-chambers consisted of 100mL vials of test-solution with five neonates each. The concentration of algae (*R. subcapitata*) set on ASTM media in the beginning of the test was $5x10^5$ cells/mL and the test-chambers were left in the dark for 24h. Blank controls (media with no daphnids) for each concentration were carried out to monitor algae growth. The concentrations of algae cells were measured by spectrophotometry (absorbance 440nm) (Jenway model UV-VIS 6505) at the end of the 24h of exposure (Allen et al., 1995). Afterwards daphnids were moved to new vials containing clean media, with the same algae concentration ($5x10^5$) for 4h also in the dark to evaluate feeding rates in post-exposure period. This enabled the recovery evaluation from chemical exposure. Feeding rates were calculated as in Allen et al. (1995) and expressed as cells per individual per hour.

2.4.3. Respiration rate

Based on the method described by Pestana et al. (2013), respiration rate in both daphnid species was assessed by measuring the oxygen consumption (i.e., difference between initial and final oxygen values) and expressed as μg O_2 consumed per organism per hour. For that, three 50-mL gastight syringes (Hamilton, USA) filled with 30mL of test-solution with five 4th-instar daphnids each were used per experimental treatment. Syringes were maintained in a water bath at 20ºC, in the dark for 24h. Initial and final O_2 concentrations were measured with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow, UK). Blank controls (media with no daphnids) for each concentration were used to correct for natural O_2 depletion.

2.5. Acetylcholinesterase activity

Neonates (< 24h old) from both species were exposed to Pb and mancozeb for 96h, for each concentration (Table 2S.1), and each replicate consisted of a pool of 15 organisms which were frozen with liquid nitrogen and stored in 1.5mL microtubes till the analysis. Then, each sample was homogenized with phosphate buffer (0.1 M; pH = 7.2) and centrifuged at 6.000 rpm for 5 min at 4ºC. Acetylcholinesterase (AChE) activity was determined according to Ellman et al. (1961), adapted to microplate according to Guilhermino et al. (1996a). The quantification of protein followed the protocol established by Bradford (1976), adapted by BioRad's micro-assay, using bovine γ-globuline as a standard.

2.6. Statistical Analysis

The lethal concentrations (LC_{50}) and the effect concentrations (EC_{50}/EC_{20}) were obtained from nonlinear regression curves (log(dose) response curves) through Global fitting (extra sums of squares F-test) for both species, using always the best model fit (GraphPad Prism®). Data for number of juveniles produced, length, feeding and respiration rate and acetylcholinesterase activity were first checked for normality (Kolmogorov–Smirnov) and homoscedasticity (Levene's equal variance test) and then evaluated through a one-way analysis of variance (ANOVA), followed by a post-hoc test (Dunnett's) to determine statistical differences in comparison with the respective control treatments (GraphPad Prism®). The intrinsic rate of population increase (*r*) was calculated using the Euler's equation as described in Pestana et al. (2013) and the replicate pseudo values for *r* were generated using the jackknife method based on Meyer et al. (1986). In order to make a clearer comparison between the analyzed endpoints, a radar chart englobing all the

results obtained was done for each chemical using the highest concentration tested as the maximum x value and by plotting the EC_{50}/EC_{20} for each endpoint in order to achieve comparisons.

3. Results

3.1. Chemical analyses

Pb chemical analyses retrieved a >79% recovery. The limit of quantification (LOQ) for Pb was 25 µg/L and. ASTM (control) presented values <LOD (Table 2S.2). The certified material evaluated achieved above 80% of recovery.

Regarding mancozeb chemical analysis, due to technical difficulties, results are not possible to report. Therefore, and considering that toxicity results are in accordance to the ones reported in the literature, we considered nominal concentrations for all calculations and discussion.

3.2. Acute Toxicity

3.2.1. Acute Immobilization test

The $LC_{50\text{-}48h}$ derived for lead exposure were 0.46mg/L (CI= 0.41-0.52) for *D. magna* and 0.34mg/L (CI= 0.31-0.37) for *D. similis* (Figure 2.1a). The response to the acute Pb exposure showed a statistical difference between both species (p = 0.0005), with an r^2 = 0.89, F (DFn, DFd) = 13.99 (1,46). For mancozeb, the respective $LC_{50\text{-}48h}$ values were 0.19mg/L (CI=n.d) for *D. magna* and 0.27mg/L (CI=0.24-0.30) for *D. similis* (Figure 2.1b); statistical difference was also obtained when comparing both log(dose) response curves (p < 0.0001), with an r^2 = 0.95, F (DFn, DFd) = 29.20 (2,36)

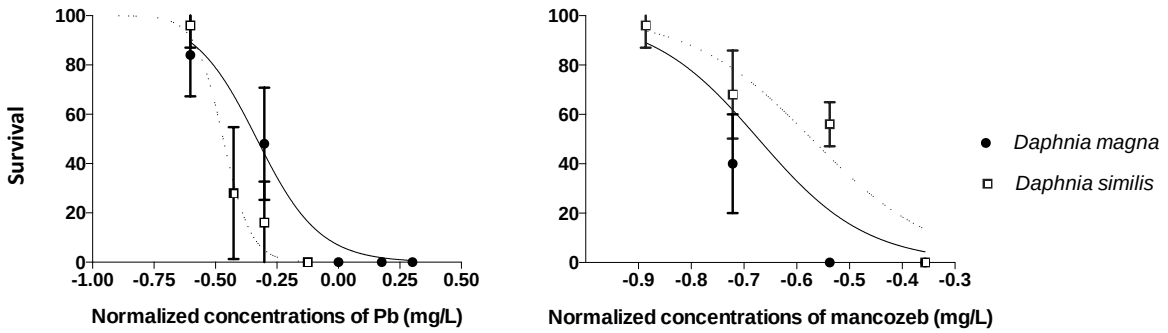

Figure 2.1: Log(dose) response curves of *daphnids* exposed to Pb and mancozeb for 48 hours. Data are expressed as average of survival ± standard deviation. Lines refer to the best model fit for survival data, with a solid line for *Daphnia magna* and a dotted line for *Daphnia similis*.

3.3. Chronic Toxicity

3.3.1. Reproductive parameters

One-way ANOVA statistical analysis for Pb exposure is detailed in Table 2S.3 An $EC_{50\text{-}21d}$ of 1mg/L (CI= 0.05-2.03) was derived for *D. magna* reproduction when exposed to Pb, although 1mg/L was the highest concentration tested (Figure 2.2). For *D. similis*, the gap window between sublethal and lethal effects for Pb exposure was very narrow. Considering the high mortality observed at concentrations >0.625mg/L, no $EC_{50\text{-}21d}$ or significant effects could be derived regarding reproduction in the concentration range used (≤0.625mg/L). When looking at daphnids' length, Pb exposure decreased the size of *D. magna* adults from 0.75mg/L onwards. For *D. similis*, control organisms were bigger than all of the others under Pb exposure. The intrinsic rate of population increase (*r*) decreased at the highest Pb concentration for *D. magna*, and the same response of diminished rate of population increase (*r*) was obtained for *D. similis* exposed to the highest Pb concentration.

For mancozeb, one-way ANOVA statistical analysis is detailed in Table 2S.4 the respective $EC_{50\text{-}21d}$ values derived for *D. magna* were 0.31mg/L (CI=0.26-0.36) and 0.26mg/L (CI= 0.08-0.78) for *D. similis* (with no significant differences between them, p = 0.13) (Figure 2.2). Mancozeb reduced the reproduction output for both daphnid species but it did not affect the growth of *D. magna* (Figure 2.2), although *D. similis* growth was altered, with significant increases being observed at 0.1, 0.15 and 0.3mg/L. Both species presented an increase in the rate of population increase (*r*) at lower mancozeb concentrations and a decrease at the highest concentrations.

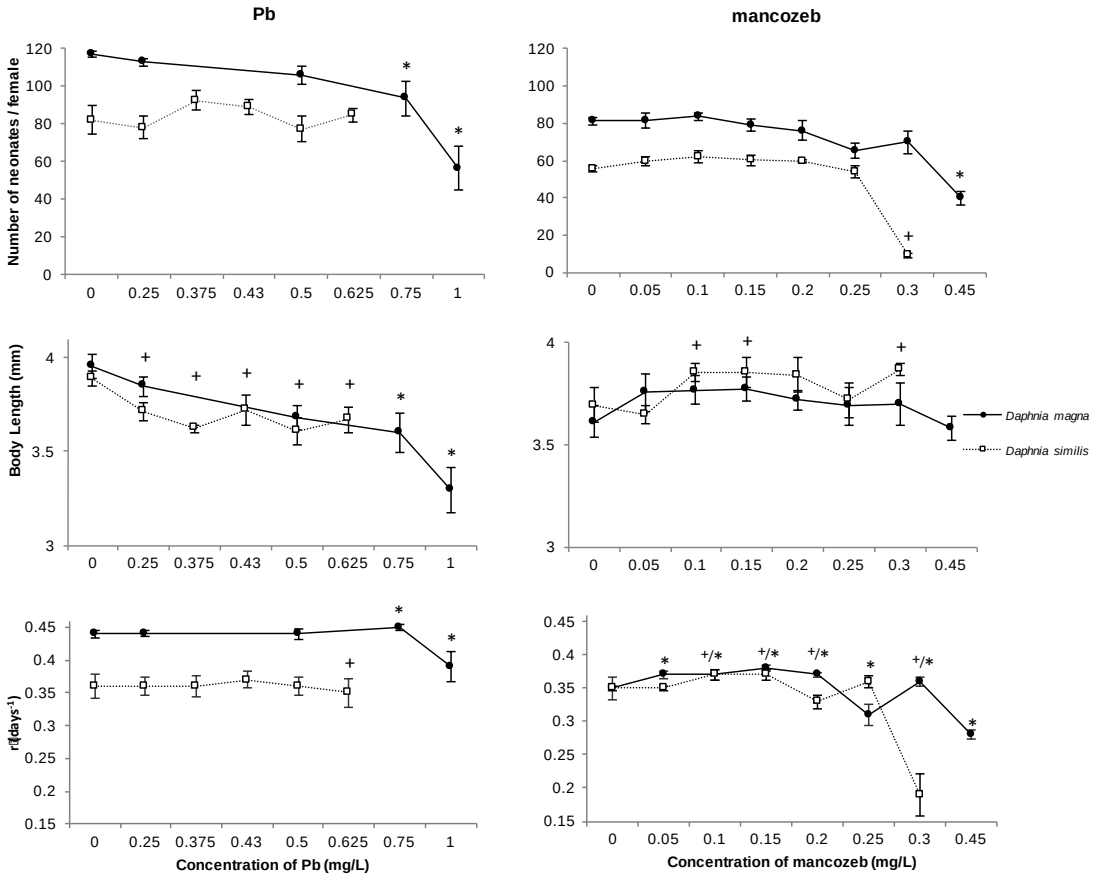

Figure 2.2: Reproductive parameters (expressed as mean ± standard error) of the number of neonates per female, body length and rate of population increase of *daphnids* exposed to Pb and mancozeb for 21 days. Asterisks and plus signs (*Daphnia magna* (*) and *Daphnia similis* (+)) mean statistical difference from control (Dunnett's, $p<0.05$).

3.3.2. Feeding inhibition

One-way ANOVA statistical analysis for feeding inhibition under Pb exposure is detailed in Table 2S.5. Feeding rates of both *Daphnia* species significantly decreased with increasing Pb concentrations (Figure 2.3). The $EC_{50\text{-}24h}$´s derived were 1.9mg/L (CI= 1.72-2.08) for *D. magna* and 0.9mg/L (CI= 0.4-1.4) for *D. similis*, with no significant difference among species ($p = 0.15$). The same pattern of diminished feeding rates was shown during the post-exposure (4h), with organisms pre-exposed eating less than those from the control, but their feeding rate was higher than during their exposure period.

The one-way ANOVA statistical analysis for mancozeb is detailed in Table 2S.6. During the 24h mancozeb exposure, *D. magna* showed a significant decrease in food uptake in all the concentrations tested (Figure 2.3). The same pattern occurred for *D. similis*, although a significant inhibition occurred only at 0.9mg/L and the estimated $EC_{50\text{-}24h}$ was

0.68mg/L (CI=n.d.). During the post-exposure period (4h), only *D. similis* exhibited a decrease on food uptake.

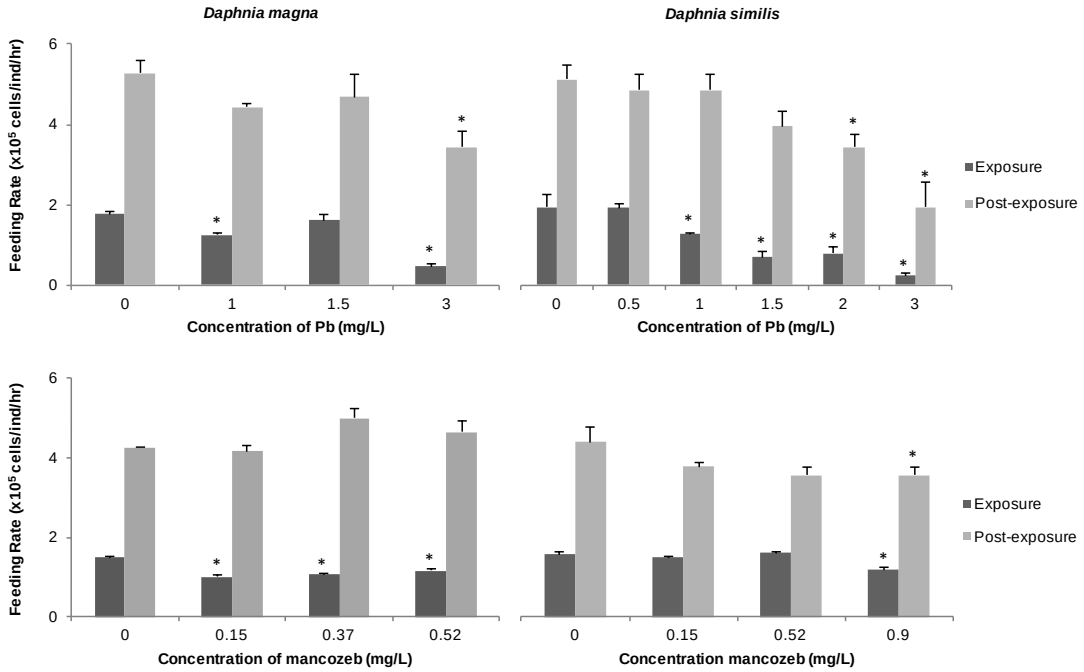

Figure 2.3: Feeding inhibition of *Daphnia magna* and *Daphnia similis* (expressed as average value ± standard error) exposed for 24h to Pb and mancozeb. Post-exposure in clean media (for 4h) is also shown. Asterisks (*) mean statistical difference from control (Dunnett's, $p<0.05$).

3.3.3. Respirometry

Regarding respirometry, one-way ANOVA statistical analysis is detailed in Tables 2S.7 (Pb) and 2S.8 (mancozeb). Results showed a significant decrease on O_2 consumption by *D. magna* and *D. similis* caused by exposure to Pb (Figure 2.4). No accurate EC_{50} could be calculated in the respiration test, and therefore an $EC_{20\text{-}24h}$ was derived for both species: 0.16mg/L (CI= 0.14-0.2) and 0.14mg/L (CI= 0.05-0.19) for *D. magna* and *D. similis*, respectively. An opposite pattern was shown for *D. magna* exposed to mancozeb; in this case, a significant respiratory stimulation occurred at the lower concentrations (0.05, 0.075 and 1mg/L), reaching values similar to those observed on control (0.125mg/L). An $EC_{20\text{-}24h}$ of 0.11mg/L (CI= 0.10-0.12) (Figure 2.4) was calculated for *D. magna*, while for *D. similis*, due to the lack of effects on the respiration rate no $EC_{20\text{-}24h}$ could be derived.

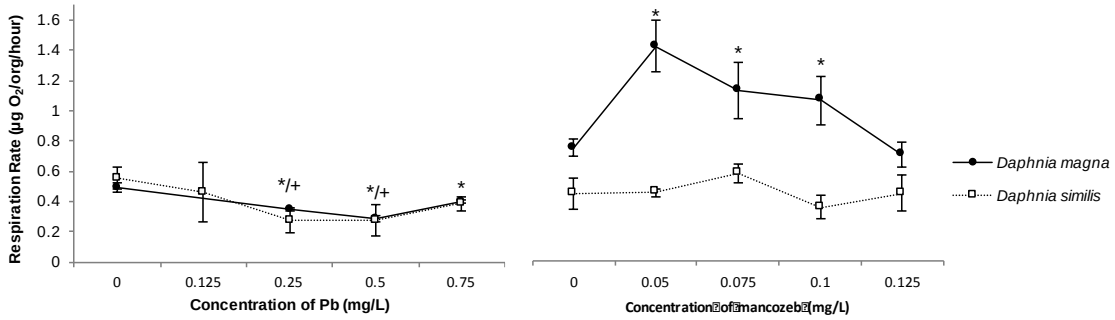

Figure 2.4: Respiration Rate on *Daphnia magna* and *Daphnia similis* (expressed as mean ± standard error) exposed to Pb and mancozeb for 24h. Asterisks and plus signs (*Daphnia magna* (*) and *Daphnia similis* (+)) mean statistical difference from control ($p<0.05$).

3.4. Acetylcholinesterase

For AChE activity, one-way ANOVA statistical analysis are presented in Tables 2S.9 (Pb) and 2S.10 (mancozeb). Regarding neurotoxic effects (evaluated using AChE activity), both species presented similar AChE activities when exposed to Pb (high standard errors) (Figure 2.5). When the two species were exposed to mancozeb, an enhanced enzymatic activity (with low standard error) was shown for all fungicide concentrations tested (both species).

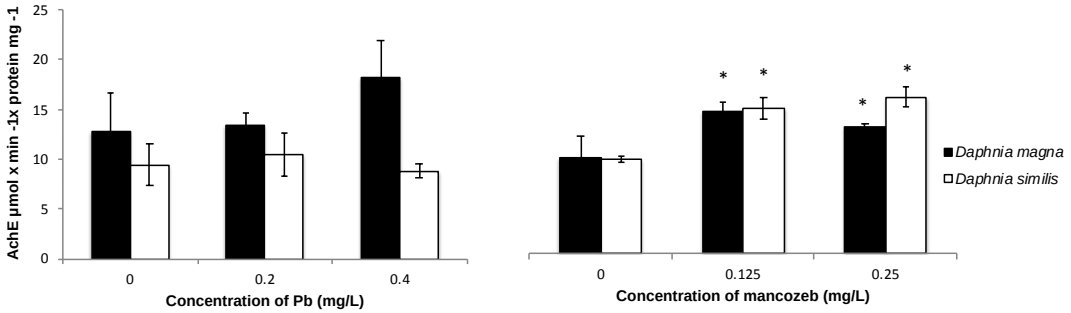

Figure 2.5: Acetylcholinesterase activity (expressed as mean ± standard error) of *Daphnia magna* and *Daphnia similis* exposed for 96h to Pb and mancozeb. Asterisks (*) mean statistical difference from control (Dunnett's, $p<0.05$), for both species.

Integrating the results obtained for each chemical exposure on life-history and physiological traits for both species in a radar chart (Figure 2.6), it is possible to see that *D. magna* has a lower sensitivity to Pb than *D. similis*. The graphs were plotted with EC_{50} for each endpoint, except for the respiration rate where the EC_{20} was used. For the organisms exposed to mancozeb, it is possible to see that *D. magna* is slightly more sensitive than *D.*

similis to this fungicide. When there was a lack of EC_{50}/EC_{20} it was plotted the highest test concentration.

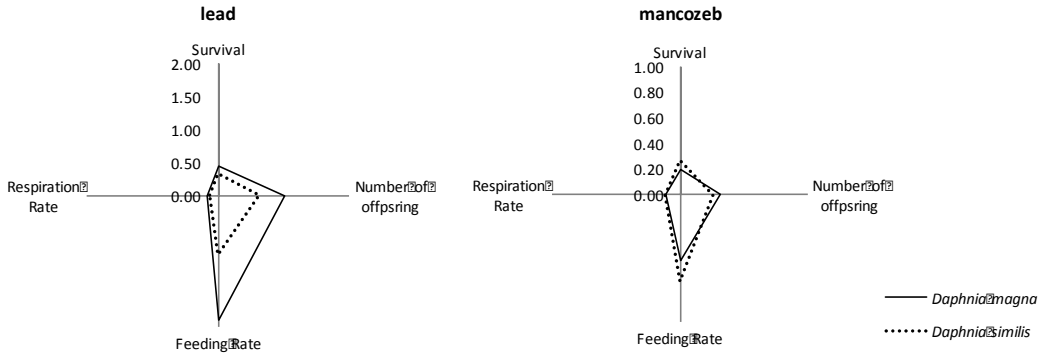

Figure 2.6: Radar chart showing the relationship of the endpoints (immobility as survival, reproduction as number of offsprings, feeding rate as algae consumption per individual per hour and respiration rate as µg of O_2 consumption per organism per hour), acquired for *Daphnia magna* and *Daphnia similis* exposed to Pb and mancozeb. The concentration values plotted (mg/L) are equivalent to the EC_{50}, except for the respiration rate where the EC_{20} was used.

4. Discussion

The comparison of metal toxicity data derived from freshwater invertebrates testing is challenging. There are some hindrances when comparing toxicity endpoints, different traits from different species, different exposure conditions, like light intensities, temperature variation, exposure time and a diverse composition of the culture medium (Rodgher et al., 2010). Those different exposure conditions can affect the sensitivity of organisms exposed to chemicals, beyond their natural genetic sensitivity. Similar, i.e. phylogenetically very close, species are expected to react in a similar pattern and with similar sensitivities to the same stressor exposure, when all other variables are controlled.

In the present study, the patterns of response along with sensitivity were compared in two monophyletic *Daphnia* species. During Pb exposure, *D. magna* and *D. similis* presented similar responses, exhibiting a decrease on reproductive related parameters, feeding and O_2 consumption, with no changes at the AChE activity level (probably due to the high standard error exhibited). Under mancozeb exposure, *D. magna* and *D. similis* displayed the same pattern in almost every endpoint such as decreased reproduction and feeding, with increased AChE activity, but both species displayed different outcomes regarding O_2 consumption.

Our results showed that Pb $LC_{50\text{-}48h}$ for *D. magna* was significantly higher than for *D. similis*, even though values are in the same order of magnitude. Other studies showed similar results for *D. magna* with values ranging from 0.41 to 0.45mg/L (Biesinger & Christensen, 1972; Hernández-Flores & Rico-Martínez, 2006; Irwin et al., 1997; Mansour et al., 2015). Regarding *D. similis,* it is difficult to compare data with the literature because, being a tropical species, most studies rely on proposed protocols (ABNT, 2016) where organisms are maintained in soft water (≈ 40 to 48mg/L of $CaCO_3$), under 20±2°C. This may represent a problem for metals, due to speciation processes, but not so much for organic chemicals. The sensitivity of aquatic organisms to metals can be altered by the media hardness, with organisms maintained in hard water being less sensitive than those in soft water solutions (Yim et al., 2006). As the aim of the present study was to compare the sensitivity of both *Daphnia* species to the tested contaminants, *D. similis* was previously acclimated in laboratory to the same conditions as *D. magna* cultures (hard water medium, 140mg/L $CaCO_3$).

D. magna reproduction impairment occurs with increasing Pb concentration while no significant effects where observed for *D. similis*. Lower reproduction in *D. magna* exposed to Pb has been previously reported at concentrations as low as 0.025mg/L (Ha & Choi, 2009) and 0.09mg/L (Regaldo et al., 2014), however with *D. magna* cultivated under a different type of media. In the case of *D. similis*, a small gap between lethal and sublethal effects could be observed when looking at a straight Pb concentration range. According to McWilliam & Baird (2002), when lethality occurs at concentrations close to sublethal responses, this acute endpoint turns to be as valid (relevant) as a sublethal endpoint. Another study has shown the negative effect of Pb, reducing growth and reproduction of *D. similis* (Soundrapandian & Venkataraman, 1990). Jardim et al. (2008) showed that *D. magna* is less sensitive to Pb than *D. similis*, although tested in a natural habitat freshwater with different hardness (Bacia do Corumbataí). In the present study, increasing exposures to Pb induced a toxicity pattern leading to smaller adult female length and decrease in population growth (given by *r*). The correlation between body length and reproduction has been already reported (e.g. Loureiro et al., 2005), indicating that the chemical effect on body length is traduced into the number of offspring. And, it can be an indicative of a possible decrease on population sizes at long-term (Pavlaki et al., 2014), which is corroborated by the decrease of the rate of population increase (*r*) in the present study.

In addition to the reproduction impairment, both species reduced the food consumption during a 24h exposure to Pb. In the study of McWilliam & Baird (2002), although essential metals such as Zn and Cu produced a feeding impairment in *D. magna,*

Pb did not affect feeding rates (during and after exposure) at 0.5 to 1.7mg/L concentration ranges. Other studies indicate feeding depression in daphnids affected by metals, such as Cd (0.08mg/L) (Allen et al., 1995; Taylor et al., 1998) and Ag ($AgNO_3$ (0.002mg/L)/AgNP (0.01mg/L)) (Ribeiro et al., 2014). Cladocera feeding activity is a critical endpoint to be used in ecotoxicology, as the food intake will mediate the energy allocation (e.g. for reproduction), and thus it may be indicative of population level effects (e.g. population growth) (Taylor et al., 1998). A long-term feeding depression is expected to negatively influence growth and reproduction (Ribeiro et al., 2014) and can reduce the number of organisms in natural populations, as demonstrated by the decreased rate of population increase (*r*). Regarding the 4h recovery period after Pb exposure, both species presented similar outcomes, with a reduction in food consumption in comparison to the control but an increase when compared to the exposure period. Ferreira et al. (2008) indicated that the increase of algae consumption by organisms transferred to clean medium after chemical exposure may indicate compensation after stress. So, the present study indicates that *D. magna* and *D. similis* tried to recover from Pb exposure by increasing algae consumption, due to the potential need for more energy to depurate or to deal with the exposure effects. The difference shown between organisms' sensitivity on experiments with and without food cannot be disregarded. Organisms fed during experiments (in the feeding inhibition test) under Pb exposure have a lower sensitivity than organisms exposed without food (acute test). This may be due to the positive charged metals (such as Pb^{2+}) that tend to adsorb to algal cell surfaces, reducing chemical toxicity, exemplified as the algal cells reducing dissolved Cd^{2+} toxicity to *D. magna* shown by Taylor et al. (1998). As organisms do not consume all the algae available and a considerable amount of Pb^{2+} tend to be attached to the cell wall, or allocated at the flask bottom with time, Pb available concentration tends to be lower than the expected.

Oxygen consumption rate can represent also a crucial endpoint to understand organisms' physiological and behavioral processes, being a valuable biomarker and enabling early warning detections of xenobiotics effects in aquatic ecosystems (Martins et al., 2007). Our results indicate that both species tend to reduce the oxygen consumption at high Pb concentrations. Roy (2009) found no effect on O_2 consumption by *D. magna* exposed to low Pb concentration (0.12mg/L). However, Chinni et al. (2002) showed decreased respiration rates caused by Pb exposure on post larvae of the crustacean *Penaeus indicus* (1.4mg/L) and several authors already reported lower O_2 consumption triggered by increasing metal concentrations on *D. magna*, such as Cd^{2+} (0.3mg/L) and Zn^{2+} (4.4mg/L) (Zitova et al., 2009), Cu (0.056mg/L) (Khangarot & Rathore, 2003) and to other

species such as the crustacean *Litopenaeus vannamei* exposed to Cd (3mg/L) and Ni (3mg/L) (Wu & Chen, 2004). According to Muyssen et al. (2006), a reason for these observations may be the structural damage in organisms' gas exchange surface caused by metals' exposure, corroborating with other studies that found structural changes in the mitochondria of *D. magna* exposed to metals (Bodar et al., 1990a; Griffiths, 1980). Moreover, the decreased filtration rate (feeding) evidenced by both daphnid species at the feeding test induced by Pb exposure could also decrease the respiration rate due to poor nutritive state (Muyssen et al., 2006). Since these organisms were feeding less under Pb exposure probably they were experiencing a poor nutritional state, which in consequence, negatively affected the offspring production, body length, rate of population increase (*r*) as well as O_2 consumption, affecting the physiological performance in both species.

Despite the probable Pb neurotoxic effect, *D. magna* and *D. similis* displayed similar outcomes and showed no effect on AChE activity under the concentrations of exposure used (although, presenting a high standard deviation). Although in the study of Guilhermino et al. (1996b) it was suggested that metals have no influence on *D. magna* AChE activity, the interaction of metals and AChE receptors (Bainy et al., 2006) has been observed, either resulting in cholinesterase's activity inhibition (Diamantino et al., 2003, 2000; Frasco et al., 2005; Labrot et al., 1996) or in its induction (Dethloff et al., 1999; Löf et al., 2016; Romani et al., 2003).

The fungicide mancozeb was previously identified as toxic to different species, inducing effects related to reproduction, or impairment of the endocrine and immune systems (Bassfeld, 2001; Corsini et al., 2005; Samussone, 2014; Tsang and Trombetta, 2007). The comparison of the mancozeb $LC_{50\text{-}48h}$ curves shows that *D. similis* is less sensitive to mancozeb than *D. magna*. Only few studies are available for comparison, but Lyu et al. (2013) also indicated a lower sensitivity of *D. similis* in comparison to *D. magna* regarding exposure to nitrites. Regarding mancozeb toxicity, similar $LC_{50\text{-}48h}$ were found for different daphnid species, with values ranging from 0.17 to 0.26mg/L (Bassfeld, 2001; Samussone, 2014).

Mancozeb exposure diminished the reproductive outcome of both species, affecting the number of offspring and decreasing the rate of population increase (*r*). An impairment on the reproduction of *D. magna* under exposure to several fungicides has been widely reported: carbamate fenoxycarb (0.045mg/L) (Hosmer et al., 1998); epoxiconazole (0.00058mg/L) and pyraclostrobin (0.0036mg/L) (isolated and mixture) (Prestes et al., 2013); dithiocarbamate disulfiram (0.018mg/L, affecting also growth) (Leeuwen, 1986); tebuconazole (1.14mg/L, affecting also survival) (Sancho et al., 2016); and also to the

pesticide carbofuran (environmental sample) (Castillo et al., 2006). Besides its effects on reproduction, mancozeb exposure also caused inhibition of feeding rates in both species. Ferreira et al. (2008) showed a decrease of food consumption on *D. magna* exposed to the carbamate fungicide carbendazim (EC_{50} = 0.097mg/L) and other studies have already shown feeding impairment on *D. magna* exposed to organic compounds such as a mixture of a neurotoxic insecticide (imidacloprid; EC_{50} = 7.88mg/L) and organophosphate pesticide (chlorpyrifos; EC_{50} = 0.34mg/L) (Loureiro et al., 2010) and to ammonium perchlorate (>150mg/L) (Loureiro et al., 2012). Regarding recovery during post-exposure (4h), *D. similis* showed again the same pattern, with a lower food intake revealing a possible non-recovery from mancozeb exposure. On the other hand, *D. magna* presented a different outcome, with organisms showing similar feeding rates when compared to the control exposures, indicating a possible success on their recovery process. This demonstrates that both species react in different ways. Xenobiotics may interact with organisms' food (algae cells), being so, a lower mancozeb sensitivity on organisms fed during experiments (feeding inhibition test) than organisms not fed (acute test) was observed. This may be explained by the molecular structure of mancozeb, that has two cationic metals (Mg^{2+} and Zn^{2+}) that by adsorbing to algae cells could diminish its bioavailability.

Despite the similar outcomes for both species regarding reproduction and feeding activity (24h), mancozeb exposure generated different effects on the recovery process and O_2 consumption when comparing *D. magna* and *D. similis*. *D. magna* increased the O_2 consumption under mancozeb exposure while no effects were observed for *D. similis*. Muyssen et al. (2006) stated that respiration rates can increase as a result of an enlarged metabolic activity (depuration attempt from chemical exposure), which probably occurred for *D. magna*. Therefore, this compensation in order to achieve depuration is limited, and at higher chemical concentrations the metabolism rates tend to be depressed (reducing O_2 consumption), as evidenced for *D. magna* at high mancozeb concentrations (approaching control values). Martins et al. (2007) showed an increase on *D. magna* oxygen consumption under only 15 minutes of pesticide (lindane) contamination ($EC_{50\text{-}48h}$ = 0.0064mg/L). Pan et al. (2017) stated that arrhythmic heartbeat rate due to contamination could reduce respiratory capacity while increase its demand and consequently the O_2 consumption. Moreover, the increased respiration rate under mancozeb exposure intensifies the swimming activity to maximize oxygen uptake from water surface (Pan et al., 2017), which could be linked to the increased AChE activity shown by *D. magna*. Jemec et al. (2017) compared cave and surface populations of the crustacea *Asellus aquaticus* and showed a

lower AChE activity in the cave population, suggesting a correlation on AChE and muscle activities.

