Universidade de Aveiro 2010 Departamento de Biologia

Cátia Alexandra Ribeiro Venâncio

Co-resistência a metais e recuperação fisiológica em clones de *Daphnia longispina*

Co-resistance and physiological recovery in *Daphnia longispina* clones

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Auxiliar do Centro de Estudos do Ambiente e do Mar e co-orientação do Doutor Amadeu Mortágua Velho da Maia Soares, professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Prof. Doutor António José Arsénia Nogueira
Professor associado com agregação, Departamento de Biologia, Universidade de Aveiro

Doutor João Luís Teixeira Pestana
Investigador, Centro de Estudos do Ambiente e do MAR, Departamento de Biologia, Universidade de Aveiro

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
Professor Catedrático, Departamento de Biologia, Universidade de Aveiro

Doutora Isabel Maria Cunha Antunes Lopes
Investigadora Auxiliar, Centro de Estudos do Ambiente e do Mar, Departamento de Biologia, Universidade de Aveiro.

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palavras-chave Co-tolerância, Taxa recuperação, *Daphnia longispina*, metais

resumo

A avaliação do risco ecológico de compostos químicos é baseada na realização de ensaios padronizados, que, usualmente, envolvem exposições contínuas dos organismos-teste a concentrações constantes de uma solução de um único tóxico e por um período de tempo fixo. Todavia, esta abordagem não considera aspectos ambientais naturais, como: a variabilidade genética de populações naturais, a possibilidade de cenários reais de exposição a misturas de contaminantes, e/ou pulsos sequenciais com diferentes concentrações; pelo que a extrapolação a partir de resultados obtidos em ensaios laboratoriais para padrões de exposição mais realísticos tem de ser efectuada através da aplicação de modelos. No entanto, esta extrapolação pode envolver inconsistências entre os resultados obtidos em laboratório e o modo como os organismos são afectados por substâncias químicas na natureza. Portanto, com o objectivo de obter uma avaliação do risco ecológico mais exacta, é necessário integrar, em ensaios de laboratório, os cenários de exposição que mais se aproximam de situações reais. Assim, este trabalho de pesquisa teve como objectivo verificar se a avaliação do risco ecológico subestima a existência de: (i) mecanismos de co-tolerância a um conjunto de metais, e de (ii) associação entre resistência a metais e tempo de recuperação fisiológica entre pulsos sequenciais de metais. Para alcançar estes dois objectivos, foram seleccionadas sete linhagens de *Daphnia longispina* O.F. Müller, de acordo com a sua sensibilidade a níveis letais de cobre. Para identificar a existência de co-tolerância, foi quantificada a sensibilidade das sete linhagens de *D. longispina* a níveis letais de quatro metais (cobre, zinco, cobalto e crómio). Não foi encontrada correlação ($r \leq 0.51$; $p \geq 0.30$) entre qualquer par de metais testados. No entanto, foi registada uma sensibilidade inversa entre metais para alguns clones. Por exemplo, o clone N116 foi o mais tolerante ao crómio, mas o mais sensível a zinco e cobalto.

Para avaliar se uma maior resistência à poluição está associada a um período de rápida recuperação fisiológica, quatro dessas sete linhagens clonadas foram expostas a cenários de exposição por pulsos sequenciais de cobre (pulsos de 24 horas), com intervalos de 72 e 24 h horas de recuperação. Os resultados não mostraram qualquer correlação entre a sensibilidade com a resposta fisiológica rápida. Estudos deste tipo são de grande importância para compreender melhor as situações reais: os factores de stress não agem ou surgem isolados, mas sim interagem e exercem stress em grupo.

keywords Co-tolerance, Recovery rates, *Daphnia longispina*, metals

abstract

Ecological risk assessment usually relies on standard toxicity tests, which currently involve the continuous exposure of organisms under constant concentrations of a single chemical. As this approach does not take into consideration natural environmental aspects, namely: the genetic variability of natural populations, the possibility of real scenarios of exposure involving a mixture of contaminants, and/or sequential pulses with fluctuating concentrations; extrapolation from laboratory assays to such more realistic patterns of exposure must rely on modelling. But, this extrapolation may involve inconsistencies between laboratory based results and how organisms are affected by chemicals in nature. Therefore, aiming for a more accurate ecological risk assessment, it is necessary to integrate, in laboratory assays, exposure scenarios that come closer to real exposures.

Accordingly, this research work aimed to evaluate if ecological risk assessment underestimates the existence of: (i) multiple/co-tolerance mechanisms to a set of metals, and of (ii) association between tolerance to metals and time to recover after sequential pulses of metals. To achieve these two objectives, seven and four, respectively, cloned lineages of *Daphnia longispina* O.F. Müller were selected accordingly to their sensitivity to lethal levels of copper. To identify the existence of multiple/co-tolerance, the sensitivity of the seven cloned lineages to lethal levels of four metals (copper, zinc, cobalt, and chromium) was quantified. No correlation was found ($r \leq 0.51$; $p \geq 0.30$) between any pair of tested metals. However, an inverse sensitivity to some metals was registered for some cloned lineages. For example, cloned lineage N116 was the most tolerant to chromium but one of the most sensitive to zinc and cobalt.

To assess if a higher tolerance to pollution is associated with a fast recovery period, four cloned lineages of *D. longispina* experimented sequential pulsed exposure scenarios (pulses of 24 hours) with intervals of 72 and 24 hours of recovery. Results showed any correlation between tolerance and a fast physiological response. Studies of this kind are of much importance to understand better real situations were factors of stress do not act or appear alone but instead interact and exert stress in group.

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Chapter One

Introduction

Traditionally, ecotoxicology uses standard guidelines to perform toxicity assays where surrogate species are exposed under constant and continuous levels of a chemical to predict its effects in the environment (e.g. Rand and Petrocelli, 1985; Calow, 1994; OECD 1992, 2001, 2004, 2006). This approach is a powerful tool to set limit levels (*e.g*. predict no effect concentration) for the presence and discharge of chemicals in the environment in order to protect it (Rand and Petrocelli, 1985). However, when conducting ecological risk assessments of contaminated sites, due for example to diffuse pollution or effluent discharges, more ecological relevancy should be introduced into such approaches in order to increase the accuracy of risks identification, and, thus, the protection of ecosystems. One of such aspects that should be considered when conducting ecological risk assessment is that natural populations are genetically variable, i.e. they hold a number of different genotypes that will respond differently to chemical contamination. Actually, if the intensity of contamination is strong enough it may lead to the disappearance of the most sensitive genotypes from the initial population, causing its genetic erosion. In literature, several works have already reported the loss of genetic diversity in natural populations exposed to chemical contamination (e.g. Belfiore and Anderson, 2001; Van Straalen and Timmermans, 2002; Morgan *et al.*, 2007; Bourret *et al.*, 2008; Nowak *et al.*, 2009; Ungherese *et al*., 2010). As an example, Lopes *et al*. (2004) and Agra *et al*. (2010) observed that populations of the cladoceran *Daphnia longispina*, historically exposed to metal contamination were less genetically diverse comparatively to reference populations inhabiting the same aquatic system. In addition, these authors also reported that the extremely sensitive genotypes were not present in the impacted population of *D. longispina*.

Since the genetic diversity is the basis for adaptation of populations through natural selection, its reduction may compromise the resilience and adaptation of populations to future environmental change, increasing their extinction risk (Medina *et al*., 2007; Lopes *et al*., 2009). A population, genetically eroded due to the exposure to a certain chemical, is expected to be mainly composed by tolerant genotypes to that specific chemical (Lopes *et al*., 2004; Agra *et al*., 2010). Whether these genotypes are tolerant or sensitive to other chemicals, may determine the survival and persistence of this population in a situation of future inputs of different chemicals. In Fig. 1.1 are represented three different scenarios that may occur: (a) if a positive association exists between tolerances to different chemicals, them the tolerant

individuals remaining in the population, after the exposure to chemical 1, will also be able to cope with the exposure to a second chemical (Chemical 2 in Fig 1.1.), (b) if a negative association exist between different chemicals, then, most of the tolerant individuals remaining in the population, after exposure to chemical 1, will died after exposure to chemical 2 (to which they are sensitive) – this constituting the worst case scenario, and (c) if no association exist between tolerance to different chemicals, then exposure to chemical 1 will lead to the disappearance of the most sensitive individuals to this chemical, and exposure to chemical 2 to the disappearance of the individuals more sensitive to it. However, all the other individuals that are intermediately or highly tolerant to both chemicals will persist in the population (Fig. 1. 1).

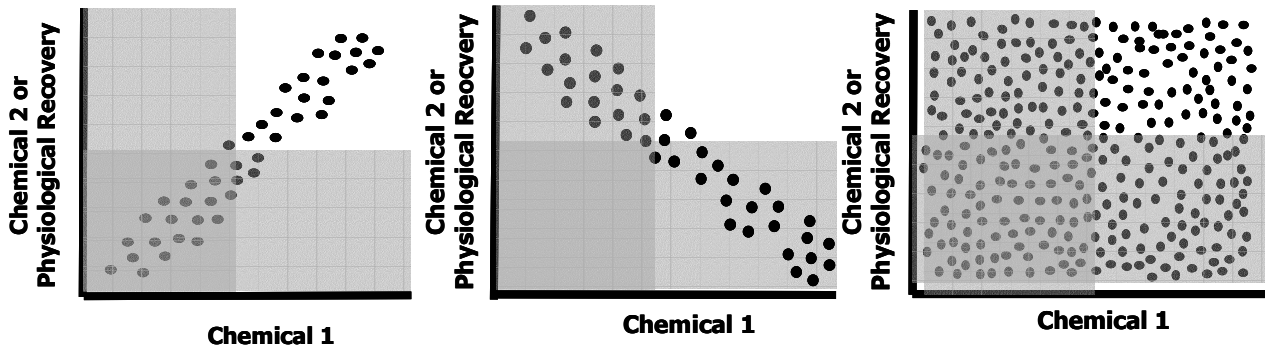

Figure 1. 1. Schematic representation of correlation scenarios: a) Positive correlation, where an increase in one response is accompanied by an increase in the other response; b) Negative correlation, where an increase in one response is accompanied by a decrease in the other response and c) No correlation, there is no association between the magnitudes of the two variables. Adapted from Vinebrooke *et al.* (2004). Shadow areas represent the part of the population that is eliminated with the input of toxicants.