The enhanced AChE activity was probably an attempt to detoxify by overproducing esterases (Hyne & Maher, 2003), which can induce a resistance trough detoxification corroborating with the daphnids attempt to depurate in result of the fungicide intoxication. Literature corroborates with our findings showing the same pattern to crustaceans and insects exposed to organothiophosphate insecticides such as the increased AChE activity shown by the crustacean *Palaemon serratus* exposed to the neurotoxic pyrethroid ester insecticide deltamethrin, at concentrations as low as $6x10^{7}$mg/L (Oliveira et al., 2012). Similarly, it happened to the stoneflie *Claassenia* sp. and to the crustacean *Hyalella azteca* exposed to the neurotoxic organophosphate insecticides fenitrothion (0.001mg/L) and azinphosmethyl (0.005mg/L), respectively (Day & Scott, 1990). Minutoli et al. (2002) analyzed AChE activity in many zooplanktonic crustaceans and stated that all the species showed a linear increase in enzyme activity with increasing amount of homogenate concentration. The cationic metals on mancozeb structure (Mg^{2+} and Zn^{2+}) may help to understand also the increased pattern of AChE activity, as well as it happened for *D. magna* under Pb exposure (although with no statistical difference). The binding of cationic metals to the anionic site of AChE has been suggested to stimulate the enzyme synthesis and therefore reflecting in an increase in activity (Guilhermino et al., 1998), even though there are more than one form or type of cholinesterases and metallothioneins that can interfere in this outcome (Romani et al., 2003; Vasák et al., 2005).

To conclude, looking at both lethal and sublethal effects, generally Pb is more toxic to *D. similis* than to *D. magna*, although the pattern of effects is similar for both species; on the other hand, mancozeb can trigger different patterns of responses/endpoints. Although of the same genus and phylogenetically close related, sensitivity and effects were not as similar as expected; differences were observed at organisms' sensitivity to both chemicals and opposite outcomes regarding O_2 consumption under mancozeb exposure. Therefore, it is crucial to take into account that when exposed to the same chemical, organisms from temperate and tropical areas can exhibit different outcomes, and also, that organisms' chemical sensitivity can vary regarding different contaminants. Therefore, it would be advisable that the non-native temperate model species *D. magna* would be replaced by the tropical species *D. similis* for regulatory purposes when it comes to mitigate and properly manage the impacts of chemicals in tropical freshwaters.

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Supplementary material

Assays nominal concentrations

Table 2S.1: Nominal assays concentrations

	Immobilization	Reproduction	Feeding inhibition	Respirometry	AChE
	Daphnia magna				
$K_2Cr_2O_7$	0; 0.3; 0.6; 1.2; 2.4; 3				
Pb ($Pb(NO_3)_2$)	0; 0.25; 0.5; 1; 1.5; 2	0; 0.25; 0.5; 0.75; 1; 1.25	0; 1; 1.5; 3	0; 0.25; 0.5; 0.75	0; 0.2; 0.4
mancozeb	0; 0.13; 0.19; 0.29; 0.44; 0.66	0; 0.05; 0.1; 0.15; 0.2; 0.25; 0.3; 0.45	0; 0.15; 0.37; 0.52		0; 0.125; 0.25
	Daphnia similis				
$K_2Cr_2O_7$	0; 0.17; 0.35; 0.7; 1.4; 2				
Pb ($Pb(NO_3)_2$)	0; 0.125; 0.25; 0.375; 0.5; 0.75	0; 0.25; 0.375; 0.43; 0.5; 0.625	0; 0.5; 1; 1.5; 2; 3	0; 0.125; 0.25; 0.5; 0.75	0; 0.2; 0.4
mancozeb	0; 0.13; 0.19; 0.29; 0.44; 0.66	0; 0.05; 0.1; 0.15; 0.2; 0.25; 0.3	0; 0.15; 0.52; 0.9		0; 0.125; 0.25

Table 2S.2: Chemical Analyses

Chemical Analyses			
Nominal concentrations	Stock solution (46.54 mg/L)	0.125 mg/L	3 mg/L
Pb ($Pb(NO_3)_2$)	48 mg/L	0.099 mg/L	2.6 mg/L
Recovery (%)	103	79.2	86.7

Table 2S.3: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding reproductive endpoints (reproduction rate, body length and rate of population increase (*r*). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Reproductive rate					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	23629	4	5907	F (4, 40) = 15.43	P < 0.0001
Residual (within Pb_concentrations)	15314	40	382.8		
Total	38943	44			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	1652	5	330.4	F (5, 44) = 1.239	P = 0.3075
Residual (within Pb_concentrations)	11739	44	266.8		
Total	13392	49			
Body lenght					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	2.567	4	0.6418	F (4, 40) = 11.00	P < 0.0001
Residual (within Pb_concentrations)	2.333	40	0.05833		
Total	4.9	44			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	0.5539	5	0.1108	F (5, 45) = 3.917	P = 0.0049
Residual (within Pb_concentrations)	1.273	45	0.02828		
Total	1.826	50			
rate of population increase (r)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	0.02553	4	0.006383	F (4, 45) = 417.2	P < 0.0001
Residual (within Pb_concentrations)	0.0006885	45	0.0000153		
Total	0.02622	49			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	0.001633	5	0.0003266	F (5, 54) = 9.950	P < 0.0001
Residual (within Pb_concentrations)	0.001772	54	0.00003282		
Total	0.003405	59			

Table 2S.4: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding reproductive endpoints (reproduction rate, body length and rate of population increase (*r*). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Reproductive rate					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	4939	7	705.6	F (7, 23) = 6.184	P = 0.0004
Residual (within mcz_concentrations)	2624	23	114.1		
Total	7564	30			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	6803	6	1134	F (6, 23) = 21.77	P < 0.0001
Residual (within mcz_concentrations)	1198	23	52.09		
Total	8001	29			
Body lenght					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	0.1611	7	0.02302	F (7, 23) = 3.067	P = 0.0196
Residual (within mcz_concentrations)	0.1726	23	0.007505		
Total	0.3338	30			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	0.5539	5	0.1108	F (5, 45) = 3.917	P = 0.0049
Residual (within mcz_concentrations)	1.273	45	0.02828		
Total	1.826	50			
rate of population increase (r)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	0.08572	7	0.01225	F (7, 72) = 1048	P < 0.0001
Residual (within mcz_concentrations)	0.0008411	72	0.00001168		
Total	0.08656	79			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	0.2422	6	0.04036	F (6, 63) = 1102	P < 0.0001
Residual (within mcz_concentrations)	0.002307	63	0.00003663		
Total	0.2445	69			

Table 2S.5: One-way ANOVA results testing for effects among Pb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding feeding inhibition (24h and 4h). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Feeding inhibition					
Exposure (24h)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	30200000000	3	10070000000	F (3, 8) = 80.57	P < 0.0001
Residual (within Pb_concentrations)	999500000	8	124900000		
Total	31200000000	11			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	68160000000	5	13630000000	F (5, 11) = 69.52	P < 0.0001
Residual (within Pb_concentrations)	2157000000	11	196100000		
Total	70320000000	16			
Post-Exposure (4h)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	53330000000	3	17780000000	F (3, 8) = 7.995	P = 0.0086
Residual (within Pb_concentrations)	17790000000	8	2224000000		
Total	71120000000	11			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	2.269E+11	5	45380000000	F (5, 12) = 17.49	P < 0.0001
Residual (within Pb_concentrations)	31140000000	12	2595000000		
Total	2.58E+11	17			

Table 2S.6: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding feeding inhibition (24h and 4h). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Feeding inhibition					
Exposure (24h)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	4304000000	3	1435000000	F (3, 8) = 53.86	P < 0.0001
Residual (within mcz_concentrations)	213100000	8	26640000		
Total	4517000000	11			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	3376000000	3	1125000000	F (3, 8) = 29.49	P = 0.0001
Residual (within mcz_concentrations)	305300000	8	38170000		
Total	3682000000	11			
Post-Exposure (4h)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	13540000000	3	4512000000	F (3, 8) = 3.334	P = 0.0769
Residual (within mcz_concentrations)	10830000000	8	1353000000		
Total	24360000000	11			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	13480000000	3	4493000000	F (3, 8) = 5.260	P = 0.0269
Residual (within mcz_concentrations)	6833000000	8	854200000		
Total	20310000000	11			

Table 2S.7: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding respirometry. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Respirometry					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	0.07042	3	0.02347	F (3, 8) = 57.98	P < 0.0001
Residual (within Pb_concentrations)	0.003239	8	0.0004049		
Total	0.07366	11			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	0.1718	4	0.04296	F (4, 10) = 3.429	P = 0.0519
Residual (within Pb_concentrations)	0.1253	10	0.01253		
Total	0.2971	14			

Table 2S.8: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding respirometry. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Respirometry					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	1.045	4	0.2611	F (4, 10) = 13.34	P = 0.0005
Residual (within mcz_concentrations)	0.1957	10	0.01957		
Total	1.24	14			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	0.07599	4	0.019	F (4, 10) = 2.727	P = 0.0903
Residual (within mcz_concentrations)	0.06966	10	0.006966		
Total	0.1456	14			

Table 2S.9: One-way ANOVA results testing for effects among Pb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding Acetylcholinesterase (AChE) activity. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Acetylcholinesterase (AChE)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	80.25	2	40.13	F (2, 12) = 3.434	P = 0.0662
Residual (within Pb_concentrations)	140.2	12	11.68		
Total	220.5	14			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between Pb_concentrations)	14.05	2	7.026	F (2, 12) = 1.784	P = 0.2097
Residual (within Pb_concentrations)	47.26	12	3.938		
Total	61.31	14			

Table 2S.10: One-way ANOVA results testing for effects among mancozeb concentrations plus a negative control of both *Daphnia magna* and *Daphnia similis* regarding Acetylcholinesterase (AChE) activity. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Acetylcholinesterase (AChE)					
Dahnia magna					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	58.71	2	29.35	F (2, 12) = 23.44	P < 0.0001
Residual (within mcz_concentrations)	15.03	12	1.252		
Total	73.74	14			
Dahnia similis					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between mcz_concentrations)	134.5	2	67.26	F (2, 12) = 66.66	P < 0.0001
Residual (within mcz_concentrations)	12.11	12	1.009		
Total	146.6	14			

Chapter 3

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

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

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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 indirectly 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' sensitivity, 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 et al., 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 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 and Trombetta, 2007)). In the environment exposure to pesticides may vary temporally and spatially, and it may often present (be applyied)

distinct pulses (e.g. spray drift or drainage/runoff events distributed in time) (Dennis et al., 2012).

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; Danielly Paiva 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 and Gonçalves, 2013). However, different outcomes from similar species (Lyu et al., 2013; Danielly Paiva 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 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 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 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, J L & Harper, 2007).

Daphnids metal acquired sensitivity is widely believed to be physiological acclimation and not genetic adaptation (Stoddard and Harper, 2007). However, some authors believe that the development of toxicant diminished sensitivity can be genetically based (Lopes et al., 2006; Rose et al., 2004). Phisiologically acclimated daphnids will present a full recovery after chemical exposure ceases. On the other hand, during a failed recovery process, organisms may be presenting epigenetic changes (Schultz et al., 2016). External stressors can alter organisms genome function, leading to epigenetic changes (DNA methylation, histone tail modification and/or microRNA expression) (Stoccoro et al., 2013; Vandegehuchte and Janssen, 2011).

With that in mind it is important to investigate the organisms' recovery by removing chemical exposure on chemical acclimated organisms to better estimate if the observed sensitivity was due to physiologicall acclimation or through epigenetic changes. 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 this goal, 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. To infer on organisms' fitness and sensitivity, tests were carried out with 1) potassium dichromate ($K_2Cr_2O_7$) to evaluate organisms' sensitivity/fitness (indirectly), 2) Pb, to monitor variation on Pb sensitivity and 3) the fungicide mancozeb, providing a pulse toxicity approach on generational Pb acclimated daphnids. To better interpret the data from the acute tests, the respective neonates' size was also measured.

2. Methodology

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 1.2). 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 □g/L of Pb in ASTM), being the legislation maximum permitted by the Brazilian federal law for fresh water bodies (CONAMA, 2005). Both setups were maintained under two different dietary regimes, 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). All generational assays started at the N3 brood of the previous generation.

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). Their respective N3 broods (aged between 6 and 24 hours) were exposed to the following chemicals: potassium dichromate ($K_2Cr_2O_7$) for 24 hours; Pb and the fungicide mancozeb for 48 hours. 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 (log(dose) response curves) and differences among LC_{50} 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)). After checked for normality (Kolmogorov–Smirnov) the differences between juvenile sizes among generations and treatments of each generation 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®).

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 3S.1). 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 $K_2Cr_2O_7$, which indirectly determines the organisms' fitness for use in ecotoxicological assays, showed in some generations changes in sensitivity for both species and for both control and Pb treatments (Figure 3.1).

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 3.1a). *D. magna* under food restriction presented the smallest variations in the calculated LC_{50} between generations showing no statistical difference towards F0 (Figure 3.1b).

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 3.1c). When under food restriction, both treatments (control and Pb) presented lower sensitivity to $K_2Cr_2O_7$ in comparison to F0.

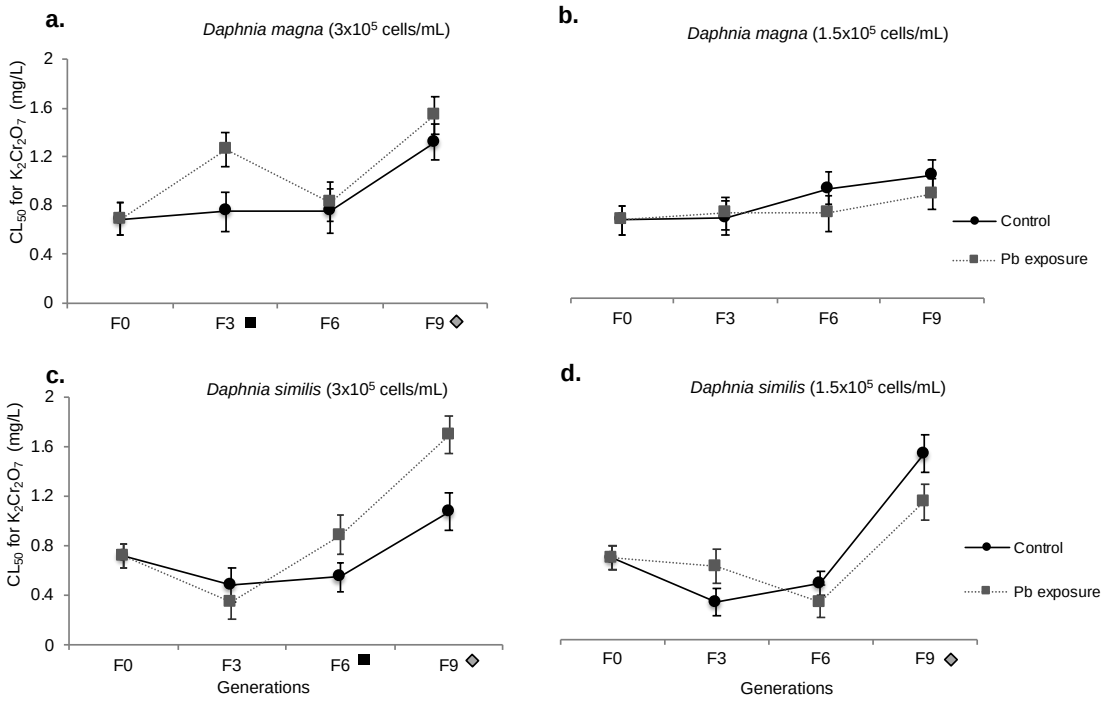

Figure 3.1: LC_{50} for a $K_2Cr_2O_7$ 24h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked 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, $p<0.05$).

Regarding the potential recovery, *D. magna* showed no relevant variations in both dietary regimes (Figure 3.2a,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 3.2c). 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 3.2d). 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.

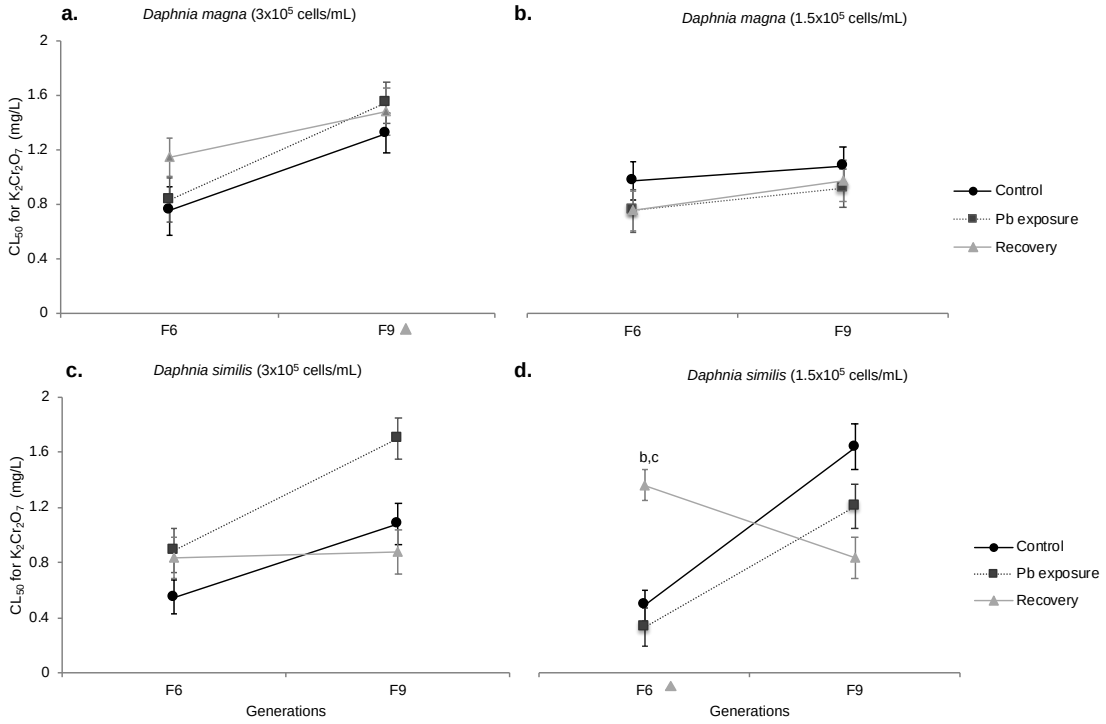

Figure 3.2: LC_{50} for a $K_2Cr_2O_7$ 24h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ 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 3.1, 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 3.3a). 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 3.3b).

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 3.3c). 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 3.3d). 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.

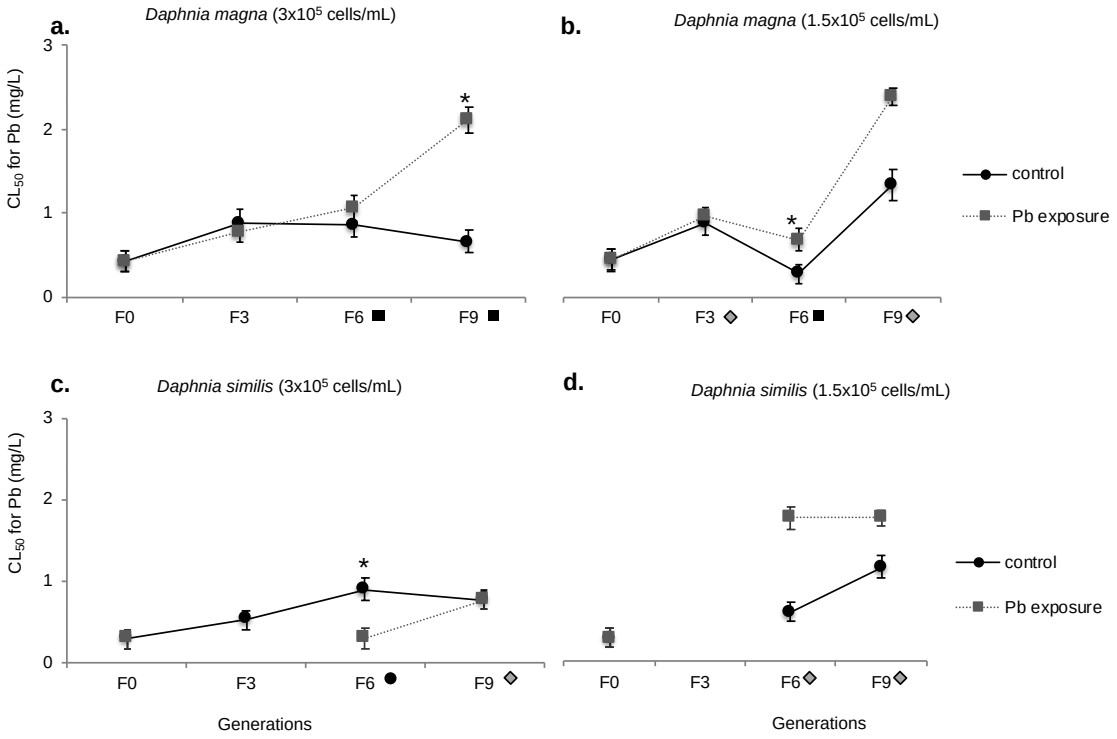

Figure 3.3: LC_{50} for a Pb 48h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with 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}.

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.

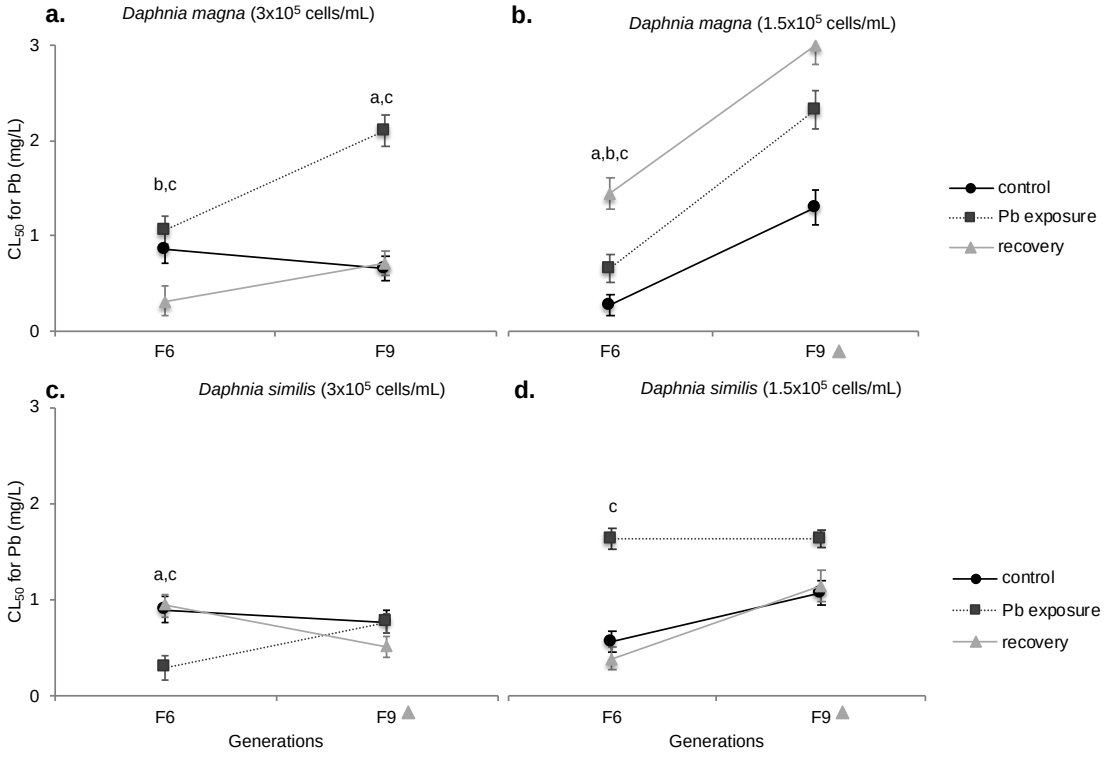

Figure 3.4: LC_{50} for a Pb 48h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ 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 3.3, just for comparison.

3.2.3. Mancozeb

The acute toxicity test with mancozeb, in which organisms from all treatments (control, Pb and recovery) 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 3.5a). 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 3.5b). 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 3.5c). However, under food restriction, organisms from control treatment showed lower sensitivity to mancozeb in comparison to continuous Pb exposure (F3 and F9) (Figure 3.5d). 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.

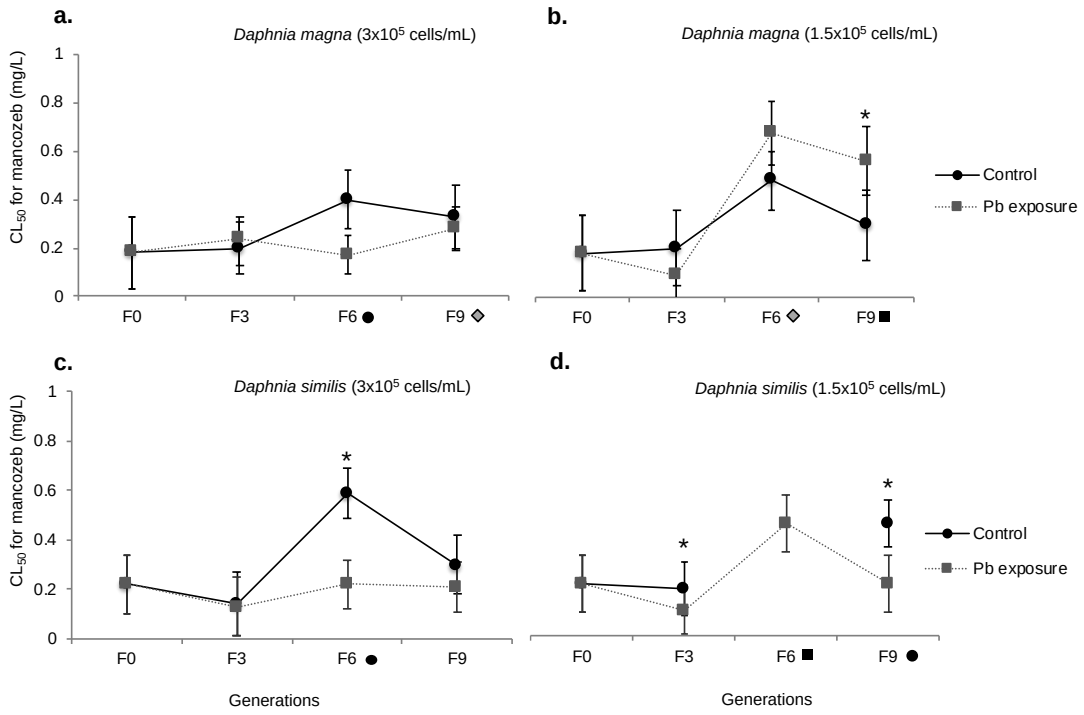

Figure 3.5: LC_{50} for a mancozeb 48h exposure of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) collected from several generations under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked 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.

During the recovery period, no difference was found between treatments on *D. magna* under usual food (Figure 3.6a). 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 3.6b). 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 3.6c). 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 3.6d). *D. similis* under continuous Pb exposure from generation F9 were significantly more sensitive to mancozeb than the respective control.

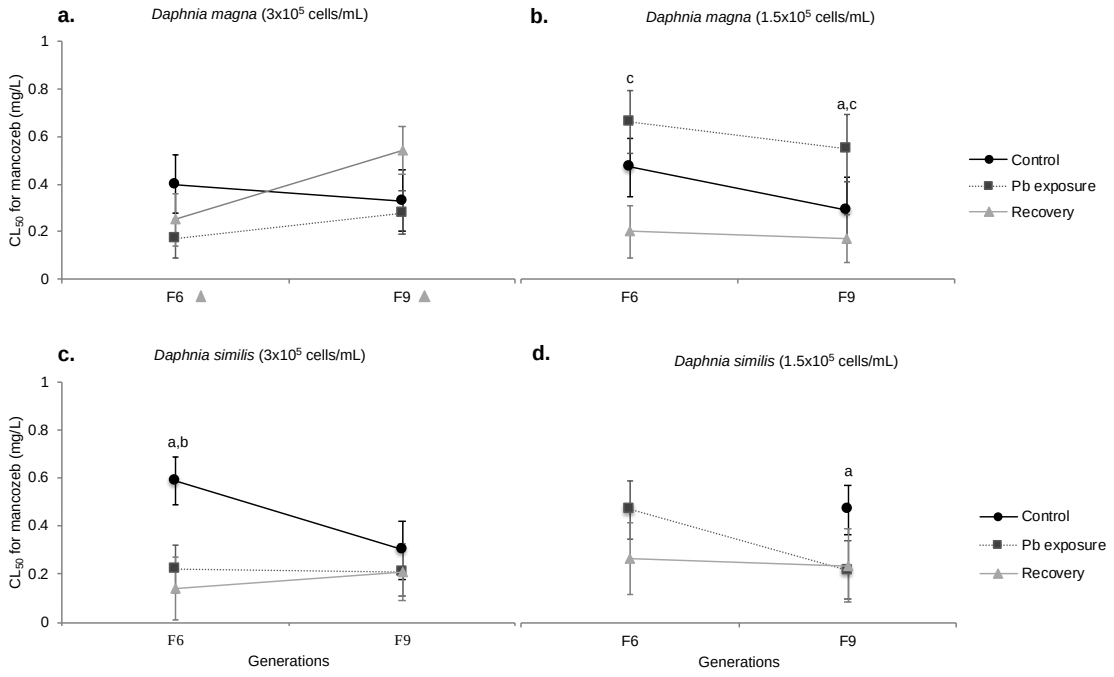

Figure 3.6: LC_{50} for a mancozeb 48h exposure of F6 and F9 *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ 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 3.5, just for comparison.

3.3. Neonate's measurement

To determine if organisms' sensitivity was related to the sensitivity 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 3.7). Two-way ANOVA statistical analysis is detailed in Tables 3S.2 (*D. magna*) and 3S.3 (*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 3.7a). 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 3.7b).

Regarding *D. similis* (under usual food), N1 control neonates were bigger than those from continuous Pb exposure in generation F3 (Figure 3.7c). 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.

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).

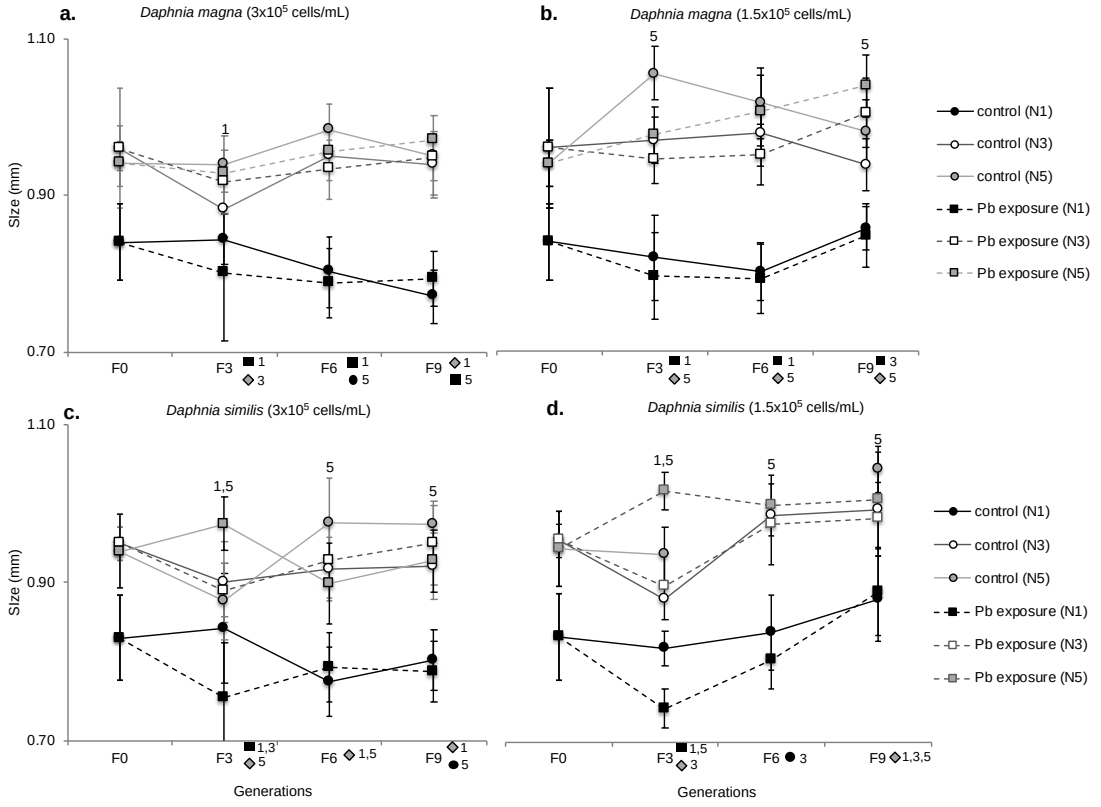

Figure 3.7: Body length of neonates from broods N1 (dark), N3 (white) and N5 (grey) of *Daphnia magna* and *Daphnia similis* in control and Pb continuous exposure through 10 generations. Generations are marked with a 1) black circle for those statistical different from F0 in the control treatment, 2) 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, $p<0.05$).

Looking at the recovery period (Figure 3.8), 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.

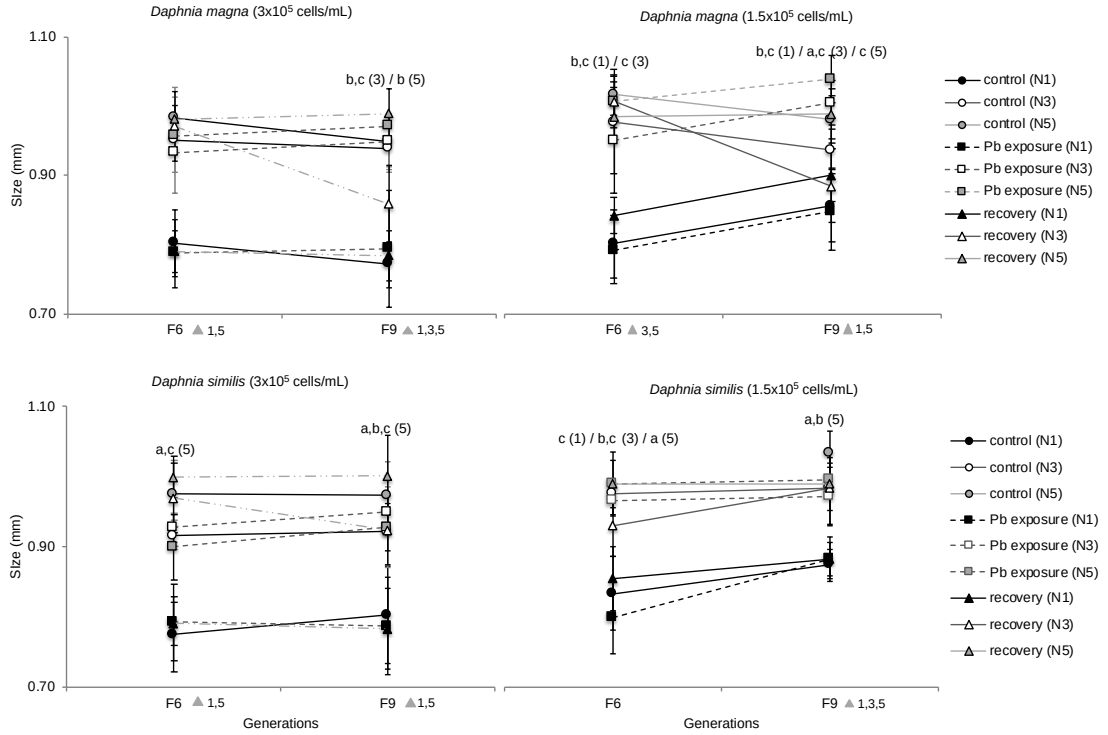

Figure 3.8: Body length of recovering neonates from broods N1 (dark), N3 (white) and N5 (grey) of *Daphnia magna* and *Daphnia similis* in control and Pb continuous exposure through 10 generations. Generations marked with a gray triangle indicate statistical difference for recovering organisms in comparison 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. Data presented for control and Pb are the same as presented in figure 3.7, just for comparison.

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*.

4.1. 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, with a higher LC_{50} in F9. This sensitivity fluctuation may be considered a natural fluctuation occurring in organisms derived from laboratory cultures. Considering the OECD guideline 202 for testing of chemicals and the ISO guideline 6341, which give an EC_{50}-24 h of $K_2Cr_2O_7$ within the range 0.6 mg/L to 2.1 mg/L, the variability acquired in this study (Cl_{50} from 0.64 up to 1.69 mg/L of $K_2Cr_2O_7$) can be considered acceptable, since it is within the proposed range. Other studies also indicate natural fluctuations on *D. magna* sensitivity to chemicals (A. R. . A. R. R. Silva et al., 2017; Stoddard, J L & 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, 2006a). 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.

In controls under usual food regimes *D. magna* shows little variation throughout generations and no difference towards F0. This linearity of control sensitivity makes even more explicit the lower Pb sensitivity developed by the continuous Pb exposure. 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 under a multi-generation control exposure tend to diminish 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., 1995b). Sustaining this data, control neonates from generation F3 showed such low Pb sensitivity that no mortality was observed under Pb exposure (0.75 mg/L at that time) and Pb concentration had to be increased (to 3 mg/L) in order to estimate *D. similis* LC_{50} to Pb on following generations.

Both species and food regimes (excepting *D. similis* food restriction due to reproduction impairment at F6) that were 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 presented increased size on brood N5 through generations, excepting *D. magna*

(usual food), that increased size on F6 and decreased (similar to F0) at generation F9. This may explain the chemical lower sensitivity pattern shown. Pavlaki et al. (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., 1995b). Other studies confirm low or no reproduction of daphnids under food restriction (Enserink et al., 1995b; Pavlaki et al., 2014; Pereira, J L & Gonçalves, 2008; Ward, T J & Robinson, 2005).

4.2. Multi-generation Pb exposure

A lower sensitivity to Pb is shown for both daphnids species through the multi-generation test, indicated by the higher LC_{50} for Pb calculated upon a sublethal exposure for several generations. 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 □g/L) under chronic Hg (3.8 □g/L) exposure for two generations (Tsui and Wang, 2005). Dietrich (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., 1990b). Other organisms, such as chironomids (*Chironomus plumosus*), also showed increased LC_{50} values when exposed to metals (8.2 to 27 mg/L for Pb) for nine generations (Vedamanikam and Shazilli, 2008). As stated before, this change in sensitivity could be acquired through physiological acclimation or epigenetic changes, underlining that physiological acclimation could develop into genetically adapted organisms along time (LeBlanc, 1982). Physiological acclimation occurs when offspring from pre-exposed parents do not endure those lower sensitivity qualities (LeBlanc, 1982), while when epigenetic changes occurs, the offspring are capable of sustain such lower sensitivity.

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. Potassium dichromate (sensitivity assay) LC_{50} shows that the previous exposure to Pb did not affect the sensitivity of daphnids to this compound. *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. A lack of sensitivity variation was found for *Daphnia pulex* throughout two generations exposed to the pharmaceutical imatinib (Borgatta, 2014). Absence of variation in the organisms sensitivity 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).

Organisms' sensitivity to specific chemicals when previously exposed to another contaminant can produce unexpected outcomes. The acute exposure (48h) to mancozeb 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 difference between treatments (excluding control from F6). 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, T J & 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*.

4.3. 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 low Pb sensitivity from F6 to F9 (differing from F0), 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 an opposite outcome in which organisms under recuperation did not differ from control (although it diverged from F0), 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. 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 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 recovery period 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, Z M & Guisande, 1992; Pieters and Liess, 2006a). 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 sensitivity than generation F0. These organisms show a trend of diminishing mancozeb sensitivity from F6 to F9, 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, T J & 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 and Janssen, 2004; LeBlanc, 1982).

4.4. Daphnia magna vs. Daphnia similis

The highlights of this study are the adverse multi-generational effects of Pb unseen in classical chronic-exposure assays such as the acclimation of both *D. magna* and *D. similis* to low concentrations of Pb under two different food regimes. And also, the adverse outcomes of both species regarding recovery period with *D. magna* relying possibly on epigenetics changes and *D. similis* on physiological acclimation. The opposite outcome under food restriction is crucial regarding natural environments and the natural fluctuating amount of nutrients. These differences between the responses of both species show that it is not straightforward to use ecotoxicological data from different species (even phylogenetically close related), common from different regions such as temperate (*D. magna*) and tropical (*D. similis*), to predict chemical hazard. We therefore state that studies

using *D. magna* to predict effects in tropical environments contamination may not have effectively represented the real sensitivity of native organisms (Flohr et al., 2012; Terra and Gonçalves, 2013). Further studies are required to increment these results such as to elucidate molecular, biochemical and physiological mechanisms. Looking at a higher level of organization, such as the rate of population increase (*r*) may also represent an important insight as reproduction was depicted as a potential adverse outcome from this multi-generational exposure (Brausch and Salice, 2011), as shown by *D. similis* under mancozeb exposure. Epigenetic assays, to infer on the phenotypic characters transgenerational transferred with no gene alteration (Kim et al., 2012), should be accomplished to clarify if the adverse effects were inherited from treated mothers, highlighting that this acclimation can increase the sensitivity to other chemicals (Ward, T J & Robinson, 2005) as shown in this study by *D. similis* exposed to mancozeb.

Acknowledgment

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Supplementary material

Table 3S.1: Chemical Analyses

Chemical Analyses				
Nominal concentrations	Stock (46.54 mg/L)	0.05 mg/L	0.125 mg/L	3 mg/L
$Pb(NO_3)_2$	48 mg/L	0.054 mg/L	0.099 mg/L	2.6 mg/L
Recovery (%)	103	108	79.2	86.7

Table 3S.2: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of *Daphnia magna* and their interaction regarding neonates' size (N1, N3 and N5). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the p value (α<0.05).

Size					
Dahnia magna (3x10^5 cells/mL) N1					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.01287	4	0.003217	F (4, 261) = 1.783	P = 0.1325
Setups	0.0006607	2	0.0003304	F (2, 261) = 0.1831	P = 0.8328
Generations	0.1458	2	0.0729	F (2, 261) = 40.41	P < 0.0001
Residual	0.4708	261	0.001804		
Dahnia magna (1.5x10^5 cells/mL) N1					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.4531	4	0.1133	F (4, 261) = 65.73	P < 0.0001
Setups	0.782	2	0.391	F (2, 261) = 226.8	P < 0.0001
Generations	0.1597	2	0.07983	F (2, 261) = 46.32	P < 0.0001
Residual	0.4498	261	0.001724		
Dahnia magna (3x10^5 cells/mL) N3					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.1435	4	0.03587	F (4, 261) = 11.35	P < 0.0001
Setups	0.02557	2	0.01279	F (2, 261) = 4.046	P = 0.0186
Generations	0.1119	2	0.05594	F (2, 261) = 17.70	P < 0.0001
Residual	0.8249	261	0.00316		
Dahnia magna (1.5x10^5 cells/mL) N3					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.05849	4	0.01462	F (4, 261) = 4.892	P = 0.0008
Setups	0.0104	2	0.0052	F (2, 261) = 1.740	P = 0.1776
Generations	0.02646	2	0.01323	F (2, 261) = 4.425	P = 0.0129
Residual	0.7802	261	0.002989		
Dahnia magna (3x10^5 cells/mL) N5					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.02667	4	0.006669	F (4, 261) = 5.163	P = 0.0005
Setups	0.01157	2	0.005785	F (2, 261) = 4.479	P = 0.0122
Generations	0.05385	2	0.02692	F (2, 261) = 20.85	P < 0.0001
Residual	0.3371	261	0.001292		
Dahnia magna (1.5x10^5 cells/mL) N5					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.04904	4	0.01226	F (4, 261) = 6.658	P < 0.0001
Setups	0.02646	2	0.01323	F (2, 261) = 7.186	P = 0.0009
Generations	0.2265	2	0.1133	F (2, 261) = 61.51	P < 0.0001
Residual	0.4806	261	0.001841		

Table 3S.3: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of *Daphnia similis* and their interaction regarding neonates' size (N1, N3 and N5). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the p value (α<0.05).

Size					
Dahnia similis (3x10^5 cells/mL) N1					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.01041	4	0.002604	F (4, 261) = 1.047	P = 0.3833
Setups	0.000343	2	0.0001715	F (2, 261) = 0.06897	P = 0.9334
Generations	0.1051	2	0.05253	F (2, 261) = 21.13	P < 0.0001
Residual	0.649	261	0.002486		
Dahnia similis (1.5x10^5 cells/mL) N1					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.07203	4	0.01801	F (4, 261) = 6.364	P < 0.0001
Setups	0.004845	2	0.002423	F (2, 261) = 0.8563	P = 0.4259
Generations	0.2481	2	0.124	F (2, 261) = 43.84	P < 0.0001
Residual	0.7384	261	0.002829		
Dahnia similis (3x10^5 cells/mL) N3					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.05644	4	0.01411	F (4, 261) = 2.763	P = 0.0281
Setups	0.01966	2	0.009829	F (2, 261) = 1.925	P = 0.1480
Generations	0.01105	2	0.005527	F (2, 261) = 1.082	P = 0.3403
Residual	1.333	261	0.005106		
Dahnia similis (1.5x10^5 cells/mL) N3					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.02789	4	0.006971	F (4, 261) = 4.086	P = 0.0031
Setups	0.01153	2	0.005766	F (2, 261) = 3.380	P = 0.0356
Generations	0.06594	2	0.03297	F (2, 261) = 19.32	P < 0.0001
Residual	0.4453	261	0.001706		
Dahnia similis (3x10^5 cells/mL) N5					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.08656	4	0.02164	F (4, 261) = 9.571	P < 0.0001
Setups	0.1569	2	0.07845	F (2, 261) = 34.70	P < 0.0001
Generations	0.03686	2	0.01843	F (2, 261) = 8.151	P = 0.0004
Residual	0.5901	261	0.002261		
Dahnia similis (1.5x10^5 cells/mL) N5					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.01702	2	0.008512	F (2, 174) = 3.912	P = 0.0218
Setups	0.01702	2	0.008512	F (2, 174) = 3.912	P = 0.0218
Generations	0.2268	1	0.2268	F (1, 174) = 104.3	P < 0.0001
Residual	0.3786	174	0.002176		

Chapter 4

Multi-generational exposure to Pb in two monophyletic *Daphnia* species: individual, functional and population related endpoints

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Multi-generational exposure to Pb in two monophyletic *Daphnia* species: individual, functional and population related endpoints

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Abstract

To better evaluate chemical damage in chronically contaminated habitats, a nine-generational exposure to Lead (Pb) was done with two monophyletic *Daphnia* species, from temperate (*Daphnia magna*) and tropical (*Daphnia similis*) environments. The multi-generational test consisted generally a continuous Pb exposed set of organisms, plus an extra control set running simultaneously. To assess daphnids recovery after Pb exposure, organisms were transferred to clean media for three extra generations (recovery period). All setups (control, Pb exposure and recovery period) were submitted to two different dietary regimes, the usual ($3x10^5$ cells/mL) and restricted food ($1.5x10^5$ cells/mL) regimes. To evaluate the effects of generational Pb exposure and food regimes, individual, functional and population related endpoints were assessed (number of offspring, body length and rate of population increase (*r*) and feeding rate (FR)). The tests were conducted on the first (F0) and last generations (F9). No difference was shown on number of offspring and feeding among control and continuous Pb exposure *D. magna*. However, an enhanced rate of population increase (*r*) was shown by Pb exposed organisms (both food regimes). *D. magna* from recovery period did not cope well with a Pb re-exposure (during the ecotoxicity test) under usual food. Food restricted Pb exposed *D. magna* showed enhanced sensitivity and recovering organisms presented the highest rate of population increase (*r*). Continuous Pb exposure *D. similis* under usual food exhibited a higher rate of population increase (*r*) and algae consumption. Recovering *D. similis* did not show a full retrieval for any of the endpoints evaluated (except for FR under food restriction). Both species presented similar patterns regarding generations. Organisms from F0 presented enhanced reproductive outputs in comparison to F9 and the contrary occurred to the FR (even in control organisms). Data show an acclimation under a generational Pb exposure, which could increase the population of less sensitive organisms in natural habitats. And, since there was not a full recovery after three generations in clean media, an indication of epigenetic changes for both species may also be considered.

Key-words: multi-generation, daphnids, Lead, reproduction endpoints, feeding rate

1. Introduction

Natural environments may be under anthropic pressure and metals are important contaminants that can generate negative impacts. Lead (Pb) is a neurotoxic non-essential metal (Reddy et al., 2003), which was used in this study as a chemical model to analyse the effects of a long-chronic generational exposure to two *Daphnia* species. This metal is

not required by any metabolic activity in organisms and its atmospheric input is due mainly to industries and vehicular exhaustion (Valavanidis & Vlachogianni, 2010), and other sources include manufacture, burning of fossil fuels and mining (Tchounwou et al., 2012). The European Chemicals Agency (ECHA) recently added (June of 2018) the metal Pb to the Candidate List of substances of very high concern (SVHCs). A great variety of metals has been studied regarding toxicity on daphnids such as *D. magna* (Komjarova and Blust, 2008), *D. similis* (Soundrapandian & Venkataraman, 1990) and *D. longispina* (Venâncio et al., 2018). Daphnids are filter feeding cladocerans that live in natural freshwater systems, that are easily cultivated in laboratory conditions, presenting a rapid life cycle and therefore widely used in ecotoxicological studies (Münzinger & Monicelli, 1992).

Existing standard test protocols are adequate to analyse acute and chronic (reproductive) short-term daphnids outputs (OECD, 2012, 2004), linking individual to population effects. In addition, their feeding performance can be also a crucial measurable endpoint as it provides a fitness and functional link between feeding behaviour, food impairment (Loureiro et al., 2012), and reproductive outputs (Kluttgen and Ratte, 1994; Pieters et al., 2005).

Standard protocols, however, may lack robustness when it comes to natural ecosystems and long-term chronically contaminated habitats. Daphnids reproduce by parthenogenesis, producing thus genetically identical individuals, which is really useful to achieve a more realistic approach through multi-generation assessments (Guan & Wang, 2006b). Assessing the long-term exposure (in terms of generations) to chemical contamination is crucial to truly understand the effects on natural biota, better mimicking the pressure situation that organisms may be living (Tsui & Wang, 2005).

Additionally, when natural habitats are under consideration, several factors can influence on the organism's responses to toxicants. Food can sometimes be scarce and this may change organisms' sensitivity to chemicals (Glazier, 1992; Heugens et al., 2001), as well as different global latitudes (often related to the temperatures) such as tropical and temperate environments (Ghilarov, 1967). Since environmental conditions may continuously change, to check for organisms' recovery is essential to determine how changes in chemical sensitivity was developed. A period of retrieval may help to distinguish if organisms' sensitivity was due to physiological acclimation or epigenetic changes. As stated before, organisms physiologically acclimated will achieve a full recovery when chemical exposure ceases (LeBlanc, 1982), while organisms that fail to recover may be presenting epigenetic changes. Epigenetic changes may affect organisms sensitivity through transgenerational transfer, being the study of changes in gene function (mitotically

and/or meiotically heritable) that do not entail a change in DNA sequence (Wu and Morris, 2001).

Considering the above mentioned, and to bridge the knowledge gap on long term exposure scenarios, the goals of this study were: 1) To evaluate the generational exposure induced chronic effects at an environmentally relevant concentration of Pb; 2) Compare those effects in two monophyletic *Daphnia* species from different climates; 3) Check for recovery after Pb exposure and; 4) Evaluate the nutrition effect on organisms' sensitivity. To accomplish that, multi-generational exposed organisms were submitted to reproduction and feeding rate (FR) tests at the first and last (ninth) generation. To the best of our knowledge, this is the first study to cover chronic endpoints after a multi-generational exposure to Pb on daphnids.

2. Methodology

2.1. Culture maintenance

Daphnids were maintained in ASTM hard water (American Society for Testing Materials) (ASTM, 2002) enriched with an organic extract (Marinure seaweed extract; Glenside Organics Ltd.) (Baird et al., 1989) and fed with *Raphidocellis subcapitata* ($3x10^5$cells/mL). Cultures were maintained under a specific photoperiod and temperature (16:8h light/dark; 20º±2ºC). Both species were maintained exactly under the same conditions to exclude the influence of other abiotic factors. ASTM medium and food were renewed every other day. New cultures were initiated with less than 24h old neonates from the third to fifth broods.

2.2. Multi-generation exposure setup

Each *Daphnia* species was submitted to four setups in total, a negative control (ASTM media, same used for culture maintenance) and continuous Pb exposure (50 μg/L of Pb in ASTM), which ran at same conditions of light and temperature (16:8h light/dark; 20º±2ºC). The sub-lethal Pb concentration (50 μg/L) is the maximum permitted by the Brazilian federal legislation (CONAMA, 2005) for fresh water bodies. Both setups were maintained at two different dietary regimes, the usual ($3x10^5$ cells/mL) algae concentration and a food restriction regime ($1.5x10^5$ cells/mL). The setups were maintained in one litter vial with 20 neonates (less than 24h old) and three replicates each (Figure 1.2). For all vials (replicates) the 3rd brood of neonates was used to start the immediate generation for the same treatment and replicate. In addition to this experimental set up, a recovery period was

settled using some of the F6 neonates from the Pb treatment (brood N3 generated from F5), by transferring them to a clean ASTM media till the end of the F9 generation. The other set of F6 Pb exposed neonates were kept exposed till the end of the F9 generation.

2.3. Chemical analyses

Stock solution for $Pb(NO_3)_2$ (CAS No. 10099-74-8, 98.5% purity, VWR chemicals®) was prepared in mili-Q water, and then used for preparing the continuous Pb exposure and the different concentrations in ASTM media for the reproduction and feeding tests.

Pb samples were acidified (nitric acid) and then analyzed by ICP-OES (Horiba Jobin Yvon, Activa M). Pb samples were evaluated in triplicate and duplicate samples of certified material were used, to ensure chemical optimum recovery during procedures.

2.4. F0 and F9 Generational testing

Ecotoxicity tests were carried out with neonates collected from F0 (which were directly collected from cultures) and F9 (both control and Pb exposure). This approach provided information on the health status of organisms considering their exposure to Pb for 9 generations, under two food regimes, and also considered a recovery period that started at F6.

2.5. Reproduction test

Neonates from each setup aging between 6 and 24 hours were collected randomly and then exposed to a range of Pb concentration for 21 days (0.25 and 0.5 mg/L for *D. magna* and 0.25, 0.43 and 0.5 mg/L for *D. similis* plus a negative control). Test concentrations were chosen based on the highest sub-lethal Pb exposure possible (for 21 days), and decreasing concentrations were established by dividing by factor 2. The test was based on the 212 guideline for testing chemicals developed by OECD (OECD, 2012). Briefly, neonates were exposed individually in 50mL glass vials, with each concentration presenting five replicates. Replicates were checked daily and the number of offspring recorded. Food (*R. subcapitata* at $3x10^5$ cells/mL) and medium were renewed at every 48 hours. Parental *Daphnia* body length was measured at the end of the test.

2.6. Feeding Inhibition Test

Neonates from each setup aging less than 24 hours were randomly separated to new vials and maintained until they in reach the 4^{th} instar, at when they were exposed to a

range of Pb concentrations for 24 hours (0.5, 1, 1.5, 2 and 3 mg/L, plus a negative control, being 3 mg/L the highest sub-lethal Pb exposure possible, for 24 h). The test was conducted according to McWilliam & Baird (2002) and feeding rate (FR) (cells per individual per hour) was estimated as described in Allen et al. (1995) and expressed as cells per individual per hour. The test vials (with 150 mL capacity) were filled with 100ml of test-solution and five neonates each. The test-solutions consisted of ASTM medium plus $5x10^5$ cells/mL of *R. subcapitata* (control) and the increasing Pb concentrations (mg/L). The vials were left in the dark for 24h. After that, the concentration of algae cells was measured by spectrophotometry (absorbance 440 nm) (Jenway model UV-VIS 6505). Blank controls with only test-solutions and no daphnids for each concentration and species were made to monitor algae population stability.

2.7. Data processing and statistical analysis

The reproductive rate was given by the amount of produced offspring. The intrinsic rate of population increase (*r*) was calculated using the Euler's equation as described in Pestana et al. (2013) and the replicate pseudo values for *r* were generated using the jackknife method based on Meyer et al. (1986). The FR was estimated as in Allen et al. (1995).

Data from each sublethal endpoint (number of juveniles, body length, rate of population increase (*r*) and FR) derived from the toxicity tests carried out with F0 and F9 neonates were first checked for normality (Kolmogorov–Smirnov) and then evaluated through a Two-Way Analysis of Variance (ANOVA), being the factors the generations and the setups. This analysis was followed by a post-hoc test (Bonferroni) when statistical differences were first highlighted (GraphPad Prism®).

3. Results

3.1. Chemical analyses

The comparison of nominal and measured concentrations retrieved a >79% recovery for Pb samples. The limit of quantification (LOQ) for Pb was 25 µg/L and ASTM (control) presented values <LOD (Table 4S.1). The certified material evaluated had >80% of recovery.

3.2. Chronic reproduction test

The chronic assay was conducted with daphnids (all setups) from F0 (equivalent to culture daphnids) and F9 (under control and Pb exposure) to evaluate their reproduction rate, body length and the rate of population increase (*r*) (Figure 4.1 and 4.2).

3.2.1. Daphnia magna

3.2.1.1. F0 vs. control F9

Comparing generation F0 to the control treatment of generation F9 (usual food) it is clear that organisms from F0 presented a higher number of juveniles, body length and rate of population increase in comparison to control F9 (for the ASTM exposure and both Pb concentrations of 0.25 and 0.5 mg/L) (Figure 4S.1). In addition, the F9 control presented higher negative responses towards Pb exposure than the F0 generation regarding body length and *r*. Under food restriction organisms followed a similar pattern and daphnids from F0 presented higher outcomes in comparison to control F9 for all concentrations and endpoints evaluated (reproduction, body length and *r*), except for body length at concentration 0.5 mg/L, that did not differ between generations.

3.2.1.2. Control vs. Pb exposure (F9)

Considering the differences between F9 control and F0, the F9 Pb exposure outcome was only compared to the F9 control set. The reproductive rates and body length of control and continuous Pb exposure from generation F9 presented no divergence among treatments (Figure 4.1). However, the rate of population increase (*r*) of control organisms was higher than for continuous Pb exposure when organisms were not exposed to Pb (0 mg/L) and control *r* gradually decreased with increasing Pb concentration. This pattern did not happen for Pb exposed organisms, which presented a higher *r* than control at concentration 0.5 mg/L. Under food restriction, females from continuous Pb exposure did not endure high Pb exposure and died at 0.5 mg/L. Control and Pb treatments differed only when organisms were not under Pb exposure (0 mg/L) considering *r*, with control exhibiting a lower *r* than continuous Pb exposure.

3.2.1.3. Recovery period

After the recovery period under usual food regime, daphnids enhanced their offspring production when compared to those from control or continuously exposed to Pb, when looking at all concentrations. However, they did not survive at high Pb exposure (0.5

mg/L). The females' body lengths showed no statistical differences among all setups (even with live recovering daphnids). Recovering organisms exhibited an enhanced *r* in comparison to Pb exposure and no difference to control at 0 mg/L. When increasing Pb concentration (0.25 mg/L), recovering daphnids' *r* was higher than the other treatments (control and Pb), until the 0.5 mg/L exposure, where lethality was induced.

Recovering organisms under food restriction showed a similar pattern as those from usual food, with a higher offspring production in comparison to the other two treatments (at 0 mg/L). This pattern did not linger at higher Pb concentrations, diminishing offsprings and being similar to control with increasing Pb concentrations. The body length indicated no statistical difference between setups. The rate of population increase (*r*) of recovering organisms was higher than control at 0 and 0.5 mg/L, being the highest *r*. Organisms kept till F9 under Pb exposure and food restriction did not survive under 0.5 mg/L.

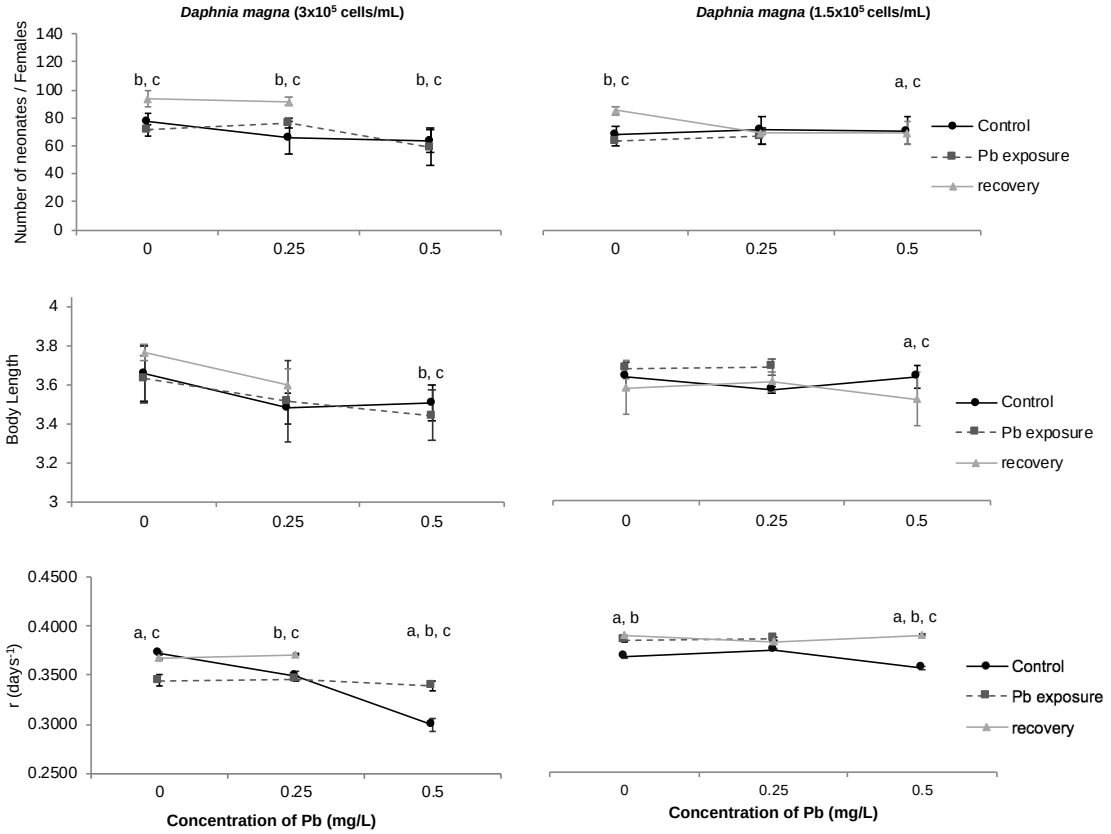

Figure 4.1: Chronic test outcome for F9 *Daphnia magna* kept under control and Pb exposure during the multi-generation test and under recovery period from F6 to F9, and under two food regimes (usual and restricted food regime). Results present: the number of offspring, body length and rate of population increase (*r*). Letters designate statistical difference among treatments, being (a) Pb vs. control, (b) recovery vs. control, (c) Pb vs. recovery (Bonferroni, $p<0.05$). No data is available for 0.5 mg/L Pb exposure *to Daphnia magna* due to the occurred mortality.

3.2.2. Daphnia similis

3.2.2.1. F0 vs. control F9

At high Pb exposure (0.5 and 0.62 mg/L), control organisms from F9 differed from F0, presenting a lower offspring production (Figure 4S.2). No divergence was seen for F0 and control F9 body length, however, control organisms (F9) died at 0.62 mg/L. However, a higher rate of population increase © was shown for F0 in comparison to control F9 for almost all Pb concentrations. When exposed to Pb, control organisms (F9) did not endure Pb exposure and died at 0.25 mg/L under food restriction. No divergence was shown for reproduction and body length between F0 and control F9. However, control organisms (F9) indicated an enhanced *r* than F0 while alive.

3.2.2.2. Control vs. Pb exposure (F9)

D. similis (usual food) from continuous Pb exposure had higher sensitivity, producing less neonates and being statistically different from control at concentrations 0.25 mg/L and 0.43 mg/L. Daphnids from both treatments died at higher Pb exposure (0.62 mg/L). No divergence between setups was shown regarding body length. The rate of population increase © of continuous Pb exposure presented the lowest rate © under no Pb exposure (0 mg/L), shifting to be higher than control until animals' death. Under food restriction patterns varied and continuous Pb exposed organisms were the only survivals at the higher Pb concentration (0.5 mg/L). Control daphnids exhibited enhanced outcomes (reproduction, body length and *r*) while alive (until 0.25 mg/L). Pb treatment presented a slight decrease of all endpoints with increasing Pb concentration.