The association between tolerance to more than one stressor has already been study by several authors, and can be explained by two different hypotheses: tolerance to more than one chemical is achieved independently, *i.e.*, if one population is tolerant to more than one chemical it is because multiple contamination selected for tolerance to more than one chemical, involving several specific resistance mechanisms. On the contrary, populations tolerant to one chemical may also be co-tolerant to other chemicals even though they have not been exposed to it, involving detoxification mechanisms more generalist (Macnair, 1997; Tilstone and Macnair, 1997). A number of works regarding the association in tolerance to different chemicals have already been published, and reported both the existence and inexistence of multiple/co-tolerance between a range of stressors (*e.g*. Backor and Váczi, 2002; Adriaensen *et*

al., 2005; Knauer *et al*., 2010). For example, Marquis *et al*. (2009) found that in a natural population of the frog species *Rana temporaria* the mechanisms involved in resistance to UV-B also protected the organisms from benzo[a]pyrene contamination. Furthermore, Gonnelli et al. (2001) studied the resistance of ecoypes of *Silene paradoxa* L. to copper and nickel, and observed that the toxic effects of these metals on root growth showed the copper-tolerant ecotypes as nickel co-tolerant, whereas the opposite was not the case, suggesting the occurrence of a non-reciprocal co-tolerance mechanism.

Besides association between tolerances to different chemicals, the relationship between tolerance to one chemical and the time needed to enable physiological recovery of organisms, between pulsed exposures, may also determine the resilience of the above mentioned genetically-eroded populations. The same three scenarios of association, as those described above for tolerance to more than one chemical, may be considered between tolerance to one chemical and time to occur physiological recovery, also involving similar outcomes at the population level in a situation of positive, negative or no correlation between these responses (Fig.1.1). This relationship becomes more important when considering scenarios of exposure to pulsed events of contamination, for example if tolerant individuals recover faster, they will exhibit a double advantage in a scenario of pulse events to the chemical to which they are tolerant. Actually, the exposure of natural populations, namely aquatic ones, to pollutants is more often pulsed (*e.g.* pulsed release of effluents from industrial activities or by non-point source pollution due to the application of pesticides in agricultural fields) rather than continuous (Siem *et al*., 1984; Parsons and Surgeoner, 1991a, b). Some studies have shown that the impact of pulsed exposure of aquatic organisms to toxicants can be different from continuous exposure. For example, *Daphnia pulex* suffered a higher mortality rate when exposed to pulsed doses of copper than when exposed to copper continuously (Ingersoll and Winner 1982). Also, Bearr *et al*. (2006), verified, with *Pimephales promelas* larvae, that mortality resultant from a double copper pulse (12 hours) was higher than the mortality registered from an equivalent continuous 24 hours test.

The works above mentioned report situations were continuous exposure would underestimate the real toxic effects; although, several other works report the inverse case. Reynaldi *et al*. (2004) working with *Daphnia magna* reported that recovery from a single pulsed exposure (24h) to lethal concentrations of copper could happen, while after a continuous exposure at the same concentrations, survival was severely affected. For sub-lethal parameters, Reynaldi *et al*. (2004), also verified that, after 21-d assays, cladocerans could recover to values

of population growth close to control levels while for continuous exposure no recovery was detected. Also, Kim *et al*. (2008) reported rapid recovery rates of *D. magna* after single short pulsed exposure (24h) to two herbicides with no significant differences on reproduction and survival 20-d after pulse. Continuous exposure in chronic tests and even in acute assays can hide the real effects of chemical in first the hours of exposure. Therefore, data published in the literature suggest that traditional continuous exposure, used in standard toxicity assays, may either overestimate or underestimate the real risk. Thus, it highlights the need for a better understanding of the factors that may influence the responses of organisms to toxicants under pulsed or continuous exposure, and the importance to integrate pulsed exposure into ecological risk assessment.

Accordingly with the above mentioned, this study intended to evaluate if: (i) multiple/co-tolerance exist to different metals among cloned lineages of the cladoceran *Daphnia longispina*, and (ii) an association exist between an increased lethal tolerance to an essential metal (copper) and a faster physiological recovery in cloned lineages of *D. longispina*. To address these objectives cloned lineages of *D. longispina* exhibiting different sensitivities to lethal levels of copper were selected and used to carry out the toxicity tests. The use of *Daphnia* species in this study is highly advantageous as it can be easily maintained in laboratorial conditions and its reproduction by parthenogenesis allows working with exactly the same cloned lineages for several generations.

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Chapter Two

Is the ecological risk of mixtures being underestimated?: inexistence of co-tolerance between pairs of metals in Daphnia longispina *clones*

Abstract

Natural populations in contaminated sites are, as a rule, exposed to mixtures of pollutants, since several sources often co-exist releasing simultaneously and/or sequentially different chemicals. Besides additivity and synergism, the co-occurrence of two pollutants can have drastic effects if the most resistant organisms to the first chemical are the most sensitive to the second one, *i.e.* if an inverse relationship exists between the tolerance to two chemicals then their mixture, even at low toxic doses each, can wipe out a population. The same output would also stand if the presence of the two chemicals is not simultaneous but, instead, their sequential release occurs, even when time intervals are large enough to allow densities to recover. Indeed, the susceptibility to extinction of a natural population would be greatly increased if the survivors of the first pulse – the most tolerant ones – are the most sensitive to the second pollutant. Such an effect at the population level cannot be predicted by the traditional approach when studying the toxicity of mixtures (*e.g.* concentration addition versus independent action), because the population genetic diversity is minimized or even suppressed (by testing organisms belonging to a single cloned lineage). Accordingly, this study evaluated and compared the lethal sensitivity to copper, zinc, cobalt and chromium of seven cloned lineages of *Daphnia longispina* O.F. Müller (Cladocera). Multiple/co-tolerance was not found between any pair of tested metals ($r \leq 0.51$; $p \geq 0.30$). Though any association was observed in the tolerance of cloned lineages to the tested metals, it was observed that the most resistant clone to chromium (N116; $LC_{50,48h}$ = 747 µg/L) was found to be the most sensitive to zinc and cobalt ($LC_{50,48h}$ = 422 and 1144 µg/L, respectively). Furthermore, the most resistant clone to copper (E99; $LC_{50,48h}$ = 317 µg/L) was the one of the most sensitive to chromium ($LC_{50,48h}$ = 2558 µg/L). In addition, though not being the main objective of this work, it was also observed that lethal responses to copper were more variable than lethal responses to the other metals, which was expected since cloned lineages were selected to have a wide range of lethal responses to copper. Finally, the obtained results suggested independency in lethal tolerance to the four tested metals.

Keywords: Multiple/Co-tolerance *Daphnia longispina* Metals Copper

Introduction

In nature it is uncommon to find only one toxicant in a polluted site; usually a mixture of contaminants or a sequential exposure to pulses of different chemicals occurs. Therefore, populations inhabiting such contaminated environments must be able to cope with the different chemicals in order to survive. The acquisition of tolerance to more than one chemical has been explained by two hypotheses: (i) **multiple tolerance** – the tolerance to more than one chemical is achieved independently, *i.e.*, if tolerance to more than one chemical occurs, it is because exposure to multiple contamination selected for tolerance to more than one chemical, involving several specific resistance mechanisms; and (ii) **co-tolerance** – the tolerance to one chemical may involve co-tolerance to other chemicals even though the organisms have not been exposed to them, involving generalist detoxifying mechanisms (Allen and Sheppard, 1971; Macnair, 1997; Tilstone and Macnair, 1997; Vinebrooke *et al*., 2004). For example, it is suggested that some metals (like copper, zinc and cobalt), share common uptake mechanisms by which they are integrated in the biological systems as well the circulation paths in the cells (Franklin *et al*., 2002).

In fact, the acquisition of tolerance to more than one chemical has been documented by several authors, namely for: insects (Nowak *et al*., 2009); oligochaetes (Klerks and Levinton, 1989; Reinecke *et al*., 1999), isopods (Brown, 1976), fish (Murdoch *et al*., 1994, Nacci et al., 2002), cladocerans (Lopes *et al*., 2005; Lopes *et al*., 2006; Martins *et al*., 2009), algae (Knauer *et al*., 2010), bacterial pathogens (Tennstedt *et al*.; 2003), plants (Eränen, 2008). For example, Soldo and Behra (2000) carried out experiences with communities of periphyton, and observed that long term exposure to copper, also induced an increase tolerance to zinc, nickel and silver. Co-tolerance between copper and zinc was also observed by Monni *et al.* (2000) for the crowberry species, *Empetrum nigrum.* However, co-tolerance between copper and zinc was tested by other authors for *Mimulus guttatus*, and, contrary to what Soldo and Behra (2000) and Monni et al. (2000) found, no association in tolerance to these metals was observed in this species (e.g. Tilstone and Macnair, 1997; Tilstone *et al*., 1997). Abd-El-Monem *et al.* (1998) verified that the growth of chromium tolerant populations of the algae *Scenedesmus acutus* was not so affected by copper but was by zinc, suggesting the existence of co-tolerance between the pair Cr/Cu and the inexistence of co-tolerance between Cr and Zn.