3.2.2.3. Recovery period

Organisms under recuperation were the only ones to survive at the highest Pb concentration (0.62 mg/L) during the reproduction test. The recovery period induced a higher offspring production than continuous Pb exposure, but similar to control. No statistical difference among setups was shown by females' body length until the highest Pb concentration, in which organisms from recovery period were the only survivors. The rate of population increase © presented a considerable variability. Recovering daphnids' *r* was higher than those from continuous Pb exposure and similar to control under no Pb exposure

(0 mg/L). At 0.43 mg/L, recovering organisms presented a lower *r* than organisms from continuous Pb exposure but higher than control. This pattern changed at 0.5 mg/l, where recovery period organisms showed a similar outcome than continuous Pb exposure and higher *r* than control organisms.

Under food restriction, recovering organisms showed a gradual decrease on their reproduction rates and did not survive at the Pb concentration of 0.5 mg/L. While alive, recovering organisms presented a lower reproduction rate than control at 0 and 0.25 mg/L and than Pb at 0.43 mg/L. The body length of control organisms was higher than those from recovery period at 0 mg/L, with no statistical differences shown for the other concentrations (despite death). The rate of population increase © for recovering organisms was the lowest in comparison to both other treatments, although they were the only survivals.

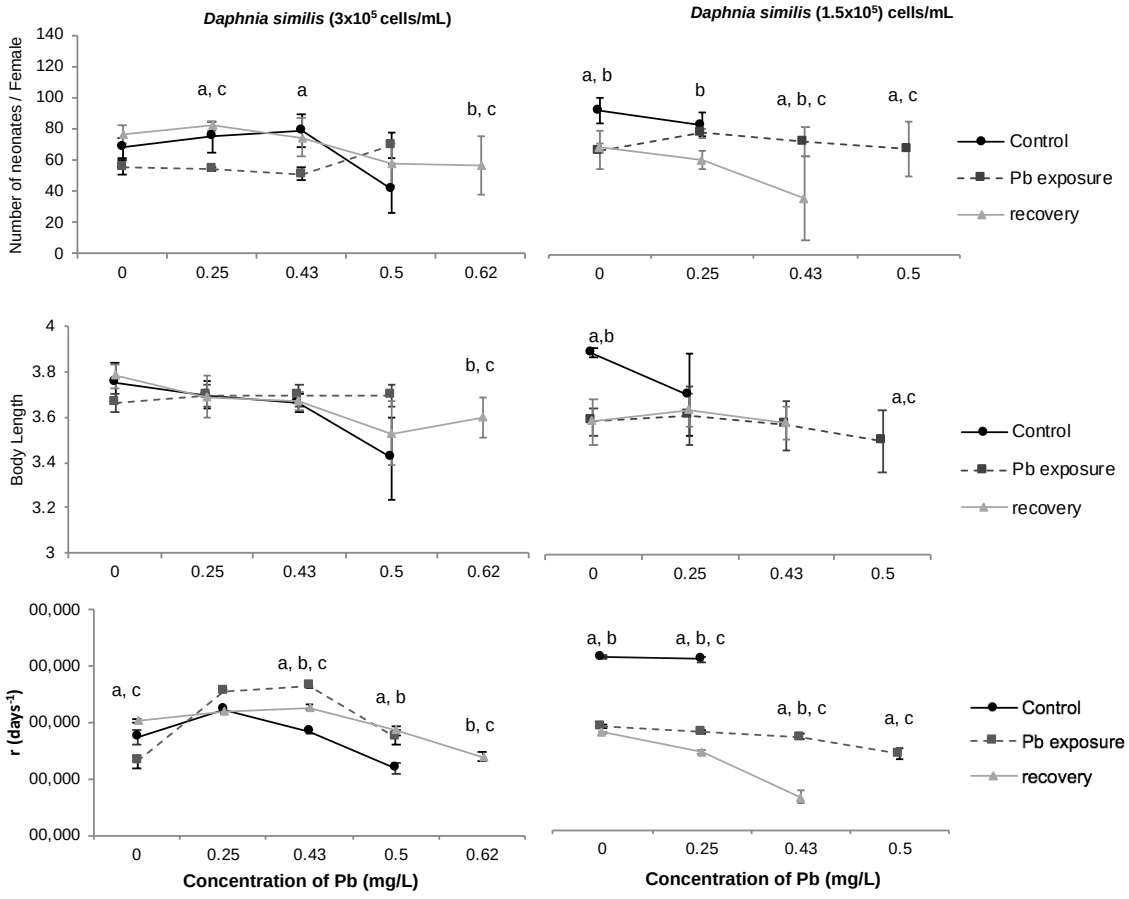

Figure 4.2: Chronic test outcome for *Daphnia similis* kept under control and Pb exposure during the multi-generation test and under recovery period from F6 to F9, and under two food regimes (usual and restricted food regime). Results present: the number of offspring, body length and rate of population increase ©. Letters designates statistical difference among treatments, being (a) Pb vs. control, (b) recovery vs. control, (c) Pb vs. recovery (Bonferroni, $p<0.05$). *Daphnia similis* missing data (control and Pb from F9 at usual food and control and recovery from F9 at restricted food regime) is due to the occurred mortality.

3.3. Feeding Inhibition Test

The feeding inhibition test was conducted with organisms from generations F0 (from culture organisms) and F9 under continuous control conditions, under continuous Pb exposure and under a recovery period from F6-F9, to evaluate the algae consumption under increasing Pb exposure (Figures 4.3 and 4.4).

3.3.1. Daphnia magna

3.3.1.1. F0 vs. control F9

Animals from F0 displayed a lower FR at concentrations 1 and 3 mg/L than control F9, decreasing algae consumption with increasing Pb concentration (Figure 4S.3). Under food restriction an opposite outcome is shown and F0, which presents an enhanced FR in comparison to control F9 at 0 mg/L. This pattern reverses and control F9 show an enhanced algae consumption at 1 and 3 mg/l compared to F0 (similar to usual food).

3.3.1.2. Control vs. Pb exposure (F9)

No divergence on FR between control and Pb treatments is shown under usual food regime. Under food restriction organisms were not evaluated due to the lack of neonate production (reproduction impairment).

3.3.1.3. Recovery period

Recovering *D. magna* under a usual food regime exhibited a lower FR in comparison to control organisms under no Pb exposure (0 mg/L) and that was the only statistical difference presented among setups. Under food restriction, control and recovering organisms presented similar algae consumption and did not exhibit any statistical differences.

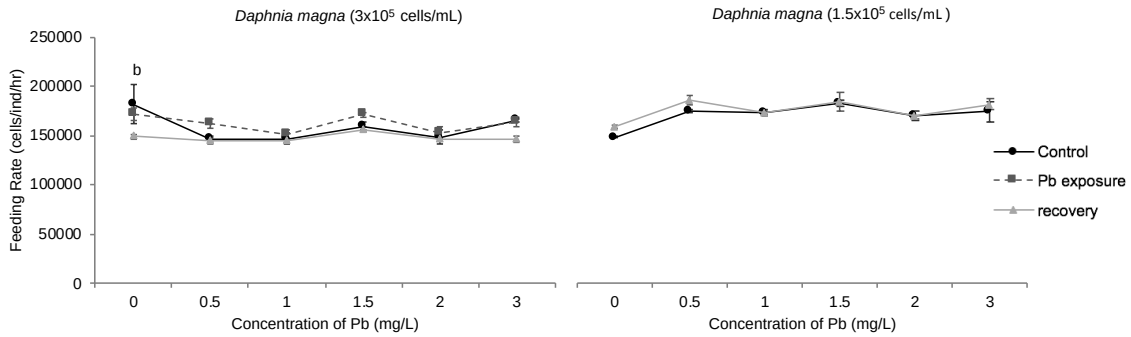

Figure 4.3: Feeding inhibition test outcome for *Daphnia magna* kept under control and Pb exposure during the multi-generation test and under recovery period from F6 to F9, and under two food regimes (usual and restricted food regime). Letters indicates statistical difference among treatments, being (a) Pb vs. control, (b) recovery vs. control, (c) Pb vs. recovery (Bonferroni, $p<0.05$). Missing data (continuous Pb exposure) was due to a lack of neonate production.

3.3.2. Daphnia similis

3.3.2.1. F0 vs. control F9

The FR of F0 organisms decreased with increasing Pb concentrations for both food regimes and exposure periods (Figure 4S.4). F0 differed from control F9 at 0.5 (higher FR), 1.5, 2 and 3 mg/L (lower FR). Under food restriction, the same pattern maintained, and F0 FR decreased with increasing Pb concentration, indicating a lower FR than control F9 at concentrations 1.5, 2 and 3 mg/L. F0 FR was higher than control F9 at 0.5 mg/L.

3.3.2.2. Control vs. Pb exposure (F9)

Continuous Pb exposure indicated a higher FR than control only at the highest Pb concentration of 3 mg/L. Under food restriction a similar outcome that occurred for *D. magna* was also observed for *D. similis* and no daphnids from the continuous Pb exposure were tested due to the lack of neonate production.

3.3.2.3. Recovery period

Recovering organisms presented a higher FR in comparison to control at concentrations 1and 3 mg/L but no difference regarding continuous Pb exposure. Under food restriction, the same outcome presented by *D. magna* was also observed for *D. similis* and no FR could be evaluated for continuous Pb exposure (due to reproduction

impairment). No difference among setups (control and recovery period) was shown under food restriction.

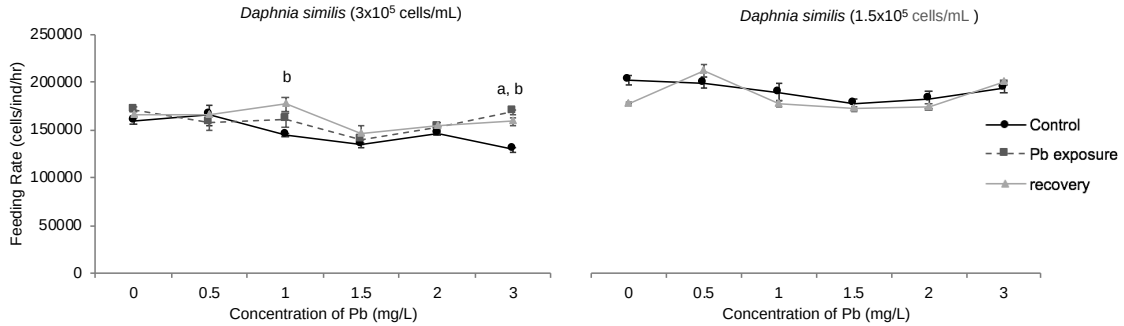

Figure 4.4: Feeding inhibition test outcome for *Daphnia similis* kept under control and Pb exposure during the multi-generation test and under recovery period from F6 to F9, and under two food regimes (usual and restricted food regime). Letters indicates statistical difference among treatments, being (a) Pb vs. control, (b) recovery vs. control, (c) Pb vs. recovery (Bonferroni, $p<0.05$). Missing data (continuous Pb exposure) was due to a lack of neonate production.

4. Discussion

4.1. Control chronic outcomes among generations (F0 vs. F9 control)

Comparing generations, higher reproductive outcomes are shown for F0 organisms in comparison to the F9 under control conditions (both food regimes and species), suggesting a natural fluctuation within generations that are kept under optimal and same laboratory conditions. This natural variance in daphnids under optimum conditions has already been reported before (A. R. . Silva et al., 2017).

F9 control *D. magna* under a usual food regime maintained the reproduction rate stable with increasing Pb concentrations, showing that the highest Pb concentration (0.5 mg/L) was not enough to cause an effect. A reduction in reproduction rate of *D. magna* was observed at 0.9mg/L of Pb by Enserink et al., (1995). However, Pb exposure decreased the body length and the rate of population increase (*r*) of F9 control organisms. Reduced body length was also observed for *D. magna* acclimated to 0.15 mg/L of Cu (Bossuyt & Janssen, 2004). Non-acclimated *D. longispina* showed a 20% lower effectiveness regarding increase in body length and 45% reduction regarding population increase (*r*) in comparison to Cu (mine drainage) acclimated organisms (Agra et al., 2011). Among individuals kept under

food restriction, no significant variations with increasing Pb concentration were seen for non-acclimated F9 control organisms, probably also due to the low Pb concentrations used.

Regarding algae consumption, F9 control *D. magna* from usual and restricted food regimes exhibited a higher FR than organisms from F0. F9 control *D. magna* presented a stable FR among Pb exposure, while F0 organisms diminished FR with increasing Pb concentration (both food regimes). Again, this variance in control populations is not uncommon. Chemical sensitivity variation was also shown by Ward & Robinson (2005) that described discrepancies in organisms' sensitivity to Cd (immobilization test), indicating a high sensitivity variance among *D. magna* cultures (from 0.02 up to 0.12 mg/L of Cd). Concerning the non-variance for F9 control *D. magna* FR, it should be noticed that at 1.7 mg/L of Pb, McWilliam & Baird (2002) found no effect on *D. magna* feeding rates, indicating that at some concentrations Pb exposure may not affect daphnids algae consumption (3 mg/L affected F0 but not F9 control).

F9 control *D. similis* (usual food) showed lower reproduction and rate of population increase (*r*) at the highest Pb concentration in comparison to F0 organisms. Here, again, it may be probably a fluctuation on organisms' sensitivity. F9 control *D. similis* (usual food) tend to reduce all reproductive outputs with increasing Pb concentration. Soundrapandian & Venkataraman (1990) reported a reduced fecundity on *D. similis* after 10 days of Pb exposure but at a much lower concentration (0.005 mg/L). The restriction of food enhances *D. similis* sensitivity to Pb and F9 control organisms dye under Pb exposure, however, an enhanced rate of population increase (*r*) was presented in comparison to F0 while F9 control were alive. Therefore, food restriction can enhance daphnids sensitivity (in terms of reproduction success and population increase), which was also shown in the study of Rodgher et al. (2010) where the presence of algae cells lowered *D. similis* sensitivity to chromium.

D. magna and *D. similis* control organisms seem to exhibit a similar feeding pattern (both food regimes): a stable algae consumption with increasing Pb concentration and a lower FR of F0 organisms in comparison to F9 control at high Pb exposure. To our knowledge, no study with *D. similis* feeding rate and Pb exposure (or any other chemical) exist to corroborate such outcomes.

4.2. Continuous Pb exposure

Regarding the number of offspring, no divergence among F9 control and F9 continuous Pb exposure *D. magna* (usual food) was shown. Corroborating with our results, Ward & Robinson (2005) did not find alterations regarding reproduction on Cd-adapted (60

µg/L) *D. magna* in an eight generation test. In our study, a lack of disparities between setups was also shown for body length. Results change regarding rate of population increase (*r*), and F9 Pb acclimated organisms present a higher rate of population increase (*r*) than F9 control. Therefore, regarding this outcome, Pb acclimated *D. magna* have a better response at higher Pb exposure than non-acclimated organisms (F9 control). Münzinger & Monicelli (1992) obtained increased *r* for chromium adapted *D. magna* in comparison to control organisms for seven generations under 5 µg/L of Cr and six generations under 10 µg/L of Cr.

A higher Pb sensitivity was shown under food restriction and F9 Pb exposed *D. magna* died at the highest Pb concentration, indicating that food affects organisms' response to Pb exposure. Body length did not differ among setups and the rate of population increase (*r*) of F9 continuous Pb exposure was higher than F9 control (before death). This may highlight an investment in reproduction with increasing Pb concentration, but such ability is quite limited, and at a certain concentration daphnids may lack energy to survive (as shown by F9 Pb continuous exposure). Increased *r* for acclimated daphnids was shown before (Münzinger and Monicelli, 1992) as well as enhanced chemical sensitivity due to low food content (Rodgher et al., 2010).

The feeding inhibition test with *D. magna* (usual food) indicated that F9 control and F9 Pb exposure presented similar results, implying that organisms under a multi-generational exposure to Pb are not significantly affected when compared to the control. Tsui & Wang (2005) affirmed that if organisms are exposed to a certain contaminant for many generations the FR of exposed organisms may not differ from control due to acclimation. This absence of divergence between treatments was also shown regarding number of offspring, although, a higher rate of population increase (*r*) was exhibited by organisms under continuous Pb exposure (F9). A higher rate of population increase (*r*) of acclimated organisms may lead to less sensitive populations.

The FR of F9 continuous Pb exposed *D. magna* under food restriction could not be evaluated due to reproduction impairment. This outcome is supported by the reproduction test, in which Pb exposed daphnids did not endure high Pb concentrations, indicating higher sensitivity under food restriction and long chronic Pb exposure. Diminished reproduction in organisms under food restriction and Pb exposure (up to 3.20 mg/L) was also shown by Enserink et al. (1995).

The reproduction patterns (number of offspring) of usual food regime F9 continuous Pb exposure *D. similis* died at the highest Pb concentration and presented lower number of offspring than F9 control on lower Pb exposure. Sotero-Santos et al. (2005) indicated that

sludge exposure in the presence of metals reduced *D. similis* reproduction. Jardim et al. (2008) indicated *D. similis* reproduction impairment when exposed to sediments contaminated with metals. Despite the reproduction discrepancies, adult females body length presented no statistical difference among setups. F9 continuous Pb exposure presented a lower *r* than F9 control under no Pb exposure (0 mg/L), suggesting that in a non-contaminated environment, non-exposed organism may have a better performance. However, an increased *r* under low Pb concentration was evidenced for *D. similis* (as well as *D. magna*), but cannot be sustained as organisms died in higher concentrations exposures. Concerning low Pb concentrations, enhanced rates of population increase (*r*) from metal exposed daphnids has already been shown in previous studies (Agra et al., 2011; Munzinger, 1990).

Regarding food restriction, only F9 continuous Pb exposure *D. similis* survived high Pb concentration. F9 continuous Pb exposure exhibited higher reproduction and rate of population increase (*r*) than F9 control, due to control`s death (as said before, low food content enhanced *D. similis* F9 control sensitivity to Pb). These outcomes indicate that a continuous Pb exposure for generations diminishes *D. similis* sensitivity in comparison to control under nutrient restriction. To our knowledge, no study exposed *D. similis* over generations to chemicals for a similar comparison to this study. In addition, a medium with higher food concentration may accelerate organisms metabolism and consequently increase metal accumulation (Heugens et al., 2001).

D. similis and *D. magna* diverged also regarding algae consumption. F9 continuous Pb exposure *D. similis* (usual food) presented a higher algae consumption in comparison to F9 control. This data differs from the low reproduction shown by F9 Pb exposed *D. similis* but supports the higher *r* displayed (until death). Boersma & Vijverberg (1995) stated that the rate of population increase (*r*) enhances with enlarged food concentration. To enhance food consumption is a way to accelerate gut clearance (Skjolding et al., 2014) and consequently help depuration.

As well as it occurred for *D. magna*, no feeding toxicity test could be performed with neonates from F9 continuous Pb exposure *D. similis* under food restriction due to reproduction impairment. During the reproduction test, F9 control *D. similis* under food restriction died when exposed to Pb (low food content enhances Pb sensitivity) and F9 Pb exposed food restricted *D. similis* were the only survivors. However, the lack of neonates to accomplish the feeding toxicity test is in accordance with the reproduction test, where under no Pb exposure (0 mg/L), F9 control presented higher reproduction than F9 Pb exposure. Therefore, although F9 control food restricted *D. similis* died when exposed to Pb, it was

able to give neonates to perform the feeding toxicity test (under no Pb exposure), unlike F9 food restricted continuous Pb exposure. Such results suggests a powerful effect on *D. similis* sensitivity when Pb exposure and food restriction act together, also shown by (Enserink et al., 1995b).

4.3. Recovery period

Recovering *D. magna* (usual food) showed enhanced reproductive outputs (number of offspring and rate of population increase (*r*)) and similar body lengths compared to F9 control and continuous Pb exposure, however, it did not cope well to a Pb re-exposure, inducing a lethal effect. Increased reproduction under previous metal exposure has already been shown on organisms under recovery. Massarin et al. (2010) observed an increase of 28% in the reproduction rate of *D. magna* offspring on clean media after parental exposure to uranium (ranging from 10 to 75 µg/L) for three generations. *D. magna* pre-exposed to Ni (160 µg/L) for seven-generations also indicated higher reproduction under a Ni re-exposure (Munzinger, 1990). Since control and recovery period treatments exhibit different outcomes, a failed retrieval (may be due to epigenetics) is suggested. As stated before, organisms chronically exposed to chemicals may undergo trough physiological acclimation (full retrieval) or epigenetic changes (failed retrieval). In another study with *D. magna* acclimated for two generations to nanosilver (AgNP), organisms did not recover when transferred to a clean media for two generations (Völker et al., 2013).

Differing from usual food regime, recovering food restricted *D. magna* exhibited a higher number of offspring under low Pb exposure than F9 control, reducing reproduction with increasing Pb concentration (becoming similar to F9 control). Despite achieving similar results as F9 control, the enhanced number of offspring at low Pb concentrations of recovering *D. magna* indicates that no full retrieval occurred and, consequently, such outcomes are related to epigenetic changes or organisms need more time achieve a full recovery (Morgan et al., 1990). Increased reproduction may be designated as a “catch-up reproduction”, which occurs as a way to compensate for the reduced offspring production shown during the period of stress (Hayashi et al., 2008). Despite the reproduction differences, body length did not differ among setups. However, recovering *D. magna* presented a high rate of population increase (*r*). Turko et al. (2016) indicated that *D. magna* under low contamination present a significantly reduced *r* under a Pb re-exposure whereas those from high Pb pollution indicate the opposite. Our results suggest that non-exposed *D.*

magna displays lower reproductive outputs than exposed organisms. This is crucial in natural environments, since it can lead to a population growth of less sensitive organisms, and, since they can remain less sensitive to specific contaminants (recovery period), such data can be essential to risk assessment studies (Lopes et al., 2006).

D. magna from recovery period (usual food) presented the lowest FR, indicating that organisms under a previous Pb exposure do not cope well to a re-exposure, reinforcing the reproduction data obtained (death at high Pb exposure). No evidence of recovery regarding feeding was shown by the isopod *Cyathura carinata* after 24h in clean media (48h and 96h of Cd exposure; 500 and 5000 µg/L) (Pais-Costa et al., 2015). Guan & Wang (2006a) analysed *D. magna* effects under a six-generational exposure to Cd (3 µg/L) and indicated that a re-exposure to Cd inhibited feeding. Comparing results from F9 continuous Pb exposure and recovering daphnids, we noticed that if organisms are still under Pb exposure, the FR is not disturbed (due to acclimation, as stated before by Tsui & Wang (2005)), on the other hand, when organisms from recovery period are re-exposed to Pb, FR is reduced, suggesting that no retrieval occurred. Food consumption reduction can lead to a diminished reproduction, growth and physiological responses (Guan & Wang, 2006a) keeping a low maintenance metabolism, suggesting a survival adaptive feature in polluted areas (Boisson et al., 1998).

Outcomes vary under food restriction and no difference in algae consumption between F9 control and recovering *D. magna* was shown. Similar FR of control and organisms under recuperation have already been found in a six-generational Cd exposure after 12 days in a clean environment (Guan & Wang, 2006a). Since F9 control and recovery period did not differ we can assume that probably a full retrieval occurred (physiological acclimation). Cd acclimated (1 and 5 µg/L) *D. magna* fully recovered after 21 days in clean media (Bodar et al., 1990), which was a pattern also reported by Brausch & Smith (2009), with an exposure of *D. magna* to a pyrethroid insecticide (cyfluthrin) and to the polycyclic aromatic hydrocarbon (naphthalene) for 12 generations followed by 12 recovery generations, where organisms reverted to the original sensitivity during the recovery period.

Regarding *D. similis* reproduction (usual food), F9 control and recovery period exhibited similar responses among themselves, however, F9 control died at high Pb exposure (as well as F9 Pb setup), being recovering organisms the only survivors. Ward & Robinson (2005) stated that if pre-exposed organisms have a similar outcome as the control population, their existence would be expected to be similar in a non-contaminated environment (shown by control and recovery period, until control's death). Meanwhile, organisms pre-exposed could have an advantage in contaminated habitats, which was

shown by recovering *D. similis* that was less sensitive than the control, presenting opposite outcomes as *D. magna* (recovery period organisms died when re-exposed to Pb). To our knowledge, no recovery assays after metal exposure with *D. similis* exist in the literature to corroborate such results. The lower recovering sensitivity to Pb in comparison to F9 control suggests epigenetic changes. No variances among setups were exhibited regarding recovering organisms body length. However, recovery period rate of population increase (*r*) was higher than F9 control and lower than F9 continuous Pb exposure (while alive). Enhanced *r* in a Pb re-exposure can be interpreted as an adjustment to the metal exposure (Turko et al., 2016).

Concerning food restriction, *D. similis* under recovery showed lower sensitivity to high Pb concentrations than F9 control but not than F9 Pb exposure. F9 Pb exposed *D. similis* exhibited enhanced reproduction and rate of population increase (*r*) than recovering organisms. Individuals recovering from metal exposure tend to reduce their metallothionein production, which may increase metal sensitivity leading to a poorer performance (e.g. reproduction, feeding) than acclimated organisms (Guan & Wang, 2006b; Tsui & Wang, 2005). As recovering *D. similis* presented lower sensitivity to Pb in comparison to F9 control, it is clear that such organisms did not fully recover and probably epigenetic changes may have occurred, even though they were more sensitive than F9 continuous Pb exposure.

When it comes to FR, recovering *D. similis* (usual food) presents a smaller FR than F9 continuous Pb exposure. F9 control and recovery period differed only at the highest Pb concentration, with recovering organisms exhibiting higher algae consumption. This supports reproduction endpoints, where recovering organisms had increased *r* in comparison to F9 control, as a way to compensate for chemical stress (Hayashi et al., 2008). Zalizniak & Nugegoda (2006) stated that *r* alone is appropriate to measure the effect in populations and recuperation after exposure. Therefore, regarding the enhanced rate of population increase (*r*) and FR (than F9 control), we can assume that recovering organisms did not fully recover. Being so, *D. similis* seems to exhibit epigenetic changes when exposed to Pb. Zn exposure also triggered DNA methylation (potentially epigenetic changes) of *D. magna* after a generational exposure (Vandegehuchte et al., 2009b). Moreover, *D. longispina* developed genetic adaptation to Cu after decades of exposure due to an abandoned mine (Lopes et al., 2006).

Under food restriction, no differences were found among F9 control and recovering organisms, highlighting that F9 continuous Pb exposure was not tested due to reproduction impairment. This outcome suggests that food restricted recovery period *D. similis* were able

to achieve a full recovery, leading to the conclusion that this was a physiological acclimation when it comes to algae consumption.

4.4. Conclusion

Nine generations of Pb exposure decreased the sensitivity of both *D. magna* and *D. similis* considering the rate of population increase (*r*), except for food restricted *D. magna*. These are crucial outcomes, since it could lead to changes in populations' dynamics upon Pb exposure. Since F9 control food restricted *D.* similis died when exposed to Pb, continuous Pb exposed (F9) *D. similis* exhibited lower Pb sensitivity. However, both species under the same conditions (continuous Pb exposure and food restriction) did not breed enough neonates to accomplish the feeding inhibition test. Non-essential metals (e.g.: Pb) are more likely to induce genetic variability than essential ones (Barata et al., 1998). Epigenetic changes seemed to have occurred for both species (reproduction outcomes), except for F9 continuous Pb exposure *D. magna* (usual food), which died during a Pb re-exposure (high sensitivity). A physiological acclimation (recovery period similar to F9 control) was suggested concerning FR, except for *D. similis* at usual food regime (recovery period showed a higher FR than the F9 control), indicating then epigenetic changes. Therefore, a lower sensitivity to Pb is acquired by both species throughout generations under usual food content, however, such outcomes vary under food restriction. Since natural habitats usually do not have the perfect nutrient quantity, difference in results among species under food restriction imply that *D. magna* outcomes not necessarily can be extrapolated to *D. similis* and guidelines of tropical areas should take this issue into account. And also, food content has a big impact on organisms' sensitivity to Pb and its recovery process. Further bioaccumulation, epigenetics and gene expression analysis should be taken into consideration to reassure physiological acclimation or if it is epigenetic changes (transgenerational transfer) affecting organisms recovery.

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. grant 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. Denis Abessa thanks CNPq (grant No. 311609/2014-7). The authors are grateful to Abel Ferreira for all the laboratorial support. The authors declare that they have no conflict of interest.

Supplementary material

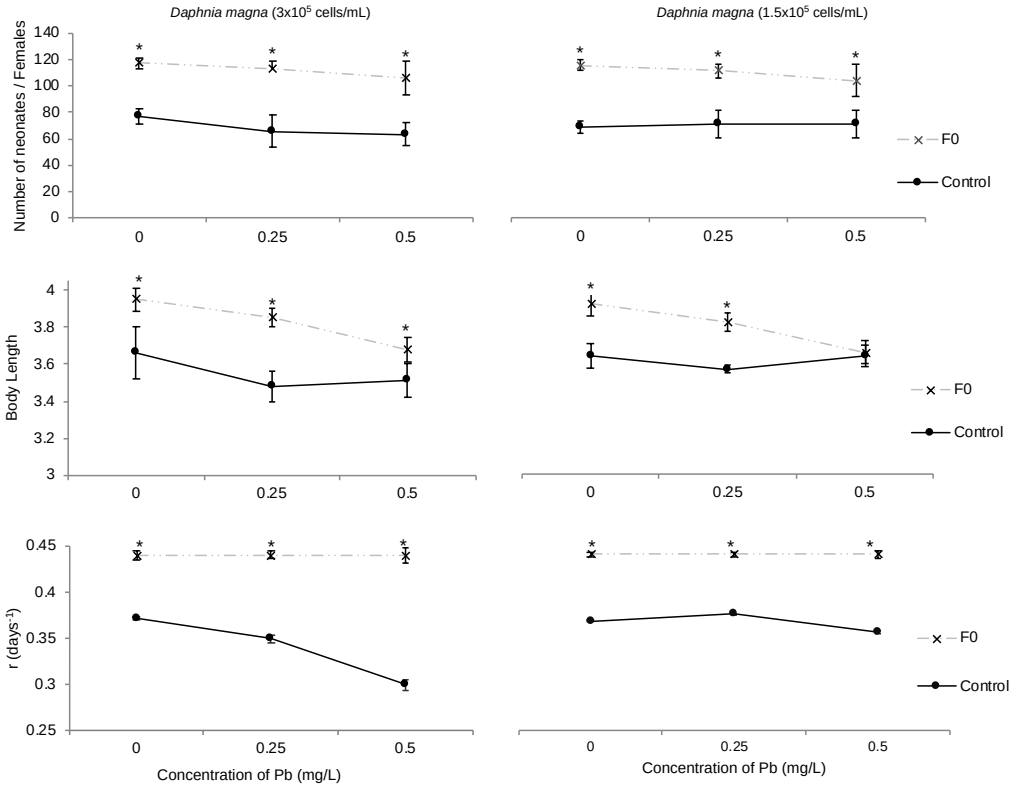

Figure 4S.1: Chronic test *Daphnia magna* of organisms from F0 and control from F9, under two different for regimes (usual and re food regime). Results present: the number of offsprings, body length and rate of population increase (*r*). Asterisk indicates statistical difference between F0 and control F9 (Bonferroni, $p<0.05$). Data presented for control is the same as presented in figure 4.1, just for comparison.