In the present study, multiple/co-tolerance (*sensu* association between tolerance to several chemicals) in different cloned lineages of the cladoceran *Daphnia longispina* was

evaluated between the following pairs of metals: copper/zinc, copper/cobalt, copper/chromium, chromium/cobalt, chromium/zinc, zinc/cobalt. The occurrence of an association in tolerance to several chemicals (i.e. the most tolerant individual to one chemical is also the most tolerant one to other chemicals) would be advantageous in scenarios of exposure to sequential pulses of different chemicals: as the survivors of the exposure to a first chemical will also be the most resistant to a second pulse of a different chemical, thus, minimising the probability of extinction of the populations. But, if an inverse relationship exists in tolerance to different chemicals (i.e. an individual that is the most resistant to one metal is the most sensitive to another one), the risk of extinction of a population would be increased: as the survivors of the exposure to a first pulse (the most tolerant to the first chemical) will be the most sensitive to a second pulse of a different chemical.

Materials and Methods

Test organisms

Seven cloned lineages of *Daphnia longispina* O.F. Müller, known to exhibit different genetically determined resistance to lethal levels of copper, were selected to perform this study. *Daphnia* is a genus commonly used in ecotoxicological studies because it reproduces by cyclic parthenogenesis, which allows maintaining in the laboratory exactly the same clone for several generations, in addition these selected clones of *D. longispina* have long been studied by some of the authors of the present work Lopes *et al*. (2004).

The seven cloned lineages were isolated from two natural populations (Lopes *et al*., 2004) inhabiting a metal impacted and another a reference site. Cultures, of each cloned lineage, were maintained in laboratory, for more than 1000 generations, under controlled conditions of temperature (19 to 21ºC) and photoperiod (14:10 h L:D) in ASTM hardwater (American Society for Testing and Materials (ASTM, 2002), with vitamins and the organic additive Marinure 25 (an extract from the algae *Ascophyllum nodosum*; Pann Britannica Industries Ltd., Waltham Abbey, UK) (Baird *et al*., 1989); medium being changed every other day and fed daily with the green algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (formerly known as *Selenastrum capricornutum*) (1.5×10^5 cells/mL/d). All lineages were maintained by asexual reproduction being selected neonates from the third, fourth or fifth brood to maintain laboratory cultures and carry out the toxicity assays.

Tested chemicals

Four essential metals (copper, zinc, cobalt, and chromium) were chosen to study the hypothesis supporting the existence of multiple/co-tolerance to metals in the cloned lineages of *D. longispina*. Copper was selected because in addition of being an essential metal, the cloned lineages were chosen accordingly to their lethal sensitivity to this metal and is known to be an important component of haemocyanin (White and Rainbow, 1985; Rainbow, 2002) ; zinc, is also an essential metal (is required for many important functions, *e.g*., reproduction, protein synthesis, cell differentiation, central nervous system differentiation), and, furthermore, the Cu, Zn superoxide dismutase is an enzyme involved in the protection of oxidative free radical damage (Apjar, 1992; O'Halloran, 1993); cobalt, is only an essential metal when in trace levels to humans and other mammals (is and integral component of the vitamin B12 complex) and for some blue-green and marine algae (Holm-Hansen *et al*., 1954; Bruland *et al*. 1991). Its toxicological effects are widely studied in fish (Diamond *et al*., 1992; Dave and Xiu, 1991), earthworms (Lock *et al*., 2006), zooplankton (Diamond *et al*., 1992; Baudouin and Scoppa, 1974) and more recently in plants (Li *et al*., 2009). Chromium, as a trivalent ion is considered essential to humans. This metal, especially in its hexavalent state (industrially the most important form), is highly toxic and is considered as a mutagen and carcinogen (USEPA; 1998) for humans. Severe effects have been studied namely on algae (Bidwell *et al*., 1998), feeding behaviour of silkworms (Tucker *et al*., 2003), and plants growth (Lopez-Luna *et al*., 2009).

Metal stock solutions were obtained by adding copper sulphate ($CuSO_4$), zinc sulphate ($ZnSO_4.7H_2O$), cobalt chloride ($CoCl_2.6H_2O$) and potassium dichromate ($K_2Cr_2O_7$) (all supplied by Sigma-Aldrich, St. Luis, USA) to nano-pure water (80 mg Cu/L, 1.0 g/L Zn, 1.0 g/L Co, and 1.0 g/L Cr).

Experimental Design

Cumulative mortality assays were carried, out according to OECD (2004) guidelines, for each cloned lineage exposed to each of the four selected metals.

No information on the lethal sensitivity of this cladoceran species to the studied metals was available (excepting for copper, Martins *et al*., 2007). Median lethal effective concentrations (LC50s) values for similar cladocerans were searched for in the literature (Co, Cr, Zn; Baudouin and Scoppa, 1974; Wong, 1992; Wong, 1992, respectively) to define

gradients of metals concentrations to be tested. After performing preliminary assays the gradient of definitive concentrations were set for each metal and for each cloned lineage. Therefore, each cloned lineage was exposed to a control (ASTM hardwater) and to a series of five increasing concentrations, with a dilution factor of 1.4×, within the following ranges: copper (13.0 to 695 µg/L), zinc (500.0 to 3246.9 µg/L), cobalt (2058 to 13 446 µg/L) and chromium (120.0 to 2305 µg/L). Dilutions were made with ASTM hardwater medium to the required concentrations. All assays were carried out under controlled temperature (19 to 21ºC) and photoperiod (16h:8h light:dark). Five neonates (> 6 and < 24 hours old), from the third or fourth broods, were introduced in 50 ml vessels containing 30 ml of test solution with four replicates per each chemical concentration. Control vessels contained 30 ml of ASTM hardwater. During the 48-h of the assay, organisms were not fed and the medium was not renewed. ASTM was prepared according to American Society for Testing and Materials (2002) without the addition of vitamins. Mortality was registered after 24 and 48 hours of exposure, an organism being considered dead when it remained immobile during 15 s after gentle prodding. Conductivity (µS/cm; LF 330/SET, best nr. 300 204), pH (pH 330/SET-2, best nr. 100 788) and dissolved oxygen (mg/L; OXI 330/SET, best nr. 200 232) parameters were registered at the beginning and end of each assay.

Data analysis

Median lethal concentrations (causing 50% of immobility; LC_{50}), with the respective 95% confidence limits, after 24 and 48h of exposure were calculated, for each cloned lineage, using the software Priprobit (Sakuma, 1998). In order to study the existence of multiple/co-tolerance among the tested metals, correlation coefficients were determined for the median effective concentrations of the seven cloned lineages (Zar, 1996), using the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

Coefficients of variation were computed for the LC_{50} values, in order to compare variability associated with the responses to the different metals. Homogeneity between coefficients of variation was analysed using the Miller and Feltz equation (Zar, 1996), and, whenever significant differences were found, the homogeneity was tested for each pair of chemical (Z-value) in order to identify where differences existed (Zar, 1996).

Results

The physico-chemical parameters measured exhibited very small variations during the lethal toxicity assays: dissolved oxygen was always above 7.2 mg/L (well above 2mg/L, the limit value referred in the OECD guideline for acute toxicity assays with *Daphnia* sp.), pH values were close to neutrality (the lowest value measured was 7.58), and conductivity values ranged from 500 (in ASTM hardwater-control) to 571 µS/cm (in the highest zinc concentration). Copper registered the highest variation of pH (7.71-8.5) and conductivity (526-540 µS/cm). The highest variation of dissolved oxygen registered was in cobalt (ranging between 8.22 at the beginning and 8.09 at the end of the test).

Lethal sensitivity to metals

In general the seven tested cloned lineages of *D. longispina* were more sensitive to copper, followed by chromium, zinc and cobalt (Fig. 2. 1., 2. 2., 2. 3., and 2. 4.). The differences in sensitivity among the four metals reached more than two orders of magnitude. Furthermore, after exposing the seven cloned lineages to the different metals, for 24 h, it was observed that, among the all tested pairs, variability in the LC_{50} was significantly higher for copper relatively to cobalt (Table 2. 1.). However no significant differences were found in the variability of responses of the four metals (Table 2. 1.).

Table 2. 1. Values of coefficients of variation (%) calculated for the seven *Daphnia longispina* cloned lineages exposed to copper, zinc, cobalt, and chromium. a and b represent homogeneous groups.

	Cu	Zn	Co	Cr	Miller and Feltz
$LC_{50,24h}$	77	35	19	42	$\chi^2_3 = 62;\ p < 0.05$
	a	a	b	a	
$LC_{50,48h}$	77	36	46	41	$\chi^2_3 = 8.4;\ p < 0.05$
	a	a	a	a	

Copper

The cloned lineages E84 and E89 were the most sensitive to copper, while N91 and E99 exhibited the lowest sensitivity to lethal levels of copper (Fig. 2.1.). The observed differences in sensitivities to copper, between the seven tested cloned lineages, achieved one order of magnitude: clone E84 exhibiting an LC_{50} (95% CI) of 20.2 (18.1 - 22.6) and of 11.8 (6.6 - 14.5) µg/L; and clone E99 exhibiting LC_{50s} of 317 (276 - 454) and 163 (156 - 170) µg/L, after 24 and 48 h of exposure, respectively (Fig. 2. 1.). Thus, the most sensitive cloned lineage being fifteen times more sensitive to copper than the most resistant one.