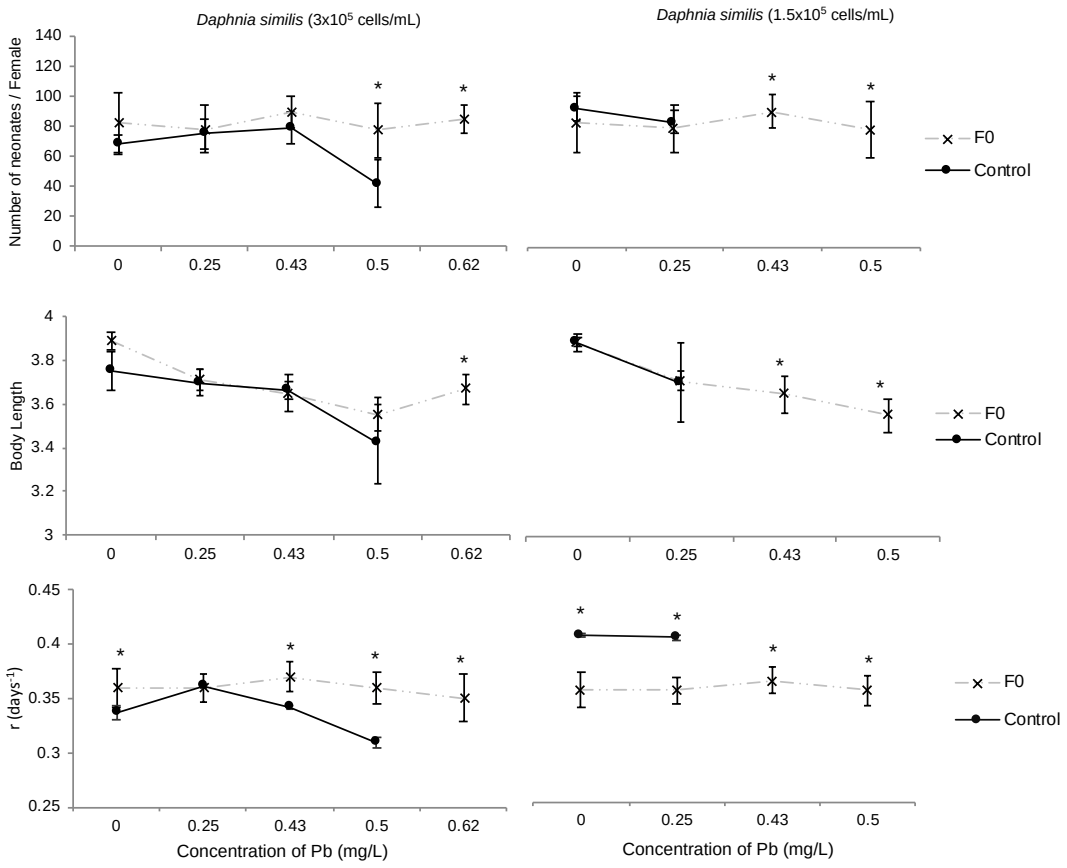

Figure 4S.2: Chronic test outcome for *Daphnia similis* of organisms from F0 and control from F9, under two food regimes (usual and restricted food regime). Results present: the number of offsprings, body length and rate of population increase (*r*). Asterisk indicates statistical difference between F0 and control F9 (Bonferroni, $p<0.05$). Data presented for control is the same as presented in figure 4.2, just for comparison. *Daphnia similis* control (F9) missing data is due to the occurred mortality.

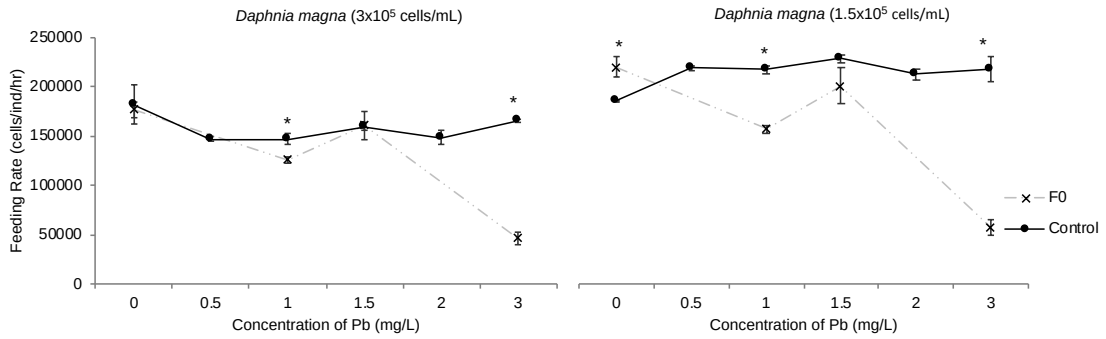

Figure 4S.3: Feeding inhibition test outcome for *Daphnia magna* evaluating the feeding rate of organisms from F0 and control from F9 under two different food regimes. Asterisk indicates statistical difference between F0 and control F9 (Bonferroni, p<0.05). Data presented for control is the same as presented in figure 4.3, just for comparison.

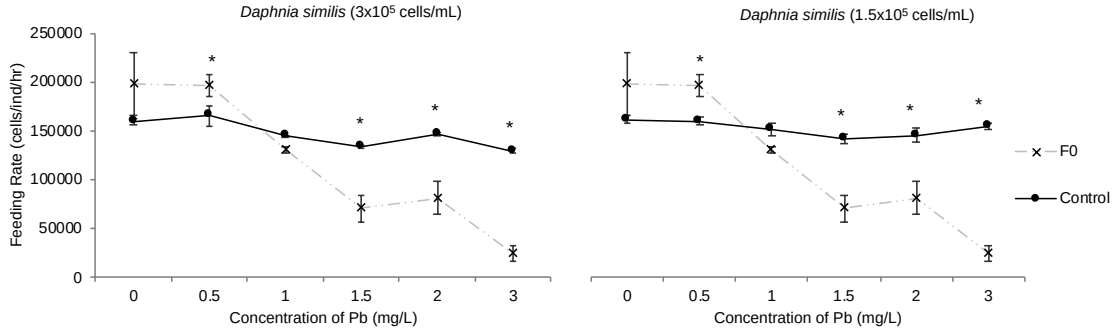

Figure 4S.4: Feeding inhibition test outcome for *Daphnia similis* evaluating the feeding rate of organisms from F0 and control from F9 under two different food regimes. Asterisk indicates statistical difference between F0 and control F9 (Bonferroni, p<0.05). Data presented for control is the same as presented in figure 4.4, just for comparison.

Table 4S.1: Chemical Analyses

Chemical Analyses				
Nominal concentrations	Stock (46.54 mg/L)	0.05 mg/L	0.125 mg/L	3 mg/L
$Pb(NO_3)_2$	48 mg/L	0.054 mg/L	0.099 mg/L	2.6 mg/L
Recovery (%)	103	108	79.2	86.7

Table 4S.2: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding the feedinng inhibition test. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Reproductive rate					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	3200	4	799.9	F (4, 8) = 1.561	P = 0.2169
Generations	30236	2	5039	F (2, 8) = 9.832	P < 0.0001
Residual	12300	8	512.5		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	2788	4	697	F (4, 8) = 1.465	P = 0.2731
Generations	20434	2	6811	F (2, 8) = 14.32	P = 0.0003
Residual	5708	8	475.7		
Body lenght					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.00108	4	0.00027	F (4, 8) = 0.01208	P = 0.9997
Generations	0.2511	2	0.0837	F (2,8) = 3.745	P = 0.0415
Residual	0.2682	8	0.02235		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.05112	4	0.01278	F (4, 8) = 0.8419	P = 0.5248
Generations	0.2525	2	0.08418	F (2, 8) = 5.545	P = 0.0127
Residual	0.1822	8	0.01518		
rate of population increase (r)					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.0002845	4	0.00003162	F (4, 8) = 1.612	P = 0.1856
Generations	0.1126	2	0.05629	F (2, 8) = 2870	P < 0.0001
Residual	0.000353	8	0.00001961		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.000039	9	4.334E-06	F (9, 27) = 0.4568	P = 0.8905
Generations	0.1543	3	0.05142	F (3, 27) = 5420	P < 0.0001
Residual	0.0002562	27	9.488E-06		

Reproductive rate					
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	207.3	4	51.83	F (4, 8) = 0.07097	P = 0.9890
Generations	12461	2	6230	F (2, 8) = 8.530	P = 0.0104
Residual	5843	8	730.4		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	814.9	4	203.7	F (4, 8) = 0.4045	P = 0.8007
Generations	7417	2	3709	F (2, 8) = 7.363	P = 0.0154
Residual	4029	8	503.7		
Body lenght					
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.0624	4	0.0156	F (4, 8) = 0.6161	P = 0.6634
Generations	0.09264	2	0.04632	F (2, 8) = 1.829	P = 0.2217
Residual	0.2026	8	0.02532		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.03643	4	0.009107	F (4, 8) = 0.6605	P = 0.6365
Generations	0.04197	2	0.02099	F (2, 8) = 1.522	P = 0.2753
Residual	0.1103	8	0.01379		
rate of population increase (r)					
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.0002076	4	0.00002307	F (4, 8) = 0.7334	P = 0.6752
Generations	0.01367	2	0.004555	F (2, 8) = 144.8	P < 0.0001
Residual	0.0008491	8	0.00003145		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Setups	0.0001316	4	0.00001462	F (4, 8) = 0.5993	P = 0.7815
Generations	0.04014	2	0.02007	F (2, 8) = 822.5	P < 0.0001
Residual	0.0004393	8	0.0000244		

Table 4S.3: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding the feedinng inhibition test. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Feeding inhibition					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	354700000	2	177300000	F (2, 6) = 0.1714	P = 0.8465
Setups	69320000000	3	23110000000	F (3, 6) = 22.33	P = 0.0012
Generations	6208000000	6	1035000000		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	760700000	2	380300000	F (2, 4) = 0.2859	P = 0.7655
Setups	42040000000	2	21020000000	F (2, 4) = 15.80	P = 0.0126
Generations	5321000000	4	1330000000		
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3713000000	2	1856000000	F (2, 6) = 1.372	P = 0.3231
Setups	2.199E+11	3	73300000000	F (3, 6) = 54.17	P < 0.0001
Generations	8118000000	6	1353000000		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6621000000	2	3310000000	F (2, 4) = 2.365	P = 0.2099
Setups	1.896E+11	2	94800000000	F (2, 4) = 67.73	P = 0.0008
Generations	5599000000	4	1400000000		

Chapter 5

Multi-generation effects of Pb on two *Daphnia* species: looking at different levels of biological organization

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Multi-generation effects of Pb on two *Daphnia* species: looking at different levels of biological organization

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Abstract

The metal lead (Pb) can be highly toxic and induce negative effects to the natural biota. To understand how a long-term Pb exposure can trigger population effects on aquatic organisms, two monophyletic *Daphnia* species (*Daphnia magna* and *Daphnia similis*) were exposed to a sub-lethal concentration of Pb (50 µg/L) for nine generations, under two different food regimes (usual and restricted). Several endpoints at different levels of biological organization were chosen, where AChE activity was measured at a subcellular level, hatching delay and lifespan at the individual level, and Net Reproductive Rate (R_0). At the sixth generation, Pb acclimated neonates were moved to a clean media for three more generations to check for recovery. Both daphnids showed different outcomes. *D. magna* showed no Pb effect on Net Reproductive Rate (R_0), however, Pb did accelerate reproduction, reduced lifespan and decreased AChE activity. Some endpoints (hatching delay and lifespan) did not show retrieval during the recovery process, indicating possibly epigenetic changes. Food restriction reduced the R_0, lifespan, delayed hatching, while enhanced AChE activity of such organisms. Opposite outcomes were shown for *D. similis* in comparison to *D. magna*, such as reduction of R_0 and the lack of Pb influence on other endpoints (hatching delay, lifespan and AChE). The full recovery shown by R_0 suggests a physiological acclimation of *D. similis*. Under food restriction, the animals exhibited reduction of R_0 and lifespan, delayed hatching and enhanced AChE activity, however, with no Pb effect (except for the decreased hatching delay). The recovery process under food restriction presented some variations and showed that *D. similis* could not cope with Pb exposure (shorter lifespan and accelerated reproduction), indicating failed recovery. Such outcomes indicate that it may be not appropriate to rely in a model species (*D. magna*) to estimate the sensitiveness of another (*D. similis*), even for phylogenetically close species. It also suggests that *D. magna* does not fully recover after Pb exposure and, for *D. similis*, failed recover occurs only under food restriction.

Key-words: Multi-generation, Net Reproductive Rate (R_0), Hatching delay, Lifespan, AChE.

1. Introduction

Natural ecosystems can be under pressure due to xenobiotics' release by anthropogenic activities, which may reach water bodies through run-off (Gandhi, 2010), effluents, percolation from solid wastes, and atmospheric deposition. Lead (Pb) is a non-essential metal that can be highly toxic to natural biota (Komjarova & Blust, 2008), and which is originated from industrial emissions, fuels (such as leaded gasoline), lead-based

paints, among others (Irwin, 1997). This metal can be of natural or anthropic sources, its emission to the atmosphere may range between 19 (natural source) and 450 (anthropic source) thousands of tons per year, along with Ni, Zn and Cu, which are considered the most important contaminants from human activities (Valavanidis & Vlachogianni, 2010). Pb can cause neurotoxic effects in some organisms (e.g. Hashim et al. (2014)), such as rats, fish and crustaceans, and can also affect organisms' cholinergic neurons (e.g. Reddy et al. (2003)). Due to its high toxicity, it was recently (June of 2018) added to the Candidate List of substances of very high concern (SVHCs) by the European Chemicals Agency (ECHA).

To evaluate a water body quality, in terms of chemical and the related toxicity, standard organisms have been used, such as microcrustaceans of the genus *Daphnia*. As daphnids are primary grazers, a toxic effect on such organisms can cause a cascading response which may be propagated through the food web (Tessier et al., 2000; Wiklund et al., 2014). Daphnids are model organisms with standardized acute and chronic tests available (OECD, 2012, 2004). However, laboratory standard tests, which expose organisms for short terms, may not properly reproduce natural ecosystems and thus they cannot adequately assess the long-term effects of chemicals on natural populations. Being so, exposing such organisms for longer periods or generations can be a more representative approach and provide extra information for risk assessment (Tanaka & Nakanishi, 2002).

Individuals from the same species or close related species can respond in different ways to a certain chemical (Dudycha & Tessier, 1999; Printes et al., 2008; Rellstab & Spaak, 2009; Tessier et al., 1983). Having that in mind, two monophyletic *Daphnia* species, from two different climate regions, *D. magna* (temperate) and *D. similis* (tropical) were used to compare the chronic Pb effects. Also, natural ecosystems may vary in food quality and quantity in a temporal scale (Wacker & Martin-Creuzburg, 2007), and, high and low-food may alter organisms sensitivity to certain chemicals (Smolders et al., 2005).

The goal of this research was to evaluate if a generational Pb exposure (nine generations) in two *Daphnia* species kept under two different food regimes (usual and restricted) would cause effects at different levels of biological organization to both species. To accomplish our goal, we evaluated the activity of acetylcholinesterase (AChE), as a low level of organization, hatching delay, lifespan and Net Reproductive Rate (R_0), to derive information from the individual to the population level.

2. Materials and Methods

2.1. Culture maintenance

Both daphnid species were maintained in ASTM hard water (ASTM, 2002), at 20 ± 2 °C and a photoperiod of 16:8h (light/dark). Organisms were fed with *Raphidocellis subcapitata* ($3x10^5$ cells/mL), enriched with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). Media and food were renewed every other day. To start new cultures, neonates from third to fifth brood (less than 24 h old) were used.

2.2. Multi-generation

The multi-generation test lasted nine generations (F0 to F9) in total (Figure 1.2). Each generation started with the third brood (N3) of the previous generation, with neonates younger than 24 hours old. Both species were divided into four setups. Two of the setups included the control, with clean ASTM media and the Pb exposure, composed by ASTM spiked with 50 μg/L of Pb ($Pb(NO_3)_2$). Both setups were maintained under two different food regimes, a normal/usual regime provided in bioassays, with $3x10^5$ cells/mL of algae and, the restricted, with half of the algae concentration ($1.5x10^5$ cells/mL). The setups consisted of three replicates of 20 neonates in one litter vial each. To check for recovery, Pb exposed organisms at generation F6 were divided into two sets. One of the sets was maintained under Pb exposure and kept the same as the previous generation (i.e., exposed to 50 μg/L of Pb) and, the other set, Pb pre-exposed organisms were transferred to clean ASTM media (similar conditions as control organisms) for extra three generations (F6 to F9).

In F0, F3, F6 and F9 several endpoints were evaluated to infer on effects due to long-term exposure to Pb and posterior recovery.

2.3. Chemical Solutions and Analyses

$Pb(NO_3)_2$ stock solution (CAS No. 10099-74-8, 98.5% purity, VWR chemicals®) was prepared in mili-Q water, and then used for preparing the Pb exposure treatment. Pb samples were acidified (nitric acid) and then analyzed by ICP-OES (Horiba Jobin Yvon, Activa M). Samples were evaluated in triplicate and duplicate samples of certified material were used, to ensure chemical optimum recovery during procedures.

2.4. Acetylcholinesterase activity

The AChE enzymatic activity was estimated for all treatments in organisms aged 96h old for both species at generations F0, F3 and F9. For that, a 96 h period (after birth) was waited and neonates from continuous Pb exposure were divided into two sets. In the first set neonates were kept in continuous Pb exposure (as Pb exposure treatment) until they reach 96 h old. On the second set, denominated as Pb ($clean_{96h}$), neonates (<24 h) from the continuous Pb exposure treatment were moved to clean ASTM media until they reach 96 h old. This may enable to evaluate the AChE activity of retrieving organisms in a 96 h period. Each replicate consisted of a pool of 15 neonates (96 hours old), which were frozen with liquid nitrogen in a 1.5 mL microtube till enzymatic analysis. All samples were homogenized with a phosphate buffer, under a pH of 7.2 and at 0.1 M. Samples were centrifuged at 6.000 rpm for 5 minutes and at 4ºC. The enzymatic activity was estimated as Ellman et al. (1961), adapted to microplate as Guilhermino et al. (1996a). Protein quantification to normalize enzymatic activity was carried out as in Bradford (1976), adapted by BioRad's micro-assay and using bovine γ-globuline as standard.

2.5. Net Reproductive Rate (R_0)

Daphnids female offspring are designated as broods. Neonates produced from the first to the fifth brood (N1 to N5) were counted in order to estimate the Net Reproductive Rate (R_0) of all setups (negative control, Pb exposure and recovery for both food regimes). Along with the total number of offspring produced (N1 to N5), adult survival was also recorded to estimate R_0, which was estimated through the formula $R_0 = \Sigma (lx.mx)$, where lx stands for survival and mx for fecundity, detailed in Coen and Janssen (2003).

2.6. Hatching delay

To determine the hatching delay, the time of release of the F control first brood (N1) was used to determine and designate time 0. The time that the other treatments took to start giving the first brood was designated as the delayed (or accelerated) time to start reproducing (hatching delay). *Daphnia* usually give the first brood on 8-10 days after they are born (normal conditions). The hatching delay was evaluated for all treatments (control and Pb) and both food regimes, including the recovery process.

2.7. Lifespan

Being r-selected organisms, daphnids reproduce early and have short lifespans. To estimate the generational Pb effect on daphnids longevity, all treatments were maintained until "natural" death occurred. Every day all cultures of treatments (negative control, Pb

exposure and recovery for both food regimes) were checked to search for dead organisms and to estimate organisms' lifespan.

2.8. Statistical analysis

All endpoints acquired through the multi-generation test were checked for normality (Kolmogorov–Smirnov) and homoscedasticity (Levene's equal variance test) and the differences among generations and treatments of each generation were evaluated through a Two-Way Analysis of Variance (ANOVA). When statistical differences were first highlighted, a post-hoc test (Bonferroni) was made (GraphPad Prism®). In order to integrate and highlight associations among variables, a multivariate Principal Component Analysis (PCA) was made. The multivariate analysis was run with the help of SPSS® software.

3. Results

3.1. Chemical analyses

A >79% recovery was achieved comparing nominal and measured concentrations for Pb samples. The limit of quantification (LOQ) for Pb was 25 µg/L and ASTM (control) presented values <LOD (Table 5S.1). The certified material evaluated had >80% of recovery.

3.2. Acetylcholinesterase activity (AChE)

The Two-Way ANOVA statistical analysis for the AChE activity is shown at Table 1S. The neurotoxic effects were evaluated based on the analysis of AChE activity in 96h old daphnids. Among generations, control *D. magna* presented higher AChE activities at generations F3 and F9 than that exhibited by F0 organisms (Figure 5.1a). Among treatments, control showed an enhanced enzymatic activity in comparison to continuous Pb exposure, but similar to that exhibited by Pb ($clean_{96h}$) organisms (Pb exposed neonates moved for 96h to a clean ASTM media). Under food restriction, no differences among treatments were shown, however, all treatments at generation F9 presented an enhanced AChE activity compared to F0 (Figure 5.1b).

Control and Pb treatment *D. similis* presented increased AChE activity throughout generations, differing from F0 at generation F9 (Figure 5.1c). Both treatments also had

higher enzymatic activity than Pb (clean$_{96h}$) organisms (F9). Food restriction showed a similar outcome for control and Pb treatment (Figure 5.1d), however, Pb (clean$_{96h}$) organisms enhanced AChE activity from F3 to F9 and no statistical difference among treatments is shown.

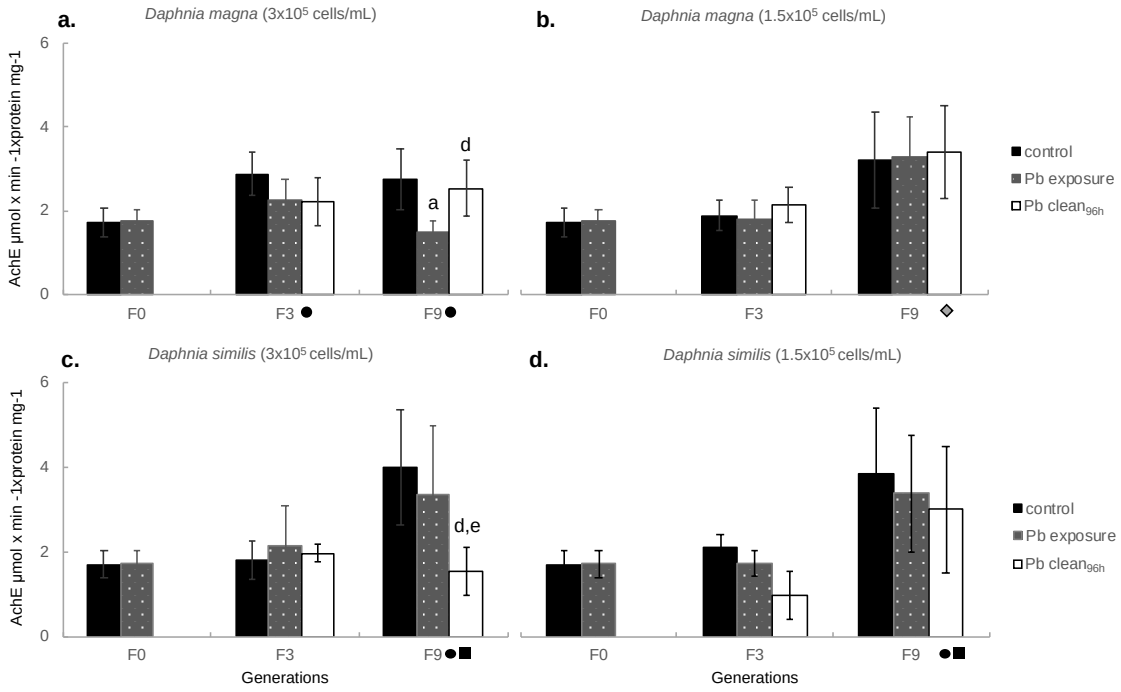

Figure 5.1: Acetylcholinesterase activity of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) under a continuous exposure to a negative control (ASTM) and Pb exposure, in two different food regimes (3x10^5 and 1.5x10^5 cells/mL). Additionally Pb (clean$_{96h}$) daphnids were also assessed and refer to a 96h exposure to clean media after Pb continuous exposure. Generations in the X axis are marked with a 1) black circle for those statistical different from F0, in control treatment, 2) a black square for those statistical different from F0, in Pb treatment and 3) a gray diamond for those statistical different from F0 in all treatments (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control, (d) for Pb vs. Pb (clean$_{96h}$) and (e) for Pb (clean$_{96h}$) vs. Pb (Bonferroni, $p<0.05$).

Regarding recovery, organisms under usual food presented increased variations among treatments than organisms under food restriction (both species). Recovery *D. magna* (usual food, F9) showed enhanced AChE activity compared to F0 and to both Pb treatments (Pb and Pb (clean$_{96h}$)) (Figure 5.2a). Under food restriction, no difference among treatments was spotted, however, recovery organisms presented a significantly higher enzymatic activity in comparison to F0 (Figure 5.2b). Recovery *D. similis* (usual food) presented a lower enzymatic activity when compared to control organisms (Figure 5.2c). Under food

restriction different outcomes are shown and no difference among treatments or generations was indicated (Figure 5.2d).

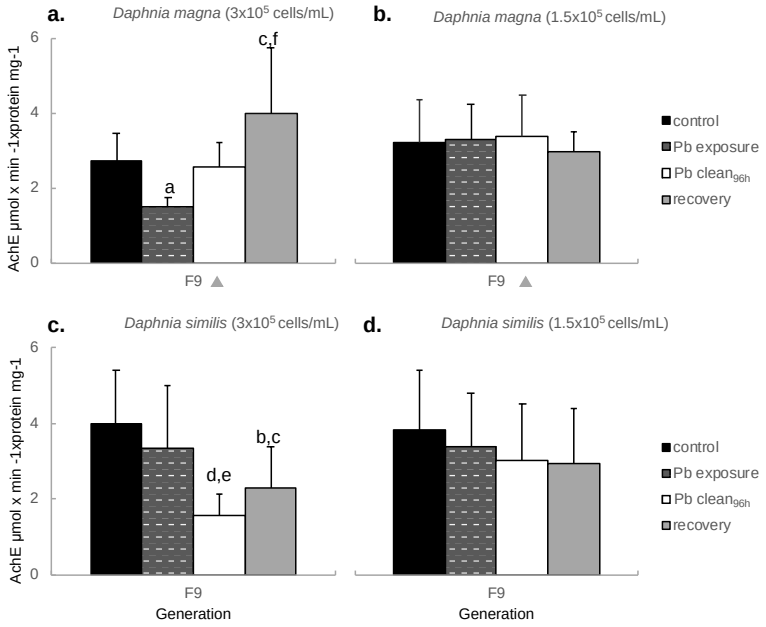

Figure 5.2: Acetylcholinesterase activity of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) under a continuous exposure to a negative control (ASTM) and Pb, a clean media (Pb ($clean_{96h}$)) for 96 h after Pb pre-exposure, and in a recovery exposure (3 generations in clean media) after Pb pre-exposure, in two different 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 (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, (d) for Pb vs. Pb ($clean_{96h}$), (e) for Pb ($clean_{96h}$) vs. control and (f) for recovery vs. Pb ($clean_{96h}$) (Bonferroni, $p<0.05$). Data presented for control and Pb are the same as presented in figure 5.1, just for comparison.

3.3. Net Reproductive Rate (R_0)

The Two-Way ANOVA statistical analysis for the Net Reproduction Rate (R_0) is shown at Table 5S.2. The quality criteria (> 60 total neonates/female, OECD (2012)) was achieved for control treatment at every generation under usual food regime. Under food restriction, both *Daphnia* from the control treatment achieved lower offspring quantity then the recommended by the OECD quality criteria at generations F6 and F9. The Net Reproductive Rate (R_0) was determined as the sum of neonates given by each brood (from N1 to N5) obtained by the living adult females of each treatment replicate. The average reproductive rate of *D. magna* under usual food regime decreased through generations in both control and Pb exposure, with F0 values being statistically higher than F6 and F9

(Figure 5.3a). In addition, no differences between treatments were attained. Under food restriction (Figure 5.3b), R_0 decreased drastically, with both treatments (control and Pb exposure) being different from F0 at generations F6 and F9.

D. similis showed a lower R_0 in comparison to *D. magna*, with averages of 82 and 120 (F0) neonates per living female, respectively. Control *D. similis* (usual food) decreased R_0 at generation F6, being lower than F0 (Figure 5.3c). Continuous Pb exposure decreased R_0 at generation F9, being lower than control treatment and generation F0. No difference among treatments was shown for *D. similis* under food restriction (Figure 5.3d), however, both treatments presented reduced R_0 at generations F6 and F9 in comparison to F0.

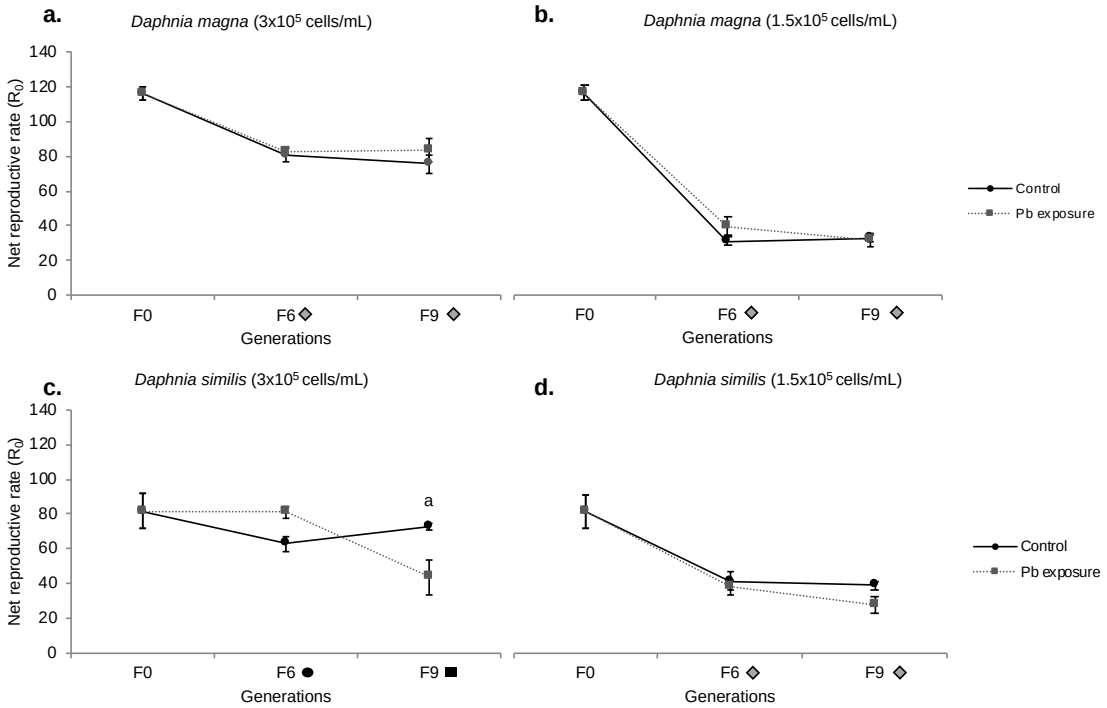

Figure 5.3: Net Reproductive Rate (R_0) of *Daphnia magna* (a. and b.) and *Daphnia similis* (c. and d.) under a continuous exposure to a negative control (ASTM) and Pb, in two different food regimes: $3x10^5$ cells/mL (left graphs) and $1.5x10^5$ cells/mL (right graphs). Generations in the X axis are marked with a 1) black circle for those statistical different from F0, in control treatment, 2) a black square for those statistical different from F0, in Pb treatment and 3) a gray diamond when all treatments (ASTM, Pb and recovery) presented difference in comparison to F0 (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control (Bonferroni, $p<0.05$).

Regarding the recovery period, *D. magna* (usual food) presented no statistical difference towards other treatments (control and Pb exposure), however, a lower R_0 was shown for both F6 and F9 when compared to F0 (Figure 5.4a). Under food restriction (Figure 5.4b), recovering *D. magna* showed an enhanced R_0 in comparison to control organisms at generation F6, pattern not maintained at generation F9 (similar to both control and Pb treatments). Concerning difference among generations, F6 and F9 indicated a statistically lower R_0 compared to F0.

Recovering *D. similis* (usual food) showed no statistical divergence among generations (compared to F0) (Figure 5.4c). Organisms from the recovery period indicated a higher R_0 than Pb exposure at generation F9, but similar to F9 control. Under food restriction no difference is shown among treatments (control, Pb and recovery period), but a lower R_0 is shown for generations F6 and F9 compared to F0 (Figure 5.4d).