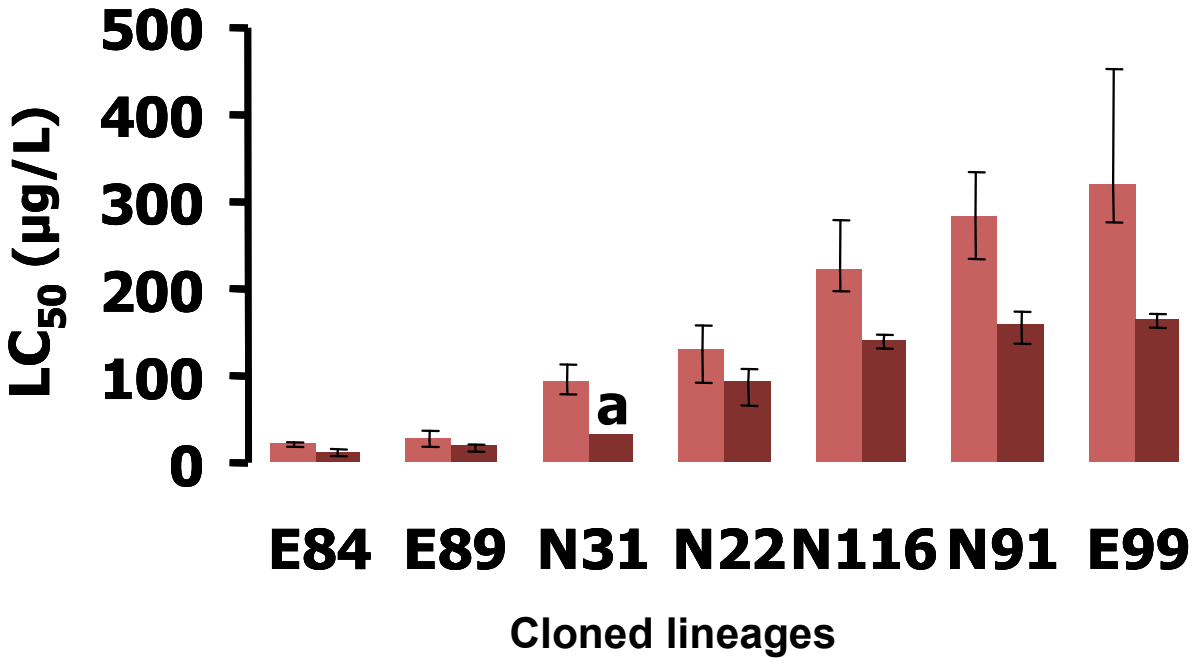

Fig. 2. 1. Values of the medial lethal concentration (LC_{50}), with the respective 95% confidence intervals (error bars), computed after 24 (light pink) and 48h (dark pink) of exposure, for the seven cloned lineages of *Daphnia longispina* exposed to copper. (a: the 95% CI could not be computed).

Zinc

The cloned lineage N116 was the most sensitive to zinc, and N31 the most tolerant one (Fig. 2. 2.). As for copper, the range of differences among clones reached one order of magnitude, although the interval was not as wide as for copper: the values of LC_{50} (95% CI) computed for N116 were 701 (314-976) and 422 (157-509) µg/L (Fig. 2.2.); and for N31 were 1806 (1478-2271) and 755 (402-896) µg/L, after 24 and 48 h of exposure, respectively (Fig. 2. 2.). Thus, the most sensitive cloned lineage being two times more sensitive to zinc than the most resistant one.