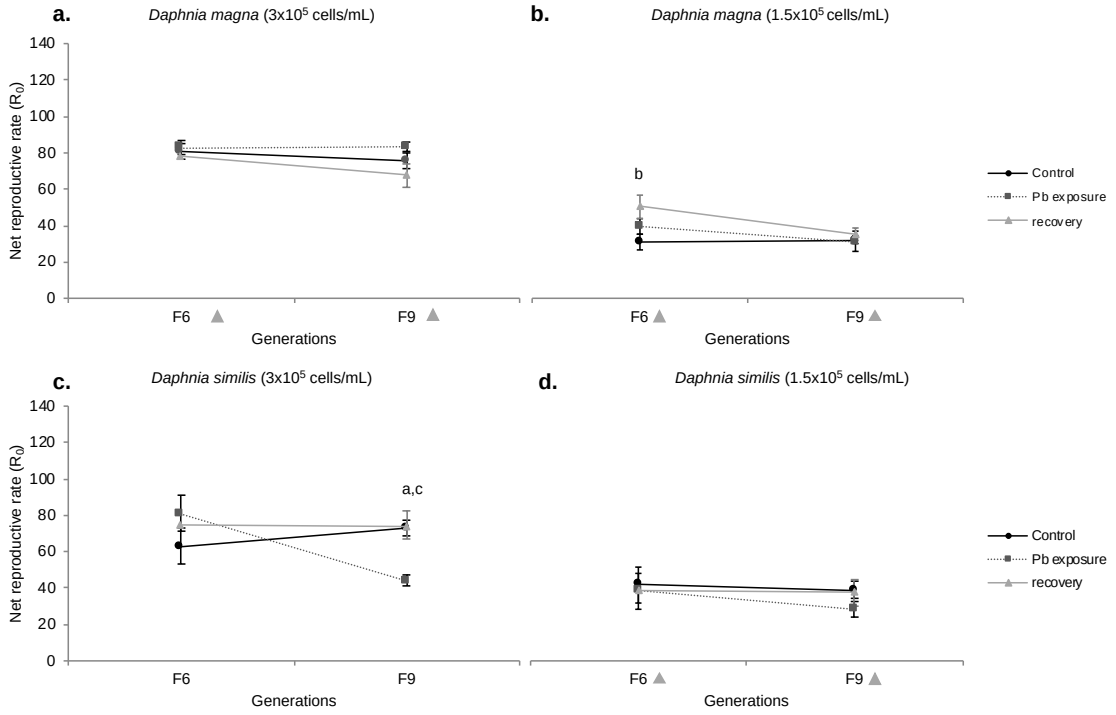

Figure 5.4: Net Reproductive Rate (R_0) of *Daphnia magna* (a. and b.) and *Daphnia similis* (c. and d.) exposed to control media, Pb continuous exposure, and the recovery period, in two different food regimes: $3x10^5$ cells/mL (left graphs) and $1.5x10^5$ cells/mL (right graphs). Generations in the X axis are marked with a gray diamond when recovery treatment presented difference 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, $p<0.05$). Data presented for control and Pb are the same as presented in figure 5.3, just for comparison.

3.4. Hatching delay

The Two-Way ANOVA statistical analysis for the hatching delay is shown in detail in Table 5S.3. The hatching delay was designated as the difference of days that organisms took to give the first brood (N1), compared to F0. Normally daphnids started giving N1 at the age of eight to ten days (normal conditions), thus the delayed days were counted from that starting point (time for 1st brood for F0 control organisms, being eight days for *D. magna* and nine days for *D. similis*). Neonates from control *D. magna* (usual food) were delayed at F9, presenting a later time for the first reproduction in comparison to F0 and to continuous Pb exposure (Figure 5.5a). Under food restriction, F9 control and Pb exposed daphnids were delayed in comparison to F0, but no statistical difference among treatments was shown (Figure 5.5b).

No difference was shown for *D. similis* (usual food) regarding F0 or treatments (Figure 5.5c). Under food restriction, control *D. similis* gradually delayed hatching through generations, being statistically different (taking longer to hatch) than Pb treatment at generation F9. Both treatments were delaying hatching N1 neonates at generation F9 when compared to F0 (Figure 5.5d).

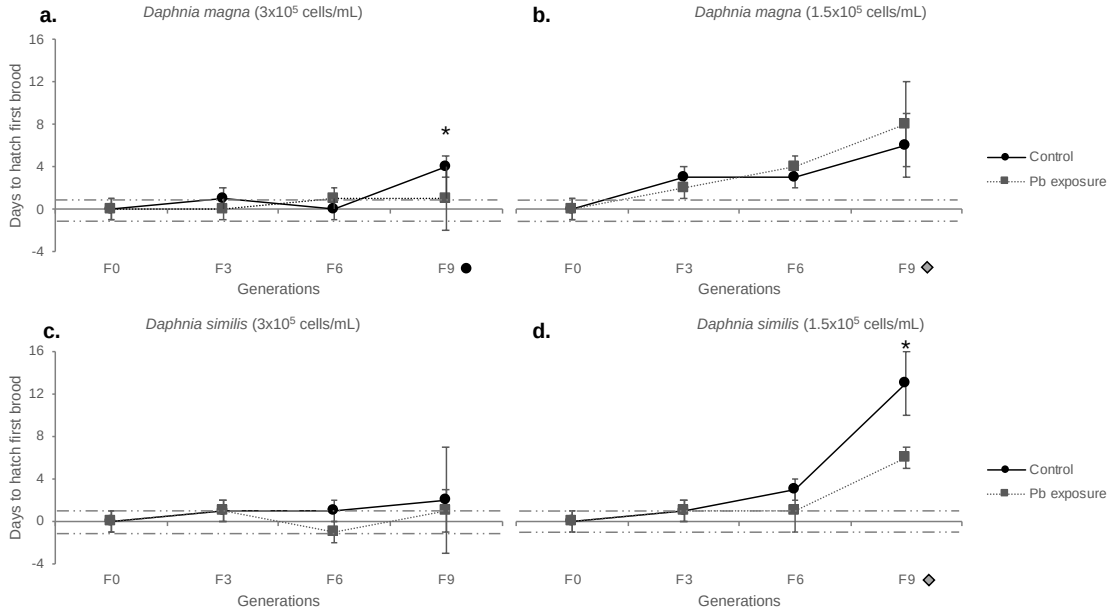

Figure 5.5: Hatching delay of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) under a continuous exposure to a negative control (ASTM) and Pb, in two different food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a 1) black circle for those statistical different from F0, in control treatment and 2) a gray diamond for those statistical different from F0 in all treatments (Bonferroni, $p<0.05$). Asterisk (*) indicate statistical difference between treatments at each generation (Bonferroni, $p<0.05$). The dotted line close to the axis determine the standard deviation for F0 control.

Recovering *D. magna* (usual food) showed no statistical difference towards F0. However, a statistically different (accelerated) reproduction was seen for recovering organisms in comparison to F9 control (Figure 5.6a). Under food restriction, the hatching delay of *D. magna* during recovery period did not show statistical differences among treatments (control and Pb exposure), but there was a reproduction delay at generation F9 in comparison to F0 (Figure 5.6b). *D. similis* (usual food) showed no significant variations regarding treatments (control and Pb exposure) or generations (compared to F0) (Figure 5.6c). Under food restriction, however, recovering organisms presented a delayed reproduction at generation F9 compared to F0. Among treatments, the 1st reproduction of recovering organisms was similar to that shown by organisms under Pb exposure, but

differed from F9 control organisms, which presented a significant delayed reproduction (Figure 5.6d).

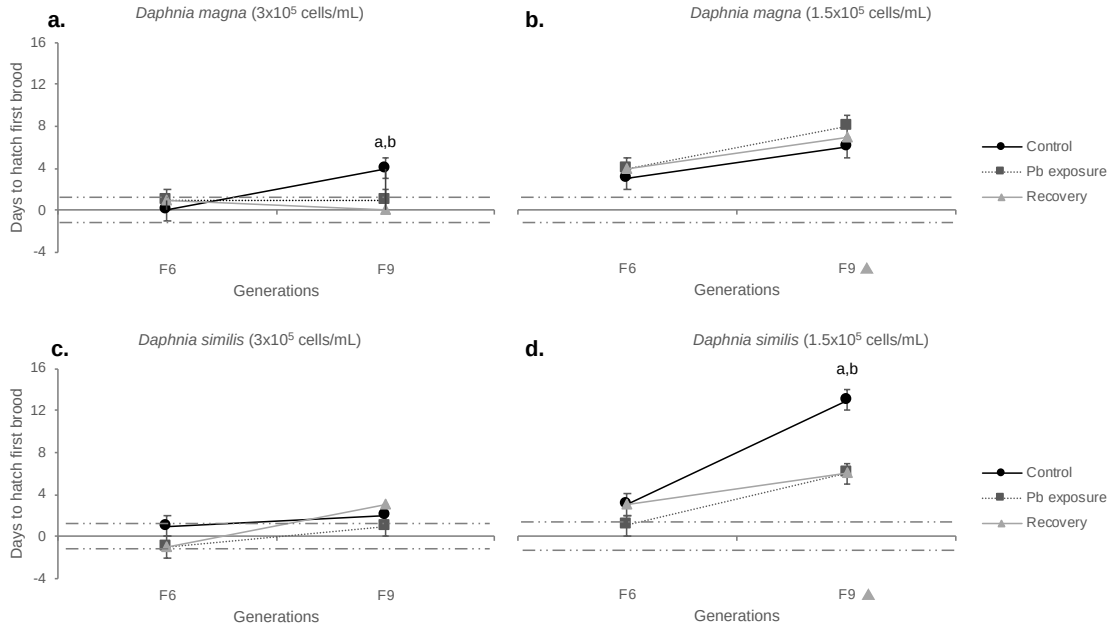

Figure 5.6: Hatching delay of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media, Pb continuous exposure, and the recovery period, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a grey triangle when recovery treatment presented difference in comparison to F0 (Bonferroni, $p<0.05$). Letters indicate statistical difference between treatments within the same generation, being (a) for Pb vs. control and (b) for recovery vs. control (Bonferroni, $p<0.05$). Data presented for control and Pb are the same as presented in figure 5.5, just for comparison. The dotted line close to the axis determine the standard deviation for F0 control.

3.5. Lifespan

The Two-Way ANOVA statistical analysis for the lifespan is detailed at Table 5S.4. To evaluate the effects of Pb exposure on daphnids' longevity, organisms' lifespan was investigated (control and Pb exposure, both food regimes). Control *D. magna* (usual food) reached ages ranging from 83 to 119 days (F0 to F9, respectively), with control organisms from generations F6 (100 d) and F9 (119 d) presenting a statistically enhanced longevity in comparison to F0 (86 d) (Figure 5.7a). *D. magna* under continuous Pb exposure had their lifespan reduced from F0 to F9 (statistically different), from 83 to 69 days. Pb exposed organisms from F6 and F9 presented statistically lower lifespan than the respective controls

from each generation. Under food restriction, such outcomes varied (Figure 5.7b). Control organisms presented an enhanced lifespan at generation F6 (110 days), being higher than those exhibited by organisms from the F6 Pb treatment; however, the lifespan of control organisms decreased (from F6 to F9), presenting similar values as F9 Pb exposed organisms (85 days). Control treatment statistically differed (lower) from F0 at generation F9. Continuous Pb exposure showed a lifespan decrease through generations F0 to F9 (from 103 to 82 days, respectively), differing from F0 at all analyzed generations.

Regarding *D. similis*, control organisms under usual food presented an increased lifespan, ranging from 84 (F0) to 112 (F6) days (Figure 5.7c), being statistically different (higher) from F0 and Pb treatment at generation F6. The lifespan of Pb exposed organisms did not exhibit significant variations among generations (usual food). Under food restriction (Figure 5.7d), control organisms showed increased lifespan at generation F6 (113 days), being the only generation that significantly differed from F0 and Pb exposed organisms. However, Pb treatment showed a lifespan decrease through generations (102 (F0) to 83 (F9) days), being lower than F0 at generations F6 and F9.

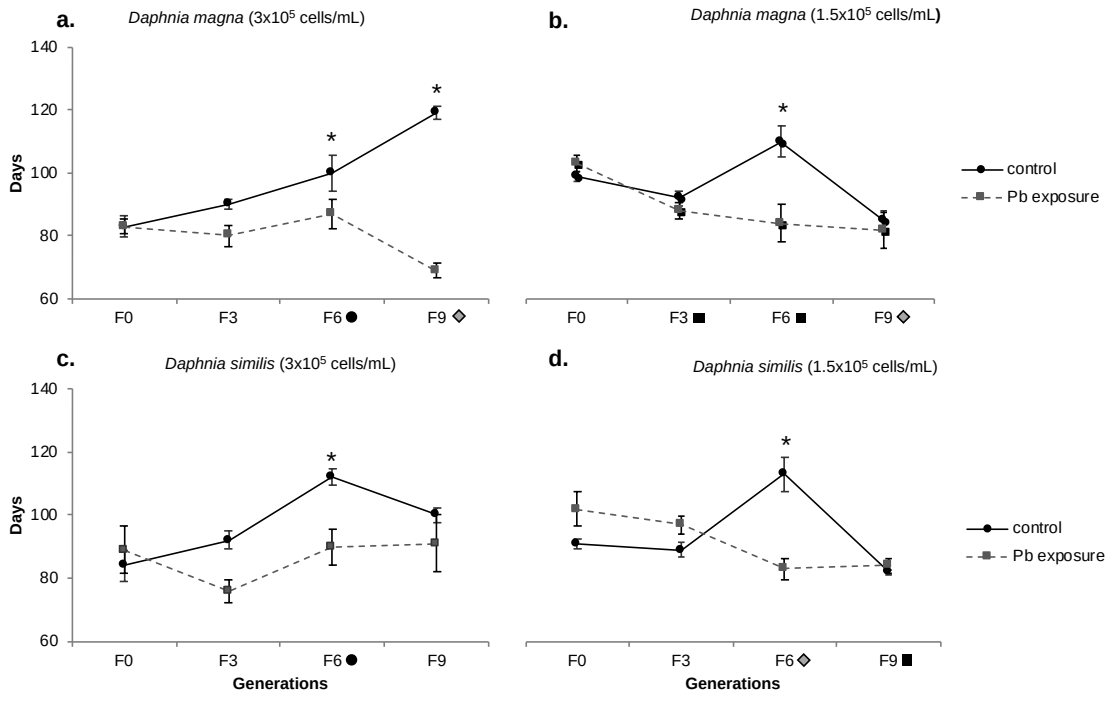

Figure 5.7: Lifespan of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) under a continuous exposure to a negative control (ASTM) and Pb, in two different food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a 1) black circle for those statistical different from F0, in control treatment, 2) a black square for those statistical different from F0, in Pb treatment and 3) a gray diamond for those statistical different from F0 in all treatments (Bonferroni, $p<0.05$).

Asterisk (*) indicate statistical difference between treatments at each generation (Bonferroni, $p<0.05$).

Regarding organisms' recovery period, *D. magna* (usual food) lifespan decreased from 99 (F6) to 86 days (F9), presenting a higher lifespan at generation F6 than F0 (Figure 5.8a). Among treatments, recovery period showed opposite outcome to the control indicating a lower lifespan, however, it was higher than continuous Pb exposure (F9). Under food restriction, recovery period organisms showed no significant statistical difference towards other treatments (control and Pb exposure) and followed the same pattern as control, exhibiting a decreased lifespan from F6 to F9 (not shown by Pb treatment, which kept stable from F6 to F9). However, a decreased lifespan at generation F9 in comparison to F0 is shown (Figure 5.8b).

Recovering *D. similis* (usual food) led to a lifespan reduction from F6 to F9 (114 to 82 days, respectively). At generation F6, recovering *D. similis* presented a statistically higher lifespan than continuous Pb exposure and F0 organisms (Figure 5.8c). A similar pattern (decreased lifespan) is shown under food restriction, with values ranging from 97 (F6) to 69 (F9). Recovery period lifespan was statistically lower than control but higher than Pb treatment at generation F6, being lower than both treatments (control and Pb exposure) and also from F0 at generation F9 (Figure 5.8d).

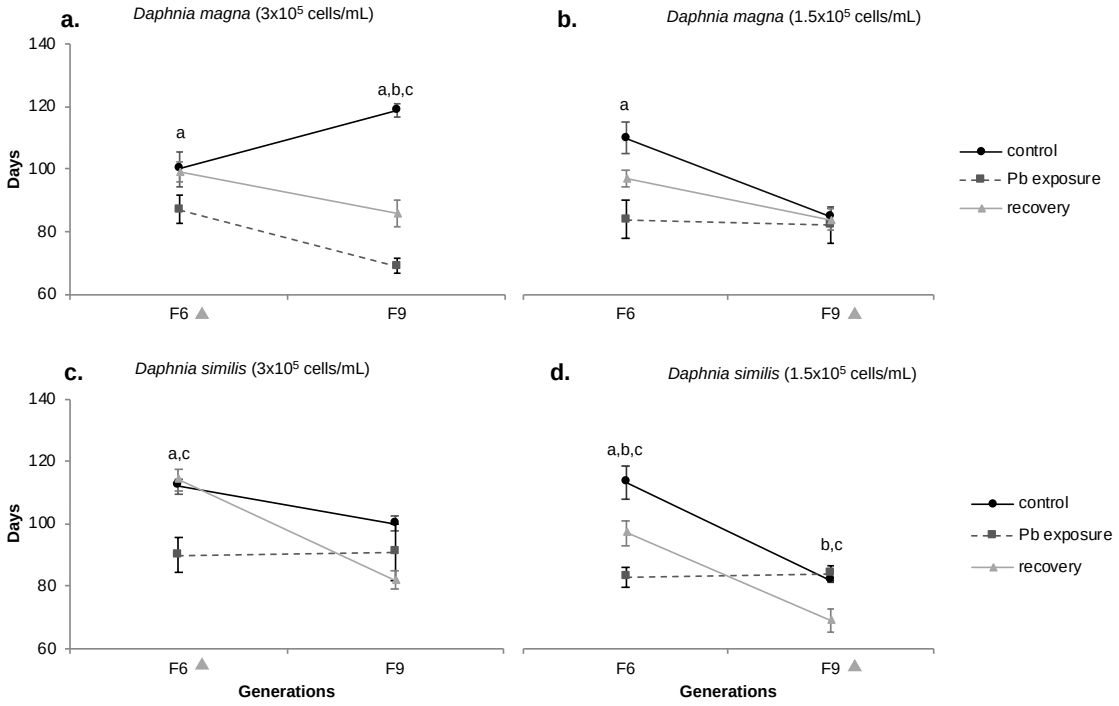

Figure 5.8: Lifespan of *Daphnia magna* (a and b) and *Daphnia similis* (c and d) exposed to control media and Pb continuous exposure for several generations, and in a recovery exposure (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Generations in the X axis are marked with a grey triangle when recovery treatment presented difference in comparison to F0 (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 5.7, just for comparison.

3.6. Principal Component Analysis (PCA)

The PCA extracted two factors as the responsible for the variances (Table 5.1). Regarding endpoints evaluated, PC1 and PC2 together explained 83.88% of variances. PC1 (56.97% of explained variables) associated the hatching delay of both species (both food regimes), associating also neurotoxicity (AChE activity) under both food regimes and life expectancy (lifespan) under usual food for *D. similis*. The R_0 of both species (both food regimes) indicated a negative (inverse) correlation to PC1, together with life expectancy of *D. magna* (food restriction).

On the second component (PC2, 26.9% of explained variables), *D. magna* related neurotoxicity (AChE) and *D. similis* associated life expectancy (lifespan), both under food restriction. A negative association was shown for neurotoxicity (AChE) of *D. magna* and hatching delay of *D. similis* (both under usual food).

Among setups (F0 and F9 (control, Pb and recovery)), such analysis showed that variables from F0 are mainly negatively associated to axis one. F9 control reveals a weak association to PC1, while F9 Pb exposure and recovery period shows a positive (more relevant) association to PC1 (indicating approximation). F9 control positively associate to PC2 while F9 Pb exposure indicates and inverse association.

Table 5.1: Principal Component Analysis (PCA) integrating *Daphnia magna* and *Daphnia similis* endpoints (Net Reproductive Rate (R_0), Lifespan, Hatching delay and Acetylcholinesterase (AChE)) exposed to control media and Pb continuous exposure for several generations, and a recovery period (clean media) after Pb pre-exposure, under two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). A cut-off value of >0.5 was used.

Initial Eigenvalues			
	Total	**% of Variance**	**Cumulative %**
1	9.116	56.97	56.97
2	4.305	26.90	83.88
		Component	
		PC1	**PC2**
AChE (*D. similis*)_restricted		0.995	
R_0 (*D. magna*)_restricted		-0.961	
Lifespan (*D. magna*)_restricted		-0.923	
Hatch. delay (*D. similis*)_restricted		0.914	
AChE (*D. similis*)		0.898	
Hatch. delay (*D. magna*)_restricted		0.892	
Lifespan (*D. similis*)		0.891	
R_0 (*D. similis*)		-0.888	
R_0 (*D. magna*)		-0.864	
Hatch. Delay (*D. magna*)		0.683	
AChE (*D. magna*)			-0.959
AChE (*D. magna*)_restricted			0.956
Lifespan (*D. similis*)_restricted			0.919
Hatch. Delay (*D. similis*)		0.652	-0.752
Lifespan (*D. magna*)			
R_0 (*D. similis*)_restricted		-0.515	
		Axis	
		1	**2**
F0		-1.428	
Control (F9)			1.496
Pb_exposure (F9)		0.785	-0.603
Recovery period (F9)		0.582	

4. Discussion

4.1. Population effects on control over generations (F0 vs. F9 control)

Comparing generations F0 and F9 control, the F9 control *D. magna* (usual food) presented lower R_0 and delayed hatching, lifespan and AChE activity (in comparison to F0). All endpoints fluctuations observed to *D. magna* (usual food) when comparing F0 to F9 under control conditions may be considered a natural laboratory culture fluctuation, since no variation exposure conditions were made. R_0 reduction and hatching delay can be linked outcomes (Ginjupalli & Baldwin, 2013), as well as late reproduction and increased lifespan (trade-off between reproduction and lifespan) (Dudycha & Tessier, 1999). Reduced R_0 and increased lifespan can be related endpoints, where a higher investment in reproduction may enhance oxidative stress, consequently reducing organisms' lifespan (Garratt et al., 2012; Speakman & Garratt, 2014). The relationship among enhanced lifespan and AChE activity is known, where an enhanced enzymatic activity increases the ability of homeostasis, and, consequently, enhances survival (Ren et al., 2017). In humans, AChE activity may increase with age (Wiklund et al., 2014) and, in other organisms (such as *D. magna*), may be linked with high protein content (enhanced body length) (Venkataraman et al., 1985). Natural enzymatic fluctuations have already been shown, and Toumi et al. (2015a) observed different AChE activities among *Daphnia* strains and also reported a high variance of proteins (0.72 to 8.9 nmol/min/mg) among control organisms from the same strain.

An opposite outcome was shown under food restriction, where control *D. magna* exhibited a decreased lifespan; however, similar results to usual food regime occurred, such as the reduction of R_0 in time combined with delayed hatching and enhanced AChE activity (F9 compared to F0). The quality criteria for control organisms (>60 total neonates/female) was not achieved under food restriction (OECD, 2012). Schwartz et al. (2017) stated that individuals at high food level tend to increase reproduction and lifespan, the opposite occurring under food restriction. Such authors exposed *D. pulex* to different food regimes and found a longer lifespan at high food content. A moderate calorie restriction is known to enhance lifespan; however, a heavy or long-period of food impairment could cause opposite effects (reduced lifespan) (Zhang & Hood, 2016). The increased delay in reproduction shown by usual food regime daphnids (F9 compared to F0) was maintained under food restriction. Frost et al. (2010) showed delayed reproduction and slow growth on *D. magna* under a transgenerational (two generations) poor food experience. Regarding food regimes and neurotoxicity, Toumi et al. (2015b) suggested that, among other factors, quality and quantity of food may also affect AChE activity.

Differently from the control *D. magna*, control *D. similis* (usual food) among generations presented similar results in time as F0 organisms, except for AChE activity (F0 showed lower activities). Since no changes were found regarding organisms maintenance, the observed AChE fluctuation is also considered as a natural fuctuaction (Toumi et al., 2015a).

Diverging from usual food treatment, food restriction affected the *D. similis* control treatment outcomes among generations; organisms showed reduced R_0 and delayed hatching and, as well as usual food, enhanced AChE activity (F9 in comparison to F0). Stige et al. (2004) indicated that at high food regime reproduction starts earlier and neonates' growth is enhanced, leading to a higher R_0. *D. magna* and *D. similis* exhibited similar outcomes under food restriction, except for the decreased lifespan of *D. magna*. Corroborating with *D. similis* outcome, Latta et al. (2011) exhibited a lack of lifespan divergence between usual and restricted food regime for *D. pulicaria*. Kim et al. (2014) evaluated *D. pulex* and *D. pulicaria* and suggested that daphnids have unique physiological mechanisms to respond to food restriction and that lifespan variations may be genotype-dependent. Regarding enzymatic activity, Xuereb et al. (2009) studied behavioral consequences of AChE inhibition in the Crustacea *Gammarus fossarum* and stated a correlation between AChE inhibition and feeding and swimming impairment. Enhanced AChE may lead to enhanced swimming *Daphnia* activity (Ren et al., 2017), which (being filter feeders) may lead to increased algae consumption.

It is important to highlight the different outcomes shown by both species and food regimes, with *D. magna* presenting outcome oscillations from F0 to F9, while *D. similis* showing no variance towards F0 (except for enhanced AChE activity). Under food restriction, both species showed similar endpoints (decreased R_0 and delayed hatching), which is expected for an environment with low nutrients resources (Chandini, 1989). However, species diverged regarding lifespan. Supporting the acquired outcomes, Latta et al. (2011) indicated divergence among two *Daphnia* species, *D. pulex* (enhanced lifespan in reduced diet) and *D. pulicaria* (no difference regarding food regime).

4.2. Multi-generation Pb exposure

A continuous Pb exposure on *D. magna* (usual food) triggered a lower R_0 and lifespan, when compared to F0. However, the R_0 of Pb exposed organisms was similar to control (all generations), indicating that this increase was not caused by Pb and thus it was probably a natural fluctuation. A similar result was found by Ward & Robinson (2005), who observed a

lack of effect on *D. magna*'s reproduction in an eight-generational Cd (60 µg/L) exposure. Gust et al. (2016) observed that reproduction of *D. magna* exposed to a concentration of 236 µg/L of Pb was not affected neither at low (1.8×10^5 cells/mL) or high (3.6×10^5 cells/mL) food concentration (*R. subcapitata*); however, they observed that the organisms fed with less quantities of algae produced less neonates. Nevertheless, a continuous Pb exposure seems to diminish organisms' lifespan (control higher than Pb treatment at generations F6 and F9) and induce early reproduction (compared to control at generation F9). A low-level toxicity can reduce lifespan and this may trigger an early reproduction investment (Roff, 2001), i.e. selecting individuals who start reproducing earlier. A similar result was observed by Heugens et al. (2006) who observed that *D. magna* kept with high amounts of food was "protected" against Cd exposure, exhibiting early reproduction and larger body size. Other authors also found reduced lifespan and shortened reproduction of *D. magna* exposed to low concentrations of chromium (Coniglio & Baudo, 1989) and Nickel (Münzinger, 1990).

A decreased AChE activity was observed among organisms from Pb treatment in comparison to control and Pb ($clean_{96h}$) at generation F9. Decreased AChE activity was found in Sprague-Dawley rats exposed to Pb (Reddy et al., 2003) and in three other species (the mollusk *Corbicula* sp., the earthworm *Eisenia fetida* and the fish *Brachydanio redo*) (Labrot et al., 1996). A recovery of AChE activity was seen by Ren et al. (2017) after *D. magna* exposure to the insecticides deltamethrin (13.36 µg/L and 33.40 µg/L) and methomyl (19.66 µg/L and 49.15 µg/L), starting 6 hours after the exposure elimination. These authors suggested that the recovery was due to a chemical stimulation of *de novo* AChE synthesis. AChE activity retrieval after the elimination of exposure was also observed to the Atlantic salmon (*Salmo salar*) exposed to fenitrothion (Morgan et al., 1990), the crabs *Barytelphusa guerini* exposed to chlorpyrifos (Narra et al., 2012), and the freshwater shrimp *Paratya australiensis* exposed to the insecticide profenofos (Abdullah et al., 1994).

D. magna from continuous Pb exposure under food restriction showed similar results than Pb exposed organisms kept under usual food regime (decreased R_0 and lifespan). However, different outcomes were also exhibited such as delayed hatching and increased AChE activity (Pb exposure in comparison with F0). No differences among treatments were spotted at generation F9 (for any of the evaluated endpoints), suggesting that Pb exposure did not affect *D. magna* under food restriction (at the exposure concentrations used in this study). Since treatments (control and Pb exposure) did not diverge, the condition that varied between F0 and F9 was the food amount, the varying outcomes probably were due to food restriction, which is known for causing negative impact on the reproduction of *D. magna* (Pieters et al., 2005; Smolders et al., 2005; Stige et al., 2004) and *D. pulex* (Kim et al.,

2014). AChE activity of control among generations and Pb treatment did not differ, and similal observations were done by Guilhermino et al., (1996b) when looking at AChE activities in control and Cd-exposed *D. magna*. In our study, food restriction enhanced F9 organisms' AChE activity (compared to F0). Severe enzymatic consequences could occur after nutritional deprivation, such as the vulnerability of AChE activity in crucial organs (such as heart) after long-term food restriction (Venkataraman et al., 1985). Other studies suggests that food variation can disturb enzymatic activities (Suchiang & Sharma, 2011; Venkataraman et al., 1985).

Pb exposed *D. similis* (usual food) for generations showed reduction of R_0 and increase of AChE activity in comparison to F0. Different from *D. magna*, Pb exposed (F9) organisms' R_0 was lower than the F9 control treatment, indicating effects of Pb on the reproduction of *D. similis*. Previous studies showed reproduction impairment of daphnids exposed to Ni in a seven-generation study (Münzinger, 1990) and to Pb (Berglind et al., 1985; Biesinger & Christensen, 1972). The AChE activity of F9 Pb treatment *D. similis* also presented a different response than that exhibited by *D. magna*, presenting a higher AChE activity for F9 Pb treatment when compared to F9 Pb ($clean_{96h}$), indicating failed retrieval. Printes & Callaghan (2006) compared the AChE activity of both *D. magna* and *D. similis* and stated that the genotype is crucial when determining AChE activity. Increased AChE activity was also found in mussels *Perna perna* exposed to Cd and Pb (Bainy et al., 2006). On the other hand, Morgan et al. (1990) indicated failed recovery of AChE activity in fish after one week in clean media.

Under food restriction, Pb treatment (among generations) *D. similis* decreased R_0 and lifespan and delayed hatching while increased AChE activity, in comparison to F0. Differing from usual food *D. similis*, Pb exposure did not affect R_0 which was similar to control (all generations); this suggests that the lower reproduction (compared to F0) was due to food restriction. Lower reproduction due to food restriction was shown for *D. similis* by Pedrozo and Bohrer (2003). Guan & Wang (2006) also observed reproduction impairment after six-generation of food restriction and Cd (3µg/L) exposure. Comparing usual and restricted food *D. similis*, it is clear that food quantity influence organisms' sensitivity to Pb (e.g. reduced R_0); similar results were obtained for food restricted daphnids exposed to Pb (Enserink et al., 1995b) and Cd (Heugens et al., 2001). Regarding hatching delay, F9 control organisms exhibited delayed reproduction compared to F9 Pb treatment, indicating that Pb (combined with food restriction) induced early reproduction. This outcome is contrary to that exhibited by *D. magna* under food restriction. Pieters & Liess (2006) suggested that low maternal food may accelerate the beginning of reproduction. This is an important outcome because

organisms from natural ecosystems usually are under food restriction (Chandini, 1989), and, when this condition is combined with Pb exposure, *D. similis* starts reproducing early while *D. magna* presents an opposite response. Thus, it may be not appropriate to use *D. magna* (temperate model species) to estimate *D. similis* (tropical) response to chemicals. Despite the difference between generations, F9 Pb exposure lifespan and AChE did not differ from F9 control, indicating that the decreased lifespan and enhanced AChE activity probably was due to food restriction. Chandini (1989) observed reduction in the lifespan of daphnids after Cd exposure combined with food restriction. Other authors reported diminished lifespan of organisms under food restriction (Kim et al., 2014; Latta et al., 2011; Schwartz et al., 2017). Regarding enzymatic activity, food restriction may regulate AChE activity (Suchiang & Sharma, 2011)

4.3. Recovery from chemical exposure

Recovering *D. magna* (usual food) presented lower R_0 and higher AChE activity than F0 organisms. However, R_0 in recovering organisms did not differ from control or Pb treatments (F9), indicating a natural fluctuation, as previously stated in this study. The hatching delay of recovering *D. magna* was similar to Pb treatment but lower than control (F9), suggesting an accelerated reproduction of pre-exposed organisms (failed retrieval). A similar result was observed by Agra et al. (2011), who found that mine drainage resistant *D. longispina* started reproducing earlier under Cu exposure. Recovery period lifespan reached an intermediary value between F9 control and Pb exposure, suggesting that the recovery period was not long or effective enough to achieve control conditions. Reduced lifespan of daphnids due to chemical exposure was previously reported to methylmercury (Doke et al., 2017), Zn and Cu (Winner, 1981) and Cd (Chandini, 1989) exposure. Regarding retrieval, Schultz et al. (2016) found a concentration-dependent reduction on nematodes (*Caenorhabditis elegans*) lifespan under silver ions and nanoparticles (Ag+ and AgNP) and also stated that the endpoints presented were more closely related to the last exposed generation than the control, as it happened for *D. magna* in this study. Failed recovery has been shown by other studies (Taylor et al., 2016; Yan et al., 2016).

As stated before, recovering organisms exhibiting outcomes that diverge from control may indicate epigenetic changes. Organisms can adapt to chemical exposure through physiological acclimation or transgenerational transfer (epigenetics). When organisms suffer epigenetic changes after a chemical exposure, they do not recover similarly to control (or to the condition exhibited previously to the exposure) (Vandegehuchte et al., 2009b),

while when they physiologically acclimate, a full recovery is shown and outcomes tend to be similar to control and the conditions prior to the exposure (Barata et al., 2002).

AChE activity had interesting results during the recovery period; an enhanced activity was shown by recovering *D. magna* (higher than F9 Pb and Pb ($clean_{96h}$) treatments. F9 control and Pb ($clean_{96h}$) presented similar enzymatic activity, indicating that the organisms can recover already after 96 h in clean media. However, recovering *D. magna* (three generations in clean media) presented the highest AChE activity, which was statistically different from F9 Pb and Pb ($clean_{96h}$) and not from F9 control; this suggests that time has a crucial role when it comes to organisms' depuration. Morgan et al. (1990) evaluated AChE of fish and observed that retrieval started one week after the end of the chemical exposure, but it was successful only after six weeks in clean media.

Under food restriction, recovering *D. magna* had lower R_0 and lifespan and delayed hatching and higher AChE activity in comparison to F0. Although endpoints differ between generations, no statistical difference was shown among treatments (recovery period to F9 control or Pb). With that in mind, such variations were probably caused by the food restriction; this factor was previously discussed in this paper, as well as its effects on reproduction and AChE. Diminished R_0 and lifespan (Kim et al., 2014; Smolders et al., 2005) and delayed reproduction (Stige et al., 2004) due to food restriction has already been shown.

On their turn, recovering *D. similis* (usual food) endpoints were similar to F0 organisms (as well as F9 control treatment). The Net Reproductive Rate (R_0) of recovering *D. similis* was higher than F9 Pb treatment and similar to F9 control, indicating that organisms were able to recover (regarding reproduction). The enhanced R_0 of recovering organisms (compared to F9 Pb treatment) could be a compensation for the Pb pre-exposure (Printes et al., 2008). *Daphnia* recuperation is time and chemical concentration dependent, and successful recovery was already presented by other studies (Duquesne et al., 2006; Li et al., 2016). The enzymatic activity of recovering *D. similis* was lower than F9 control and Pb treatments, however, it was similar to Pb ($clean_{96h}$), indicating that organisms did not fully recover. Failed AChE retrieval was already shown in the literature (Morgan et al., 1990; Reddy et al., 2003). An important highlight is that F9 control and Pb treatments did not diverge for AChE activity, while recovering organisms (both Pb ($clean_{96h}$) and recovery period) presented lower AChE activity. Such results indicate a divergence of recovering process among *D. similis* and *D. magna* (enhanced AChE activity than Pb ($clean_{96h}$)).

On the other hand, under food restriction, *D. similis* from recovery period presented similar results as *D. magna* (diminished R_0 and lifespan and delayed hatching) compared

to F0. Although the R_0 of recovering organisms was decreased in comparison to F0, it did not differ from F9 control nor Pb treatment, and it was probably due to food restriction. Diminished reproduction and lifespan due to food restriction on retrieval organisms was shown by other authors (Frost et al., 2010; Ginjupalli & Baldwin, 2013; Schwartz et al., 2017). The hatching delay of recovering *D. similis* was similar to F9 Pb treatment and both presented an accelerated reproduction in comparison to F9 control. Failed recovery was shown by daphnids under a four-generation exposure to microplastics; in that case, organisms showed lower R_0, reproduction and body length (Martins & Guilhermino, 2018). Food restriction and Pb exposure combined resulted in a shorter lifespan for *D. similis* (shorter than F9 control and Pb treatments), a type of result that was previously reported (Roff, 2001), for Cr (Coniglio & Baudo, 1989) and Ni (Münzinger, 1990) exposure.

4.4. Principal Component Analysis (PCA) evaluation

The PCA just confirmed the results obtained when the variables are evaluated individually. F0 variables presented opposite trends than F9 setups (control, Pb and recovery period). Such outcome was demonstrated by the inverse correlations to PC1 (F0 negative and F9 positive). These integrations help us to elucidate the crucial effect of food restriction on daphnids for many generations, since all treatments from F9 (including control) diverged from generation F0.

According to the results discussed before, the Net Reproductive Rate (R_0), hatching delay, lifespan and AChE of both species under food restriction differed in F0 from F9 (control, Pb and recovery period), except for the lifespan of *D. magna* (F9 control) and the AChE of *D. similis* (F9 Pb ($clean_{96h}$) and recovery period). According to the PCA analysis, it can be inferred that generation F0 indicated a higher association with R_0 while F9 Pb exposure and recovery period indicated an association with hatching delay (both species) and AChE of *D. similis*.

The inverse association of PCA regarding reproductive outputs (R_0 and hatching delay) of both species and food regimes could may be due to an association of low R_0 to delayed reproduction, indicating that the number of neonates produced (from brood N1 to N5) was reduced when females delayed reproduction, as previously discussed in this manuscript. The survival expectancy of both species was also associated to reproductive outputs (with hatching delay for *D. similis* and R_0 for *D. magna*), as well as the AChE activity of *D. similis*, which related to reproductive outputs (at both food regimes) and survival expectancy (usual food). Diminished R_0 was related to enhanced AChE activity in this study, in a similar way to the *D. magna* exposed to the insecticide Guadipyr (Qi et al., 2013).

4.5. Daphnia magna vs. Daphnia similis

To conclude, food restriction triggered similar reproductive outputs on both species (decreased R_0 and reproduction delay), however, both species differ regarding lifespan (F9 control), with *D. magna* reducing its lifespan while this parameter for *D. similis* was not affected. Regarding F9 Pb exposure, both species were affected, with *D. magna* exhibiting a more evident response, such as reduction of lifespan, delayed hatching and AChE activity (compared to F9 control); Pb exposed *D. similis*, in its turn, presented decreased R_0 (than F9 control). Under food restriction, both species showed similar responses (shorter lifespan) and only diverged for the hatching delay (Pb accelerated *D. similis* reproduction). The recovery period (usual food) presented a lot of disparities between species. *D. similis* reproduction (R_0) was fully recovered, indicating a physiological acclimation. Instead, *D. magna* suggested the occurrence of epigenetics, since this species presented early reproduction (as well as Pb treatment) and a failed attempt to recover. The recovering *D. magna* increased lifespan (not similar to F9 control nor Pb treatments) could be a sign of epigenetic changes or only a matter of time (not enough to full retrieve). Both species also differed regarding AChE activity, where *D. magna* was able to recover (similar to F9 control but higher than Pb and Pb ($clean_{96h}$)). On the other hand, AChE activity of *D. similis* failed to recover, indicating that despite three generations in clean media *D. similis* could not recover. Under food restriction the recovery process of *D. magna* showed no divergence among treatments for all endpoints evaluated, indicating that all the observed responses were due to food restriction. *D. similis* however, showed shortened lifespan after Pb exposure and accelerated reproduction, indicating a failed recovery (and possibly epigenetic changes). Therefore, the results of this study show that some caution should be taken when using daphnids of different regions (temperate and tropical) to establish sensitivity to pollutants, since differences were observed between *D. similis* and *D. magna*. Further investigation (bioaccumulation, epigenetics and gene expression) should be accomplished in order to reassure the physiological or epigenetic element on organisms' recovery process.

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Supplementary material

Table 5S.1: Chemical Analyses

Nominal concentrations	Chemical Analyses	
	Stock (46.54 mg/L)	0.05 mg/L
$Pb(NO_3)_2$	48 mg/L	0.054 mg/L
Recovery (%)	103	108

Table 5S.2: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding the acetylcholinesterase activity (AChE). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Acetylcholinesterase (AChE) activity					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	75.97	6	12.66	F (6, 108) = 30.76	P < 0.0001
Setups	26.86	3	8.954	F (3, 108) = 21.75	P < 0.0001
Generations	63.07	2	31.54	F (2, 108) = 76.61	P < 0.0001
Residual	44.46	108	0.4117		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	28.65	6	4.776	F (6, 108) = 13.15	P < 0.0001
Setups	31.16	3	10.39	F (3, 108) = 28.61	P < 0.0001
Generations	122	2	60.99	F (2, 108) = 168.0	P < 0.0001
Residual	39.21	108	0.3631		
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	24.97	6	4.162	F (6, 108) = 8.800	P < 0.0001
Setups	67.79	3	22.6	F (3, 108) = 47.78	P < 0.0001
Generations	80.51	2	40.26	F (2, 108) = 85.13	P < 0.0001
Residual	51.07	108	0.4729		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6.887	6	1.148	F (6, 88) = 1.259	P = 0.2848
Setups	47.91	3	15.97	F (3, 88) = 17.51	P < 0.0001
Generations	123.2	2	61.58	F (2, 88) = 67.53	P < 0.0001
Residual	80.25	88	0.9119		

Table 5S.3: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding the Net Reproductive Rate (R_0). Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Net Reproductive Rate (R_0)					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	18504	4	4626	F (4, 18) = 52.73	P < 0.0001
Setups	9399	2	4699	F (2, 18) = 53.56	P < 0.0001
Generations	925.2	2	462.6	F (2, 18) = 5.273	P = 0.0158
Residual	1579	18	87.73		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	22363	4	5591	F (4, 18) = 247.3	P < 0.0001
Setups	5993	2	2997	F (2, 18) = 132.6	P < 0.0001
Generations	8996	2	4498	F (2, 18) = 199.0	P < 0.0001
Residual	406.9	18	22.6		
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	13635	4	3409	F (4, 18) = 101.8	P < 0.0001
Setups	2624	2	1312	F (2, 18) = 39.17	P < 0.0001
Generations	1351	2	675.7	F (2, 18) = 20.17	P < 0.0001
Residual	602.9	18	33.5		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	10701	4	2675	F (4, 18) = 60.39	P < 0.0001
Setups	3754	2	1877	F (2, 18) = 42.37	P < 0.0001
Generations	1492	2	746.1	F (2, 18) = 16.84	P < 0.0001
Residual	797.4	18	44.3		

Table 5S.4: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding hatching delay. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Haching delay					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	64.39	6	10.73	F (6, 24) = 3.018	P = 0.0242
Setups	90.06	2	45.03	F (2, 24) = 12.66	P = 0.0002
Generations	74.44	3	24.81	F (3, 24) = 6.979	P = 0.0015
Residual	85.33	24	3.556		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	127.6	6	21.26	F (6, 24) = 3.610	P = 0.0108
Setups	113.6	2	56.78	F (2, 24) = 9.642	P = 0.0008
Generations	410.5	3	136.8	F (3, 24) = 23.24	P < 0.0001
Residual	141.3	24	5.889		
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	97.5	6	16.25	F (6, 24) = 3.232	P = 0.0180
Setups	77.39	2	38.69	F (2, 24) = 7.696	P = 0.0026
Generations	81	3	27	F (3, 24) = 5.370	P = 0.0057
Residual	120.7	24	5.028		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	121.5	6	20.25	F (6, 24) = 4.028	P = 0.0062
Setups	145.4	2	72.69	F (2, 24) = 14.46	P < 0.0001
Generations	598.8	3	199.6	F (3, 24) = 39.70	P < 0.0001
Residual	120.7	24	5.028		

Table 5S.5: Two-way ANOVA results testing for effects of setups (Control, Pb exposure and recovery period) and among generations (F0 to F9) of both *Daphnia magna* and *Daphnia similis* and their interaction regarding lifespan. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Lifespan					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2203	4	550.8	F (4, 18) = 34.19	P < 0.0001
Setups	1989	2	994.3	F (2, 18) = 61.72	P < 0.0001
Generations	712.7	2	356.3	F (2, 18) = 22.12	P < 0.0001
Residual	290	18	16.11		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	746	4	186.5	F (4, 18) = 10.23	P = 0.0002
Setups	314	2	157	F (2, 18) = 8.616	P = 0.0024
Generations	1400	2	700	F (2, 18) = 38.41	P < 0.0001
Residual	328	18	18.22		
Dahnia similis ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1236	4	309	F (4, 18) = 7.242	P = 0.0012
Setups	345.4	2	172.7	F (2, 18) = 4.048	P = 0.0353
Generations	1908	2	953.8	F (2, 18) = 22.36	P < 0.0001
Residual	768	18	42.67		
Dahnia similis ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1529	4	382.2	F (4, 18) = 24.22	P < 0.0001
Setups	422.7	2	211.4	F (2, 18) = 13.40	P = 0.0003
Generations	1973	2	986.3	F (2, 18) = 62.51	P < 0.0001
Residual	284	18	15.78		

Chapter 6

Bioaccumulation and morphological traits in a multi-generation test with two *Daphnia* species exposed to Lead

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Bioaccumulation and morphological traits in a multi-generation test with two *Daphnia* species exposed to Lead

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Abstract

Anthropic pressure negatively affects natural environments. Lead (Pb) is a non-essential highly toxic metal that is present in aquatic ecosystems. Two daphnid species from two different latitudes, the temperate *Daphnia magna* and the tropical *Daphnia similis* were used as test-organisms to evaluate a long-term Pb exposure. Both species were exposed for nine generations to a chronic concentration of Pb (50 µg/L) and the effects were explored, considering some endpoints not commonly used in toxicity tests: body burden of Pb and presence of granules in the dorsal region of neonates, hemoglobin contents, carapace deformation and morphology, production of males and ephippia (or dormant haploid egg), changes in the eggs' color and eggs abortion. This multi-generation test was conducted under two food regimes, the usual ($3x10^5$ cells/mL) and the restricted ($1.5x10^5$ cells/mL) regime. On generation F6, Pb acclimated neonates were changed to a clean media for three generations, to evaluate exposure retrieval (recovery period). Negative and adverse effects occurred through generations, but no disparity was shown between *D. magna* and *D. similis*. The *D. magna* Pb accumulation showed different patterns regarding food regime. Bioaccumulation was faster under usual food, rapidly reaching a saturation point, whereas a gradual increase occurred under food restriction. A successful retrieval happened regarding Pb in *D. magna*, since no difference between control and recovering organisms was evidenced regarding their Pb body burdens. Generational Pb exposure led to carapace malformations, Pb aggregation in neonates' dorsal region, reddish extremities, production of males, ephippia (or dormant haploid egg), and aborted eggs, and changes in the eggs' color (green and white). Food restriction also induced the production of males. Reddish extremities disappeared in recovering organisms and ephippia (or dormant haploid egg) did not occurred during the recovery period. Existent males revealed a shorter lifespan than females (under stress). *D. magna* and *D. similis* presented similar responses, for the endpoints analysed; however, it does not mean that this lack of sensitivity difference will be observed when other endpoints (e.g. survival, reproduction) are considered. Bioaccumulation of Pb and adverse effects occurred at the tested concentration of 50µg/L, although higher Pb levels are allowed in the environment as safe concentrations, as reported by the Brazilian legislation and the literature where effects are evidences above 400 □g/L of Pb. Pb effects on reproduction, respiration, malformation, and other adverse effects suggest that a chronic generational exposure can be harmful to both *D. magna* and *D. similis*, and that such chronic contaminated environments should not be disregarded when it comes to environmental monitoring.

Key-words: multi-generation, *Daphnia*, Pb, males, ephippia, reddish extremities, malformation.

1. Introduction

Lead (Pb) is a non-essential metal that can be highly toxic to aquatic biota. Non-essential elements can be even more dangerous than essential metals, since their presence may interfere in the metabolism of those essential nutrients with similar characteristics and a long exposure might result in accumulation and, consequently, transference through the food web (Hashim et al., 2014; Gašpić et al., 2002). Pb sources are mainly from anthropic actions (e.g. fuel, pigment and paint additive, industrial effluents, mining activities, wood treatment, among others) but natural sources (e.g. volcanoes, Pb ores) may also contribute to increase levels of Pb in the environment (Mager et al., 2011). Pb is a neurotoxic chemical that can affect organisms behavior (Reddy et al., 2003) and it may be the most toxic metal for aquatic organisms, affecting organisms gills (respiratory system), renal dysfunction, enzymatic inhibition and anemia (Hernández-Flores & Rico-Martínez, 2006). This metal recently entered the Candidate List of substances of very high concern (SVHCs) created by the European Chemicals Agency (ECHA).

The concentrations of Pb in the natural environment can reach high concentrations (Mahiques et al., 2012) and even exceed the legal limits (Bordon et al., 2011; CCME, 2001). Lead has also been found in soft tissues of organisms consumed by humans such as fish (Gusso-Choueri et al., 2018, 2016, 2015; Hashim et al., 2014) and crustaceans (Bordon et al., 2012). Concentrations of Pb above European limits (EC, 2008) were found in mussels in the Mediterranean, Adriatic, and Black Sea (Stankovic & Jovic, 2012), above Mercosul and European limits (ANVISA, 2013; EC, 2008) in fish (sashimi of Japanese restaurants) (Morgano et al., 2014) and seafood in Italy (Cirillo et al., 2010), as well as in European agricultural products (Dudka & Miller, 1999). Therefore, such high Pb concentration in organisms consumed by humans are of a major concern. This show the importance of chemical evaluation in chronically contaminated areas, where the contamination is not as easily perceived as in acute contaminated environments (e.g. mortality), but it can still affect native organisms, the food chain and, consequently, humans (Newton et al., 2003).

Standard toxicity tests, with well-known test-organisms, such as daphnids, have been normally used to estimate the toxicity of Pb on natural ecosystems. *Daphnia* is a well-known species, with a simple and widely used standard protocol for toxicity testing and worldwide used in ecotoxicological assessments (OECD, 2012, 2004). It is small sized and easily cultivated in laboratory, with a parthenogenetic reproduction that facilitates generational

approaches (Tsui & Wang, 2005). Standard protocols for acute and chronic tests are based on the exposure for 48 h and 21 days, respectively; consequently, they do not realistic mimic a long-term contaminated environment (Guan & Wang, 2006b), where the populations are exposed for multiple generations. Therefore, a multi-generation chemical exposure may be a more realistic approach (Tsui & Wang, 2005). Moreover, to accurately represent a natural environment, one has to consider that geographical differences may affect organisms' sensitivity to chemical exposure (Ghilarov, 1967), because they often relate to differences in some physical and chemical aspects, such as water temperature, pH, conductivity, hardness, among others. With that in mind, this study used two monophyletic *Daphnia* species, the temperate species *Daphnia magna* and its equivalent from tropical environments, *Daphnia similis*. To overcome the factor "temperature" as a key ruling factor in toxicity studies, both species were cultured and tested at 20ºC.

The goal of this study was to compare the effects of a long-chronic Pb exposure on two *Daphnia* species. Both species were submitted to a nine generation chronic Pb exposure (50 µg/L), which is the limit permitted concentration established by the Brazilian legislation for water bodies (rivers) (CONAMA, 2005), and adverse effects were recorded during this period as well as the accumulation of Pb in organisms. In order to consider the nutrient variability in the natural ecosystems, the generational exposure was prepared under two different food regimes (usual and restriction of food). Moreover, to check if exposed organisms were capable of retrieval, a recovery period was allowed, in which Pb acclimated daphnids were transferred to a clean media and monitored for more three generations.

2. Methodology

2.1. Culture maintenance

Daphnids cultures were maintained under constant photoperiod and temperature (16:8 h light/dark and 20 ± 2 ºC, respectively). Cultures were kept in one liter flasks with hard-water ASTM medium (ASTM, 2002). Food (*Raphidocellis subcapitata*, $3x10^5$ cells/mL) and media were renewed every other day. In addition to the algae, daphnids' cultures were enriched with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). Cultures were initiated with neonates from the third to fifth brood (N3 to N5). *D. magna* and *D. similis* were cultured under the same conditions, with no influence of abiotic factors during the test.

2.2. Chemical analysis

The $Pb(NO_3)_2$ (CAS No. 10099-74-8, 98.5% purity, VWR chemicals®) was used to expose daphnids during the multi-generation test. Stock solution was prepared in mili-Q water, which was diluted in ASTM medium for the sub-lethal exposure. Prior to analysis Pb samples were acidified with nitric acid and the analysis were performed by ICP-OES (Horiba Jobin Yvon, Activa M). Samples were evaluated in triplicate and duplicate samples of certified material were used, to ensure chemical optimum recovery during procedures.

2.3. Multi-generation test

The multi-generation test consisted in two main setups, the negative control (ASTM medium) and the Pb exposure (50 µg/L of Pb), which was prepared by diluting $(PbNO_3)_2$ in ASTM medium. For both situations (control and Pb exposure) organisms were submitted to two different dietary regimes, the regular ($3x10^5$ cells/mL) and the restricted, with half of the algae concentration ($1.5x10^5$ cells/mL). The test lasted nine generations (F0 to F9), as shown in Figure 1.2. All generations started with the third brood (N3) of the previous generation. Pb acclimated neonates (from continuous Pb exposure) from generation F6 were transferred to a clean media (same as control) to check for recovery, which lasted from F6 to F9 (recovery period). All setups (negative control, Pb exposure and recovery period) were kept in one litter flaks with 20 daphnids (as cultures), with three replicates for each setup. The pH (WTW pH 330i) and the dissolved oxygen (DO; WTW OXI 330i) of all treatments were measured to monitor ideal conditions during the generational test.

2.4. Pb accumulation

The accumulation of Pb was evaluated only for *D. magna*, because problems occurred with the *D. similis* samples and, as it was a long generational exposure, it was not possible to repeat such experiment. Bioaccumulation was measured in organisms (20 neonates) of generations F0, F6 and F9, from each setup (negative control, Pb exposure and recovery period) and rinsed with ultrapure water (to remove the medium excess). Afterwards, samples (20 neonates in 1.5 mL Eppendorf® flasks, 3 replicates per treatment) were freeze-dried overnight and weighed in a microbalance. Then, samples were transferred to a Teflon beaker to start digestion. The digestion was carried out with nitric acid (HNO_3, 69%, trace analysis), and heated in a hot plate until dryness, to destroy all organic material. The residues in the bottom of the Teflon beakers were re-suspended with 1000 µl of nitric acid and Pb concentration was measured by Flame Atomic Absorption Spectrometry (FAAS) Analytik Jena AG, Germany, AAS 5 FL (deuterium lamp), at the laboratory of chemistry

REQUIMITE of New University of Lisbon (NOVA). The detection limit for Pb in FAAS was 1 µg/L. Three replicates were prepared for each setup, including for the certified reference material (CRM) (TORT-3 lobster hepatopancreas) to control the method accuracy and assess recovery (Pb concentration, 0.225 ± 0.018 mg/kg). All values were calculated based on organisms' dry weight.

2.5. Endpoints evaluated

Trying to escape classical endpoints used in acute and chronic toxicity tests (e.g. mortality/immobility and reproduction rate, respectively) and obtain a broader approach, during this multi-generational exposure, daphnids were cautiously evaluated throughout generations under a stereomicroscope (MS5, Leica Microsystems, Houston, USA) and observed adverse effects produced by a long-term chronic Pb exposure were reported. We monitored some qualitative endpoints which indicate stress in organisms cultured and are not usually used in ecotoxicological analysis, such as the presence of granules in the dorsal region of neonates, reddish extremities (probably indicating increased haemoglobin content), morphology (carapace malformations) and the production of males. Other endpoints were evaluated by naked eye, such as the production of ephippia (or dormant haploid egg), changes in the eggs' color and eggs abortion. All these phenotypical characteristics were identified in a subsample of 20 neonates per replicate, per treatment. Along this, males (all) and females (20 per replicate, 3 replicates per treatment) were separated to infer on their lifespan, with organisms being maintained in similar conditions as those where they were born until "natural" death occurred.

2.6. Statistical analysis

The data was first tested for normality (Kolmogorov–Smirnov) and homoscedasticity (Levene's equal variance test). Then, a Two-Way Analysis of Variance (ANOVA) was done in order to identify differences among generations and setups (negative control, Pb exposure and recovery period), for the chosen endpoints. A post-hock (Bonferroni) test was made when differences were identified. All analyses were made using the GraphPad Prism® software.

3. Results and Discussion

The pH (7.5 ± 0.2 SD) and DO (> 7.2 mg/L in all samples) levels for all generations were within the recommended values by OECD (2012), where a value between 6 and 9 for

pH and >3 mg/L for OD is suggested. The chemical analyses of Pb retrieved a >79% recovery comparing nominal to measured concentrations. The limit of quantification (LOQ) was 25 µg/L and ASTM media (control) presented values <LOD (Table S2). The certified material evaluated achieved above 80% of recovery for Pb.

The Pb accumulation in continuous Pb exposed *D. magna* increased over the generations (Figure 6.1, Table 6S.3 (ANOVA statistical results)). Under usual food, continuous Pb exposure *D. magna* from generations F6 and F9 exhibited a statistically higher Pb concentration than F6 and F9 control treatment and F0 organisms (Figure 6.1a). *D. magna* from generation F6 presented a trend of higher Pb accumulation than generation F9, but not statistically different. Under food restriction, Pb exposed *D. magna* in F9 showed a gradual increase of Pb accumulation, being statistically different from F9 control and F0.

Pb accumulation in recovering organisms (usual food regime) decreased after three generations in clean media, being slightly higher than the control, but not statistical different (Figure 6.1c). Under food restriction, concentration of Pb in the recovering organisms decreased to values similar to the control treatment, being statistically lower than continuous Pb exposure at generation F9.

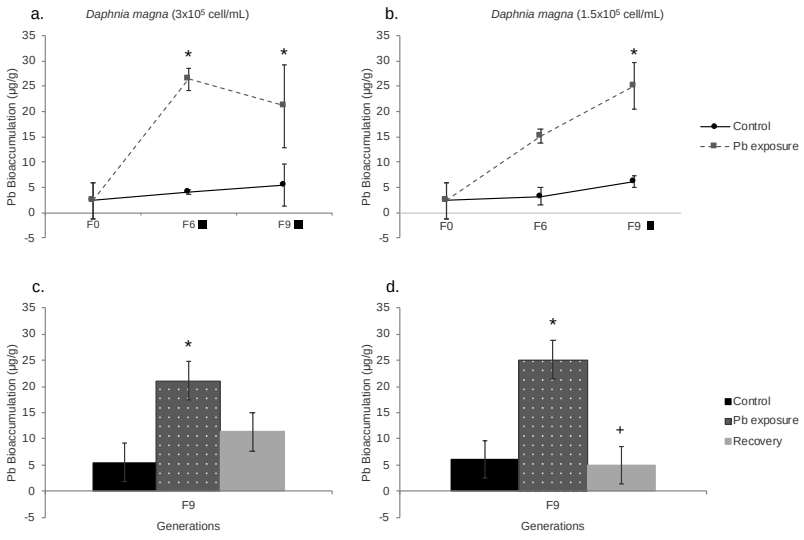

Figure 6.1: Pb bioaccumulation in *Daphnia magna* under a continuous exposure to a negative control (ASTM) and Pb, in two food regimes ($3x10^5$ and $1.5x10^5$ cells/mL). Top graphs represent F0, F6 and F9 generations under continuous exposure (Control and Pb), while bottom graphs represent F9 generations under control, Pb exposure and after recovery. Generations in the X axis are marked with a black square for those statistical different from F0, in Pb exposure (Bonferroni test, $p<0.05$). Asterisk (*) indicate statistical difference from control (within the same generation), and the plus sign (+) indicate statistical difference from continuous Pb exposure (within the same generation)

(Bonferroni test, $p<0.05$). Data presented for control and Pb (**c.** and **d.**) are the same as presented in **a.** and **b.**, just for comparison.

The different results among food regimes may be because part of Pb accumulation in daphnids could have occurred through the diet, since algae is known to be able to incorporate Pb^{2+} (Hernández-Flores & Rico-Martínez, 2006), thus we can infer that Pb uptake by D. magna was higher in organisms of usual food. The Pb accumulation in *D. magna* under usual food seems to have reached a maximum at generation F6, followed by a slight reduction at generation F9. A reduction of the Pb accumulation during the recovery period was shown. Guan & Wang (2006a) indicated that pre-exposed daphnids are capable of potentiate metal accumulation in comparison to control. However, since organisms are capable of recovery (regarding Pb accumulation), the Pb concentration on wild daphnids could indicate a recent, but not long-term exposure (MacLean et al., 1996), as shown by organisms from the recovery period.

To our knowledge, no study with Pb accumulation among *Daphnia* generation exist in the literature, therefore, the endpoint that evaluated a Pb accumulation in different organisms (among generations) cannot be compared with other under similar conditions. Despite this, some studies on *Daphnia* Pb accumulation using a short-term exposure to Pb indicate that the Pb accumulation pattern differs from those patterns reported in the literature for other metals (e.g. Cd, Ni, Zn; Guan & Wang (2004); Komjarova and Blust (2008)) and displays a hyperbolic kinetics (curvilinear), suggesting that Pb has a high initial accumulation rate and reach rapid saturation, which could sometimes be accompanied by a decrease of Pb accumulation (Komjarova & Blust, 2008; MacLean et al., 1996; Roy, 2009). Increasing metal accumulation on daphnids tissue is a usual response after metal exposure, as it was already shown for *D. magna* exposed to various metals (Cd, Cu, Ni, Zn and Pb) (Komjarova & Blust, 2008); to Cd (Guan & Wang, 2006, 2004); and Pb (Grosell et al., 2006; Hernández-Flores & Rico-Martínez, 2006; Mager et al., 2011; Roy, 2009). Pb^{2+} uptake in *Daphnia* occurs through calcium channels, disrupting Ca^{2+} and Na^{+} uptake, altering the ionic balance within the cells and affecting homeostasis (Grosell et al., 2006; Roy, 2009). This disruption caused by Pb exposure on calcium channels can have serious consequences. Since *Daphnia* carapaces have calcium in its composition, an obstruction of such channels could induce serious damage on daphnids moulting process (Roy, 2009). Hessen et al. (2000) affirmed that when calcium deficiency occurs, daphnids tend to decrease size or have a low calcium content carapace. In accordance with such statements, Pb induced carapace malformations, such as the protuberance shown in Figure 6.2b.

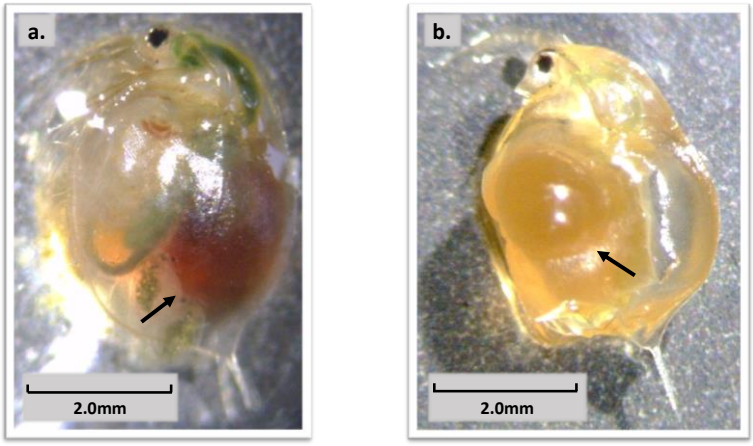

Figure 6.2: Morphological aspects of *Daphnia similis* (**a.**) and *Daphnia magna* (**b.**) from continuous Pb exposure under usual food regime a. arrow indicates red brood chamber with reddish shell gland, containing two neonates with "dry" green granules at generation F8 and b. arrow indicates carapace deformation at generation F6.

Besides carapace malformations, Pb induced other negative effects on daphnids, such as the red brood chamber presented by Pb exposed *D. similis* (Figure 6.2a) and the "dry" green granules shown by Pb exposed and recovering *D. magna* and *D. similis* (both food regimes) (Figure 6.3a,b). Malformation of young *D. magna* due to Pb and food restriction was shown by Enserink et al. (1995). Reports on malformations induced by metals are antique, with some fossils from palaeozoic plankton organisms showing increased malformations linked with enhanced metal concentrations (Fe, Mo, Pb, Mn and As) (Vandenbroucke et al., 2015). Besides, Pb exposure (3.25 mg/L) was reported to induce a curly spinal cord on Zebrafish embryos (Tu et al., 2017).

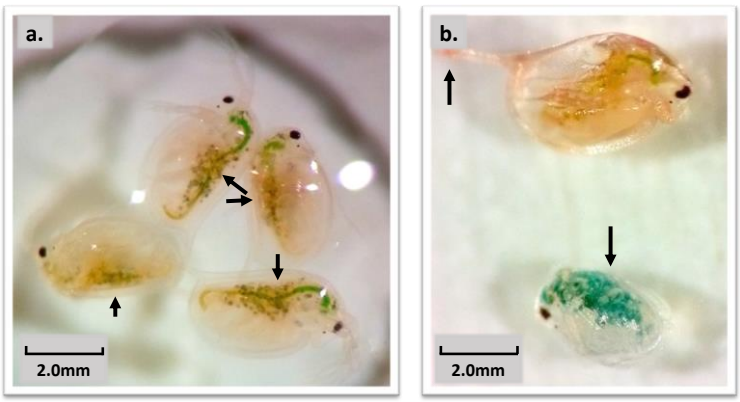

Figure 6.3: Morphological aspect of *Daphnia similis* (**a.**) from the recovery period (N1, F9) under food restriction with light "dry" green granules (arrows) and (**b.**) from continuous Pb exposure under food restriction (N3, F9), where differences in size is depicted and reddish extremities (b. top neonate) and "dry" green granules (b. bottom neonate) are indicated with arrows.

The granules observed in neonates' bodies (Figure 6.3) appear to be Pb accumulation at their dorsal region. The feeding pattern of daphnids begins by locomotion, in which particles in the water column are captured by the setae (used to filter particles) and brought to the ventral groove, where water is removed and the particles are trapped and taken to the mouth (Abbas et al., 2012). This process involves not only food but also other particles which can be further impregnated on *D. similis* gut, shell and appendices (Becaro et al., 2015), producing a similar aggregation as revealed by this study. Chemical aggregation in daphnids was previously reported it the literature, including enhanced residual metal body burden (Böhme et al., 2015; Heinlaan et al., 2017). Moreover, a crucial factor is that a Pb maternal transfer to eggs occurs in ovigerous crustaceans females (Lavradas et al., 2014).

Pb exposure may have also led to an increase of the haemoglobin content in organisms at generation F9 (Pb exposure and recovery period, both species and food regimes), which was revealed as reddish extremities in neonates (Figure 6.4a). Highlighting that the oxygen levels were within the limits recommended by OECD (2012) (>3 mg/L). Haemoglobins facilitate oxygen transportation and, in a hypoxic environment, haemoglobin production tend to increase (Kobayashi & Hoshi, 1982). However, besides haemoglobin regulation by oxygen concentrations, it might also be regulated by chemical stress (Ha & Choi, 2009), being haemoglobin and stress positively correlated (Schwerin et al., 2010). Haemoglobin can have diverse functions (besides as a respiratory protein) and also performs as a protein store, being a buffer between protein availability (food) and individuals protein demand (Schwerin et al., 2010). Pb exposure inducing haemoglobin production was seen for *D. magna* at 250 µg/L (24 h) (Ha & Choi, 2009) and 64 µg/L (41 h) (Berglind et al., 1985). The association between Pb and haemoglobin was already stated by several authors, which suggests that after Pb enters the cell it binds mainly to haemoglobin (Kutllovci-Zogaj et al., 2014; Roy et al., 2011; Simons, 1986). Roy (2009) suggested that Pb can trigger respiratory disturbances in freshwater invertebrates. However, Berglind et al. (1985) stated that, after 10 days of recovery, haemoglobin levels of *D. magna* were similar to control. This statement supports the Pb accumulation of recovering organisms presented in this study (Figure 6.1) and disappearance of reddish extremities in recovering neonates (Figure 6.4b). The exposure to Pb tend to reduce the O_2 consumption of *D. magna* and *D. similis* (Chapter 2), as did other metals (Khangarot & Rathore, 2003; Zitova et al., 2009). Muyssen et al. (2006) suggested that this reduced O_2 consumption may be due to gas exchange surface structural damage and, a depletion of gas exchange between individuals and the environment triggered by metals was implied by Pane et al. (2003). This is a crucial

endpoint, since an increase in *Daphnia* haemoglobin content can be correlated with decreased reproduction (Ha & Choi, 2009).

Pb exposure also tend to inhibit the δ-aminolevulinic acid dehydratase (ALAD) activity (Jan et al., 2015). ALAD is the most Pb-sensitive erythrocyte enzyme and participates during the haemoglobin synthesis process by condensing two molecules of aminolevulinic acid to form porphobilinogen in red blood cells (Hoffman et al., 2003; Sharma et al., 2014). Organisms exposed to Pb tend to bind this metal to the -SH group of ALAD, making it inactive (Ercal et al., 2001). This ALAD inhibition may lead to haemoglobin oxidation (Flora et al., 2012) by increasing the amount of the substrate δ-aminolevulinic acid (ALA), which may generate hydrogen peroxide and reactive oxygen species (ROS) (Ercal et al., 2001). Although, Berglind et al. (1985), indicated that *Daphnia* exposed to low sub-lethal Pb concentrations enhanced ALAD activity and haemoglobin content and, according to Gonick (2011), ALAD have different isoforms being both inhibited or induced by Pb.

A crucial point here is that ALAD activity inhibition may not lead to a decrease in haeme production, due to a compensatory increase in ALAD production (Hoffman et al., 2003). Pb intoxication causes disturbances in the haeme biosynthesis and the inhibition of ALAD activity leads to accumulation of ALA and other intermediates, such as protoporphyrin IX, in erythroid cells (Lubran, 1980; Sachar et al., 2016). Protoporphyrin IX has a dark red colour (Maere et al., 2014), which could be the reason for the neonates reddish extremities. Similar occurrences have been observed in fish, where ALAD inhibition was observed at low Pb concentrations (10 µg/L in fish) (Hodson et al., 1977), with fish intoxicated by Pb presenting a black-tail effect, which is the darkening of the dorsal region, due to selective destruction of chromatophores (Demayo et al., 1982).

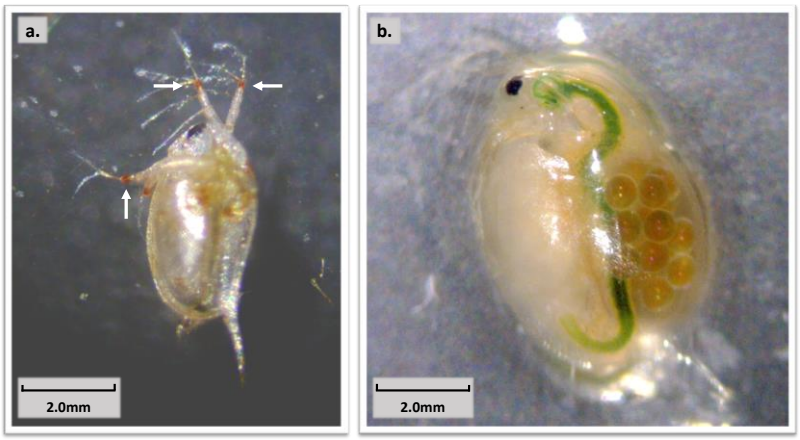

Figure 6.4: *Daphnia magna* individual (F7) with <24h old (a.) and with 7 days old (b.) obtained from the recovery set up under food restriction. **a.** arrows depicts neonate reddish extremities; **b.** with loss of reddish extremities (recuperation).

Increased levels of haemoglobin and production of male individuals are highly correlated when organisms are under stress, since these two processes are regulated by the same signalling pathway (Rider et al., 2005). Figure 6.5 shows a female and a male *D. magna*, which, in this specific case was due to Pb exposure combined with food restriction (F6). Males also appeared in the Pb exposure and control under food restriction (both species) and, during the recovery period, for *D. magna* (both food regimes) and *D. similis* (food restriction). Males and females can be distinguished by the size of the first antennae; neonatal males present a ten times more elongated antennae than females, and also, the frontal portion of the head capsule is flattened and the abdominal process is absent (Olmstead & LeBlanc, 2000). Males also present a smaller size and modified post-abdomen and first legs (Ebert, 2005).

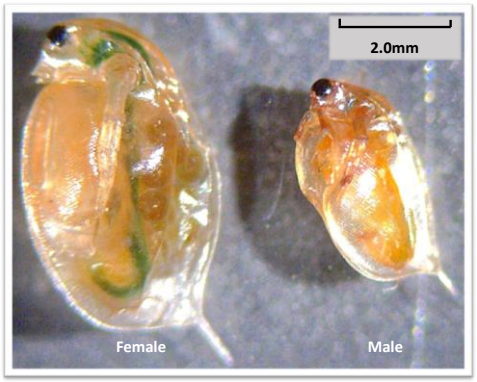

Figure 6.5: Adult female and male of *Daphnia magna* from continuous Pb exposure and food restriction at generation F6.

Male daphnids manifestation has been described as a population response to stress, such as low-food quality or quantity, crowding (Olmstead & LeBlanc, 2001) and/or to the presence of contaminants (Ginjupalli & Baldwin, 2013). The lifespan of males and females may differ. Both species under usual food regime did not exhibit male production, except for recovering *D. magna*, which (at generation F7) produced males with similar lifespan than females (95 days for males and 99 days for females; average values). Under food restriction, however, *D. magna* from all setups (control, Pb exposure and recovery period) produced males. Control *D. magna* from generation F4 produced males (due to food restriction), which showed a lifespan of 58 days, differing from females that had a 110 days long lifespan. *D. magna* from continuous Pb exposure, also from generation F4, presented disparities regarding male and females' lifespan, being 53 days for males and 84 days for females. Recovery period organisms from generation F7 exhibited a lifespan of 46 days for males and 97 days for females. *D. similis* also produced males under food restriction, with males presenting shorter lifespans than females at several setups, such as control and Pb exposure. Results show that if organisms are under stress (Pb exposure or pre-exposure and/or food impairment), males present a shorter lifespan than females and also that food impairment and Pb exposure induces male production (which does not occur under usual food). Males *D. magna* exhibit a faster heart rate and shorter longevity than females (MacArthur & Baillie, 1929; Schwarzenberger et al., 2014). However, if organisms from recovery period (*D. magna*) are returned to a regular food regime no more differences between male and female lifespan are shown, which is corroborated by Pietrzak et al. (2010).

A subsequent action after male appearance can be the production of resistant dormant eggs (Olmstead & LeBlanc, 2001). Under unfavourable environmental conditions, the production of resistant eggs is common, being protected by an ephippial case against harsh conditions (Lass et al., 2005). They may appear under temperature fluctuations, high population density, low-food, competition, temporary ponds, abiotic factors, seasonal effects and anthropic actions (Brandão et al., 2014; Maia-Barbosa et al., 2003; Paes et al., 2016). The ephippium is abandoned when daphnids molt (as shown in Figure 6.6a) (Ebert, 2005). Ephippia production due to metal contamination stress was shown for *D. magna* exposed to Cd (Bodar et al., 1988) and Cd combined with low-food (Kluttgen & Ratte, 1994). Daphnids produce ephippia mainly to facilitate dispersion, future survival under unfavourable conditions (e.g. desiccation, freezing), overcome predation and/by renewing the population genetic structure (Brendonck & De Meester, 2003; Havel & Shurin, 2004; Pereira, 2008; Rider et al., 2005). Ephippia are dependent of male fertilization, which can

produce then genetically different females (Ebert, 2005). In our study, ephippia (or dormant haploid egg) occurred for both species and food regimes (continuous Pb exposure) but not for recovering organisms. Despite ephippia appearance may be linked to males, in this study, ephippia (or dormant haploid egg) production was not directly linked with male production, since males did not appear for organisms under usual food (except for recovering *D. magna*) and occurred on organisms from the recovery period. The majority of ephippium eggs are produced sexually, but parthenogenetic *Daphnia* may produce diploid eggs asexually as well (Ebert, 2005). A personal note here will be made, it appears that smaller neonates (in comparison to healthy neonates) developed into adult females that produced ephippium (or dormant haploid egg). A total of nine ephippium (or dormant haploid eggs) were produced along the generational test (five for *D. magna* and four for *D. similis*).

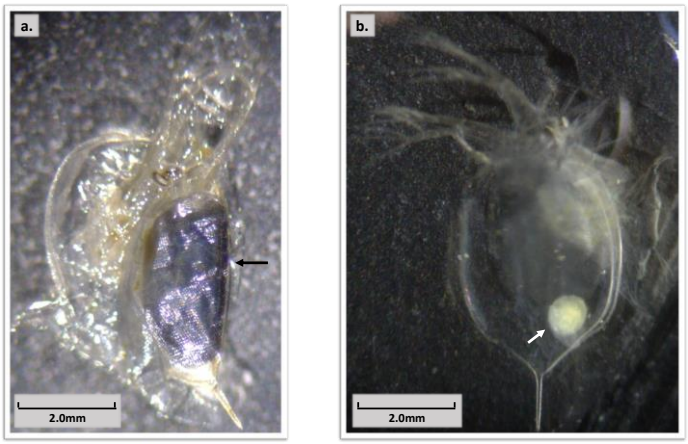

Figure 6.6: **a.** Ephippia (or dormant haploid egg) with carapace of **a.** Pb exposure food restricted *Daphnia magna* at generation F7. **b.** White egg of recovery period *Daphnia magna* under food restriction on a fresh released carapace at generation F8.

Both *D. magna* and *D. similis* chronically exposed to Pb for generations under both food regimes spawned green, white and also showed aborted eggs. Healthy daphnid eggs have a brownish/dark yellow colour (Figure 6.4b). In laboratory cultures, when daphnids are not healthy, the eggs colour varies to an olive green (personal observations). A certain amount of haemoglobin of the mother is transferred to the eggs and therefore to neonates (Green, 1955), this provides the eggs a brownish colour and consequently the mothers become more translucent. Daphnids body colour variation can also vary under chemical exposure, with *D. magna* body colour changing to white (uncoloured) after Pb exposure

(Offem & Ayotunde, 2008). *Daphnia* body is normally translucent in a healthy environment, and it has a pinkish-red colour when haemoglobin concentration increases, due to hypoxia, contamination or crowdedness (Roy, 2009) or in our case, Pb exposure. To our knowledge, no study relating white eggs (or colour variation) to toxicity has been carried out. Our hypothesis is that, since mothers pass its haemoglobin content to eggs, which provide the brownish colour (Green, 1955), the white eggs presented through the generations under Pb exposure could be due to a failure on this process, since Pb affects the haemoglobin content (Lavradas et al., 2014). Despite egg colour variation, aborted eggs also appeared under Pb exposure. Silva et al. (2017) supports our results, indicating abortion of eggs for *D. magna* under a generational exposure to carbendazim. Ribeiro et al. (2011) also indicated enhanced number of egg abortion with increasing carbendazim concentration and, suggested that the cause of the abortion was due to the effect of the chemical exposure on the mitotic phase of egg development, inducing an early release (before complete cleavage). Other metals (e.g. Cd) also triggered abortion in *D. magna* in low concentrations (>2µg/L) (Enserink, 1995).

Comparing the Pb adverse effects to *D. magna* and *D. similis*, no divergence was shown between species (see Table S1 for further clarification). However, such outcome does not implicate that a divergence in organisms' sensitivity to chemicals does not happen. Chapters 2, 3 and 4 of this thesis indicate different outcomes regarding both species, such as lower Pb sensitivity to *D. magna* regarding acute exposures and disparities concerning organisms' recuperation after Pb exposure.

4. Conclusion

Results indicate that *D. magna* is capable of bioaccumulating Pb, but the accumulation process differs depending on the food regime (endpoint not evaluated for *D. similis*). Under usual food, a faster accumulation occurs, reaching a peak; while under food restriction, the accumulation is gradual. *D. magna* was capable of retrieval after three generations in clean media, diminishing the tissue Pb concentration. Long term Pb exposure led to several effects, such as malformations (in both species), aggregation of Pb granules in the neonates' dorsal region and reddish extremities, which could be due to increased haemoglobin content (in F9, at Pb exposure and recovery period from both food regimes). Reddish extremities disappeared in recovering organisms. Male production occurred in continuous Pb exposure and control daphnids under food restriction (both species), as well as during recovery period for both species and food regimes (except for *D. similis* usual food). Males generally exhibited a shorter lifespan than females. Ephippium

(or dormant haploid egg) occurred in both species and food regimes (Pb exposure), but not in recovering organisms. On top of all these negative effects, green, white and aborted eggs were detected. In this study, the responses of *D. magna* and *D. similis* tended to be similar, for the endpoints evaluated. Finally, this study shows that qualitative endpoints can be useful to evaluate stress in daphnids exposed to contaminants in multi-generation experiments.

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Supplementary material

Table 6S.1: Adverse effects appearance in *Daphnia magna* and *Daphnia similis* exposed to a control media and Pb continuous exposure for several generations, and in a recovery period (clean media) after Pb pre-exposure (from F6 to F9), under two food regimes ($3x10^5$ (u) and $1.5x10^5$ (r) cells/mL).

		Malform.	**Granules**	**Reddish extremities**	**Males**	**Ephippia (dormant egg)**	**Eggs (colour/abortion)**
Control	*D. magna* (r)				X		
	D. similis (r)				X		
Pb exposure	*D. magna* (u)	X	X	X		X	X
	D. magna (r)		X	X	X	X	X
	D. similis (u)	X	X	X		X	X
	D. similis (r)		X	X	X	X	X
Recovery period	*D. magna* (u)		X	X	X		
	D. magna (r)		X	X	X		
	D. similis (u)		X	X			
	D. similis (r)		X	X	X		

Table 6S.2: Chemical Analyses

	Chemical Analyses	
Nominal concentrations	Stock (46.54 mg/L)	0.05 mg/L
$Pb(NO_3)_2$	48 mg/L	0.054 mg/L
Recovery (%)	103	108

Table 6S.3: Two-way ANOVA results for Pb bioaccumulation in *Daphnia magna* for all setups (Control, Pb exposure and recovery period) and among generations (F0, F6 and F9) and their interaction. Indicating the sum-of-squares (SS), degrees of freedom (DF), mean squares (MS), the F ratio (F) and the P value (α<0.05).

Pb Bioaccumulation					
Dahnia magna ($3x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	562.7	4	140.7	F (4, 17) = 7.983	P = 0.0008
Setups	893.2	2	446.6	F (2, 17) = 25.34	P < 0.0001
Generations	320.6	2	160.3	F (2, 17) = 9.097	P = 0.0021
Residual	299.6	17	17.62		
Dahnia magna ($1.5x10^5$ cells/mL)					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	327.7	4	81.93	F (4, 17) = 9.987	P = 0.0002
Setups	555.1	2	277.6	F (2, 17) = 33.83	P < 0.0001
Generations	221.8	2	110.9	F (2, 17) = 13.52	P = 0.0003
Residual	139.5	17	8.204		

Chapter 7

General Discussion and Conclusions

General discussion and conclusions

The described effects of both Pb and mancozeb during the work carried out in this thesis on *Daphnia magna* and *Daphnia similis* express the importance of evaluating the hazard and risk associated to these chemicals. The effects observed after short and long-term exposures reveal the relevance of generational assays, due to their higher sensitive responses in comparison to conventional experiments, as it will be explained below. Standard protocols focus on single generation effects, generally for a short-term, as well as frequently regard single chemical exposures, which are not the realistic scenario of those organisms living in the environment. This study highlighted the relevance of long-term exposures as well as pulse exposures on pre-acclimated organisms. Other concerns which may interfere on the organisms' sensitivity (or tolerance) were also considered in this study, as the variation of food quantity and the divergence of chemical sensitivity between monophyletic species.

Results presented in Chapter 1 showed that Pb and mancozeb are chemicals which we should be concerned about and introduced that chemical sensitivity may diverge between two monophyletic species. Chapter 2 presented single chemical effects on both daphnids, in order to state the consequences of Pb and mancozeb single exposure for both species. Chapters 3 to 6 presented the results of the Pb multi-generation exposure (and mancozeb pulse exposures on Pb pre-acclimated daphnids on chapters 3 and 4), in conditions of regular and restricted food quantity, and also evaluated the organisms' recuperation after chemical exposure. This chapter on discussion and conclusions of the thesis will synthetize the most important conclusions drawn from the data acquired as well as future perspectives. Thus, in summary, the results obtained in this study provided us some relevant statements, such as:

→ *Short term exposures of Pb and mancozeb differs among two Daphnia species*

Chapter 2 presents the results of single chemical exposures and indicate a lower Pb sensitivity for *D. magna* than *D. similis*. Despite the sensitivity variation for both species, the response patterns of the sub-lethal endpoints indicated that the responses to Pb were similar, such as lower reproduction, feeding impairment, lower oxygen consumption and no influence on AChE activity. The limit Pb concentration recommended by the World Health Organization is 10 µg/L (WHO, 2011). The limit Pb concentration for Brazilian surface waters is 50 µg/L (CONAMA, 2005) and, for the European Union, the Maximum Allowable Concentration (MAC) for Pb is not applicable, due to the Annual Average Value for the

Environmental Quality Standards (AA-EQS) found in surface waters being 7.2 µg/L (European Parliment, 2008). Nonetheless, areas containing Pb concentrations above the Brazilian limit are reported in the literature, such as in Turvo Limpo River (Minas Gerais state), where surface water reaches up to 22.5 mg/L of Pb (Jordão et al., 2007) and Monjolinho river (São Carlos, São Paulo state) that reaches up to 226 µg/L of Pb in the freshwater system (Chiba et al., 2011). European freshwater systems also presented high levels of Pb, such as Odiel (0.24 mg/L) and Tinto (0.6 mg/L) rivers (Sainz et al., 2004). In North West England, Pb concentration values in freshwater systems reached from 30 µg/L up to 345 µg/L (Rothwell et al., 2010). Pb concentration above the limit in rivers was also seen across the world, such as values assessed by Alsaffar et al. (2016) (52 µg/L of Pb in Penang, Malaysia) and Shanbehzadeh et al. (2014), reaching up to 1.91 mg/L of Pb (Tembi River, Iran). So, to evaluate the effects of Pb when it occurs above limits, but still resembling contaminated areas, short term exposure bioassays used Pb (sub-lethal) concentrations between 0.125 mg/L and 3 mg/L (Chapter 2), being the Pb LC_{50} = 0.46 mg/L (CI= 0.41-0.52) for *D. magna* and 0.34 mg/L (CI= 0.31-0.37) for *D. similis*.

Mancozeb also presented similar outcomes among species such as decreased reproduction and feeding rate and enhanced AChE activity. However, it triggered distinct results regarding oxygen consumption (increased for *D. magna* and no effect for *D. similis*). Mancozeb exposure affected *Daphnia* reproduction at concentrations such as 50 µg/L (Chapter 2). The mancozeb predicted environmental concentration (PEC) for the German risk assessment in surface waters is 12.7 µg/L (Sabero, 2017). However, such concentration can alter regarding crops and vegetables being farmed, achieving levels as high as 210.8 µg/L, for tomatoes farms for example (US EPA, 2005).

Thus, these data indicate that such chemicals should not be disregarded, since they can be introduced in the environment in large quantities and reach levels capable to cause toxic effects to aquatic organisms. Such outcomes also indicate that they are toxic to both *D. magna* and *D. similis* even in short-term exposures. This chapter also shows that metal and organic xenobiotics may induce different effects on different monophyletic *Daphnia* species. Therefore, an effort is required to replace the temperate model species *D. magna* to tropical species, such as *D. similis*, when it comes to regulatory purposes to better mimic contamination in tropical freshwater environments. Regarding different latitudes and temperature variation, the increase of temperature can enhance the metabolism (heart rate and respiration) of daphnids (Khan and Khan, 2008). As well as increase metal uptake and enhance organisms sensitivity, having a major impact on organisms response to chemical exposure (Heugens et al., 2003).

→ *Multi-generation exposure to Pb can impact daphnids in different ways*

Chapters 3 to 6 demonstrate the effects of a multi-generation exposure to Pb in a chronic legally permitted (Brazil) concentration (50 µg/L), lower than the Pb concentration (0.25 mg/L) that already triggered chronic effects on *Daphnia* (oxygen consumption), for example. Diminished Pb sensitivity (immobilization) through generations was seen for both *D. magna* and *D. similis* under both food regimes (Chapter 3). The chronic endpoints (reproduction and feeding) shown in Chapter 4 indicates similar results between *D. magna* and *D. similis*, highlighting that organisms continuously exposed to Pb presented an enhanced rate of population growth (*r*), in comparison to control. The production of less sensitive offspring can seriously impact the dynamics of a natural environment, which could be aggravated when dealing with primary consumers, since it could affect the whole trophic chain (Wong & Candolin, 2015).

Contrarily to the acute effects shown on Chapter 2, the population effects triggered by the long-term Pb exposure (Chapter 5) affected more drastically *D. magna* (reduced lifespan, hatching delay and AChE activity) than *D. similis*, which exhibited less disparities (decreased Net Reproductive Rate (R_0)) among generations (F0 to F9). However, Pb adverse effects (Chapter 6) did not distinguish between species, since malformations, Pb aggregation, increased levels of haemoglobin, alterations of the eggs' colour variation, growth in the number of eggs abortion, production of males and ephippias were observed in both species. *D. magna* revealed that Pb can accumulate within the organisms' tissues and that the accumulation pattern differs depending on the food regime. Such outcomes imply that despite of differences among *D. magna* and *D. similis* in lethal and sublethal effects, Pb negatively affect both species under a long-term exposure. That is, under a Brazilian Pb governmental authorized concentration (50 µg/L), both *D. magna* and *D. similis* suffer critical changes that likely will trigger a cascade effect and may damage the ecosystem functioning and health.

Comparing acute and chronic generational exposures, a short acute exposure can rapidly affect the organisms' life, leading to death and instantly harming the balance of aquatic biota. Such effects occur in higher concentrations and are evaluated by individual effects (Valavanidis & Vlachogianni, 2010b). Long-term chronic exposures, even though occurring at lower concentrations, are not rapidly perceived and can trigger population effects, presenting higher ecological relevance and enabling the extrapolation from laboratory to ecosystems, which may result in alterations of ecosystem structure (Heugens et al., 2001; Van der Oost et al., 2003).

→ *A pre-exposure to Pb alters mancozeb toxicity and differ among species*

Organisms exposed to more than one substance can present alterations in their sensitivity in comparison to single exposures (Mansour et al., 2015). In Chapter 3 it was demonstrated that mancozeb toxicity fluctuates due to Pb pre-exposure. Our study thus confirm the one of Barata et al. (2002) which previously stated that chemical sensitivity can vary in daphnids pre-exposed to other chemicals. Comparing control and Pb exposed *Daphnia*, *D. magna* was less sensitive to mancozeb, while the contrary was shown in *D. similis*. Our outcomes are sustained by Magalhães et al. (2014), which demonstrated that phylogenetic related species can produce different toxic responses.

Concerning natural ecosystems, it is likely that organisms will be concomitantly exposed to more than one contaminant (Cooper et al., 2009). That being said, our results showing opposite sensitivity responses for *D. similis* and *D. magna* is a key point when dealing with risk assessment issues and should be considered; in this case environmental risks should contemplate the responses of a range of species, avoiding being supported by a single species study (Brix et al., 2001; Silva et al., 2014; Van Leeuwen, 2007).

→ *Recovery from Pb exposure differs among Daphnia species*

The analysis of organisms' recuperation after chemical exposure allow us to estimate if individuals would recover after a long-term exposure to specific substances, being also a method to categorize the organisms tolerance achievement (Guan & Wang, 2006b). Moreover, such evaluation may clarify whether, if sensitivity varied, if it probably was epigenetic or physiological change. The recovery period differed among species, with *D. magna* appearing to present epigenetics changes while *D. similis* seemed to have a physiological acclimation, at least for the acute endpoints used (e.g. immobilization rate, Chapter 3).

A lack of organisms' retrieval regarding chronic endpoints (may be due to epigenetic changes or maternal transgenerational transfer) occurred for both species and food regimes (Chapter 4). Besides, *D. magna* exhibited population effects (Chapter 5), such as early reproduction and reduced lifespan (under the usual food regime used in ecotoxicological tests). However, differently from *D. magna*, *D. similis* showed a full recovery regarding Net Reproductive Rate (R_0), suggesting a physiological acclimation. The *D. magna* full recovery on AChE activity (usual food) and the enhanced enzymatic activity shown in comparison to Pb ($clean_{96h}$) organisms (96h in clean media), indicates that time has a crucial role regarding daphnids recovery, which is also supported by Morgan et al. (1990). Contrary to *D. magna*, *D. similis* AChE activity did not recover and kept on displaying a lower enzymatic activity

compared to control and Pb treatments. During food restriction, the same pattern of *D. magna* full retrieval and *D. similis* failed recovery was shown regarding population effects (early reproduction and shorter lifespan). However, other endpoints seemed to exhibit successful recover, such as Pb accumulation and haemoglobin content; in addition, ephippias were not produced during the recovery period (Chapter 6).

A lack of retrieval may also indicate that the retrieval time could have not been sufficient or that daphnids may suffered epigenetic changes due to Pb exposure (*D. magna* acute and chronic outputs and *D. similis* chronic results). This could lead to an increase of less sensitive populations in the ecosystem, which can have crucial implications on ecological restoration, as offspring born from adapted mothers can continue to generate less sensitive organisms by maternal transfer (Guan and Wang, 2006b; Martins and Guilhermino, 2018), which can be an additional route for adult females' detoxification (Tsui and Wang, 2007). The capacity of physiologically acclimate to a chemical exposure can be crucial to organisms survival (Sánchez et al., 2004), even so, such individuals may be more fragile than unexposed organisms (e.g. recovering *D. similis* from usual food shown in Chapter 3). Tsui and Wang (2005) indicated that recovering daphnids (metal Hg exposure) presented increased sensitivity under Hg re-exposure. Such outcomes suggests that chemical exposure and the respective retrieval may lead to an unknown fate under complex contaminated environments (Li et al., 2016).

→ *Food variation trigger different sensitivity and recovery responses among species*

The food regimes used altered organisms' sensitivity and responses to chemical exposure. Both food regimes were used based on culture maintenance procedures, which does not reflect what is available in the real environment. But it reflects an acclimated food regime (the usual or normal in cultures) and half of the usual, which represents a regime with lower food availability (restriction). Chemical influence on zooplankton communities depends on population sensitivity/fitness, which is related to food availability (Antunes et al., 2004) as well as maternal nutrition, which is crucial since it directly affects offspring sensitivity to stressors (Enserink et al., 1990). *Daphnia* mothers are able to alter egg size and composition in a nutrient varying environment (Wacker & Martin-Creuzburg, 2007). Therefore, organisms that better cope with food fluctuations may be favoured by natural selection (Pereira and Gonçalves, 2008).

The influences of two dietary regimes (usual and food restriction) evaluated are presented in Chapters 3 to 6. Chapter 3 demonstrated enhanced body length in low food

media, which consequently lead to diminished chemical sensitivity. Larger offspring from poor nourished mothers tend to be more resistant than smaller neonates from well feed mothers. Such outcome may be due to the increased ability of larger organisms (greater energy) to survive in environments with scarce food due to natural selection (Glazier, 1992; Goulden et al., 1987). Pieters & Liess (2006b) suggested that food quantity and quality are major variables conducting natural *Daphnia* populations, including organisms' recovery, which could be slower under nutrient restricted environments. Differences in recovery responses from both species were also indicated in Chapter 3, where *D. magna* may have presented epigenetic changes while *D. similis* a physiological acclimation to Pb. Moreover, Pb pre-exposed *D. magna* was less sensitive to mancozeb while in food restricted media, contrarily to *D. similis*. Both species under a restricted food media and continuous Pb exposure for a long period presented reproduction impairment, making it impossible to evaluate daphnids algae consumption. Also, food restriction and Pb exposure together caused death of organisms during the 21-day reproduction test (both species).

Food restriction induced reduction of R_0 and lifespan in *D. magna* and *D. similis* as well as led to delayed hatching and increase of AChE activity. *D. similis* recovery was affected by food restriction, leading to failure in recovery (shorter lifespan and early reproduction) (Chapter 5). Concerning the adverse effects explored under Chapter 6 upon Pb exposure, no disparity is shown between species, but food restriction lead to male production (also in the control treatment of both species). The Pb accumulation in *D. magna* also varied when under food restriction, presenting a more gradual accumulation throughout generations in comparison to that exhibited by organisms kept under usual food.

Food quality and quantity can seriously impact population dynamics (Wacker & Martin-Creuzburg, 2007). Therefore, the disparities on the responses of *D. magna* and *D. similis* under food restriction is of great importance, since food amount fluctuates in natural ecosystems and, consequently, *Daphnia* sensitivity to Pb also may vary among species in its habitat. These variations among *D. magna* and *D. similis* sensitivities suggest that Pb exposure can lead to less sensitive organisms (survival) and possibly population failure or in extreme consequent disappearance. Food amount can affect offspring quality through lipid resources allocation, and influence their sensitivity/fitness, therefore, this issue should be widely discussed and taken seriously (Wacker & Martin-Creuzburg, 2007).

→ *The need for generational standard protocols to improve environmental risk assessment together with adequate species*

Regarding the ecological relevance of choosing appropriate species, multiple chemical 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 contaminated populations, keeping in mind that monophyletic species may differ; 5) Food quantity has an important role regarding organisms sensitivity and may vary the phylogenetic close related species responses to chemicals 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.

Since long-term exposure to chemicals threats natural populations and consequently the trophic chain, a protocol for multi-generation should be developed. Further, epigenetic modifications (phenotypic characters transgenerational transferred) on *Daphnia* should be done to elucidate if results shown were inherited from exposed mothers.

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