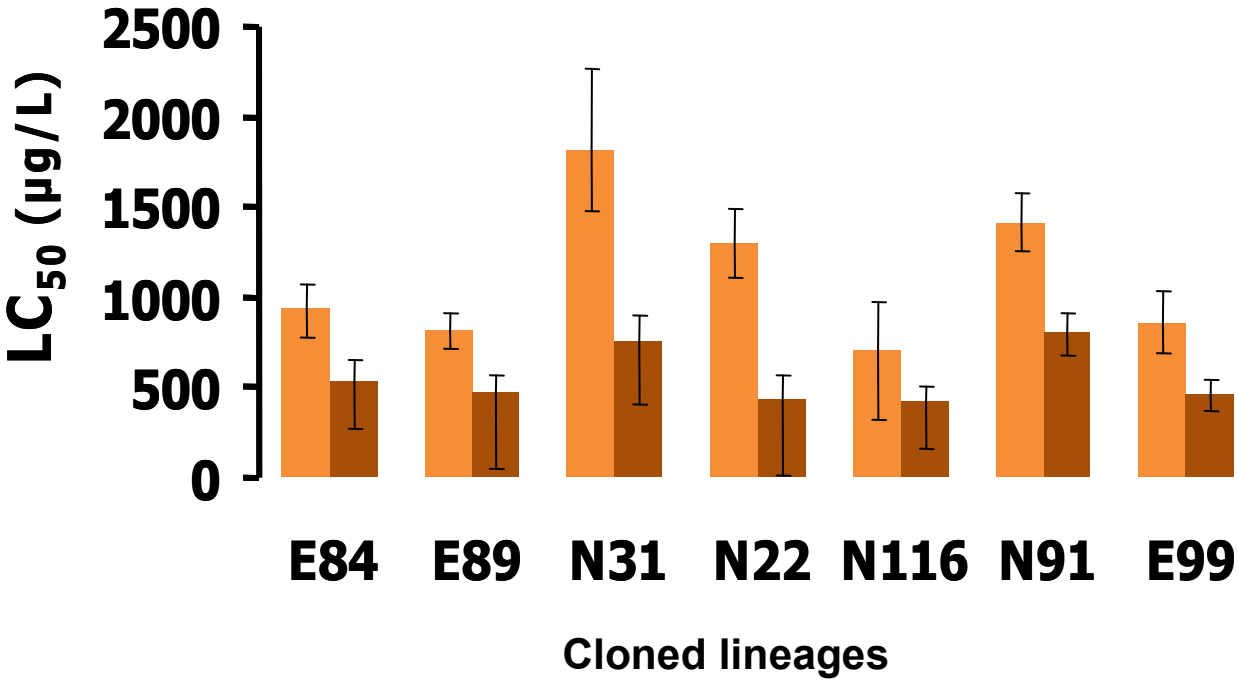

Fig. 2. 2. Values of the medial lethal concentration (LC_{50}), with the respective 95% confidence intervals (error bars), computed after 24 (light orange) and 48h (dark orange) of exposure, for the seven cloned lineages of *Daphnia.longispina* exposed to zinc.

Cobalt

Among the seven cloned lineages exposed to lethal levels of cobalt, E99 and N22 exhibited the highest and lowest sensitivity, respectively, to this metal. The interval of differences among clones was not as wide as for copper or zinc, never reaching one order of magnitude: the values of LC_{50} (95% CI) computed for E99 were 2558 (2239-2882) and 1880

(1693-2043) µg/L; and for N22 were 4635 (4154-5131) and 3942 (3388-4377) µg/L, after 24 and 48 h of exposure, respectively (Fig. 2. 3.).

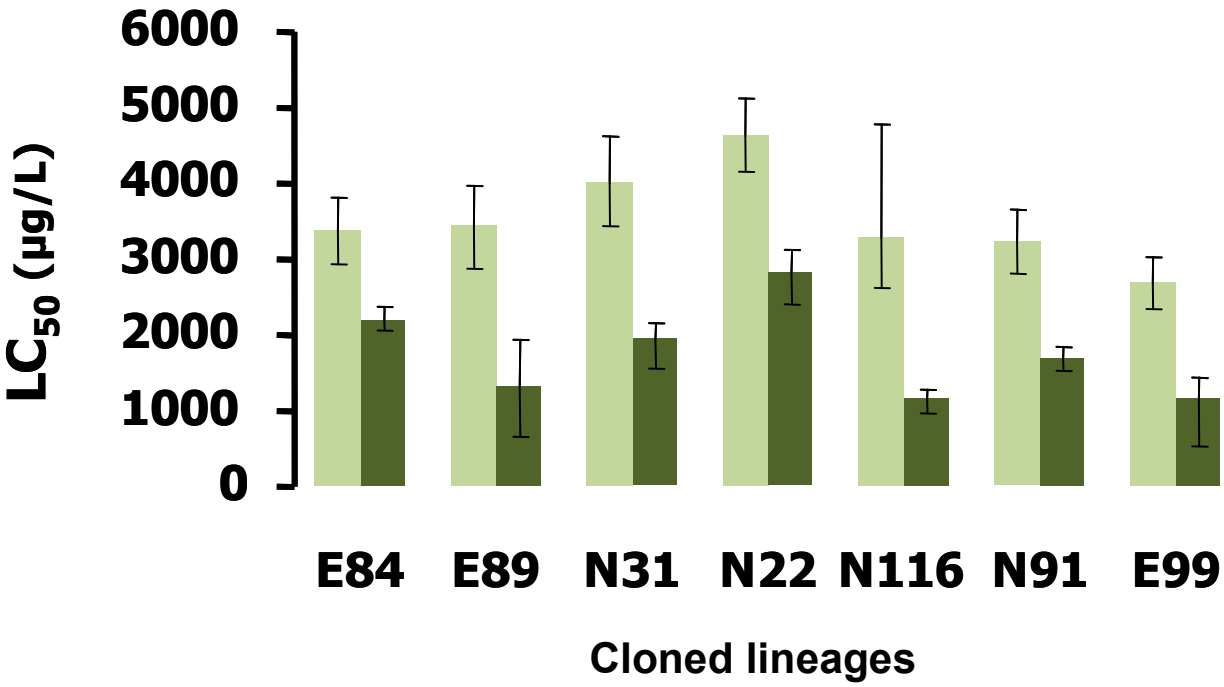

Fig. 2. 3. Values of the medial lethal concentration (LC_{50}) with the respective 95% confidence intervals (error bars), computed after 24 (light green) and 48h (dark green) of exposure, for the seven cloned lineages of *Daphnia longispina* exposed to cobalt.

Chromium

Cloned lineage N31 was the most sensitive to chromium, while N22 and N116 were the most tolerant ones (Fig. 2.4.). As for copper and zinc, the range of differences among clones reached one order of magnitude: the values of LC_{50} (95% CI) computed for N31 were 300 (242-413) and 240 (192-304) µg/L, for N22 were 1132 (1008-1271) and 729 (614.3-823.2), and for N116 were 1010 (856-1141) and 747 (714-781) µg/L; after 24 and 48 h of exposure, respectively (Fig. 2. 4.).

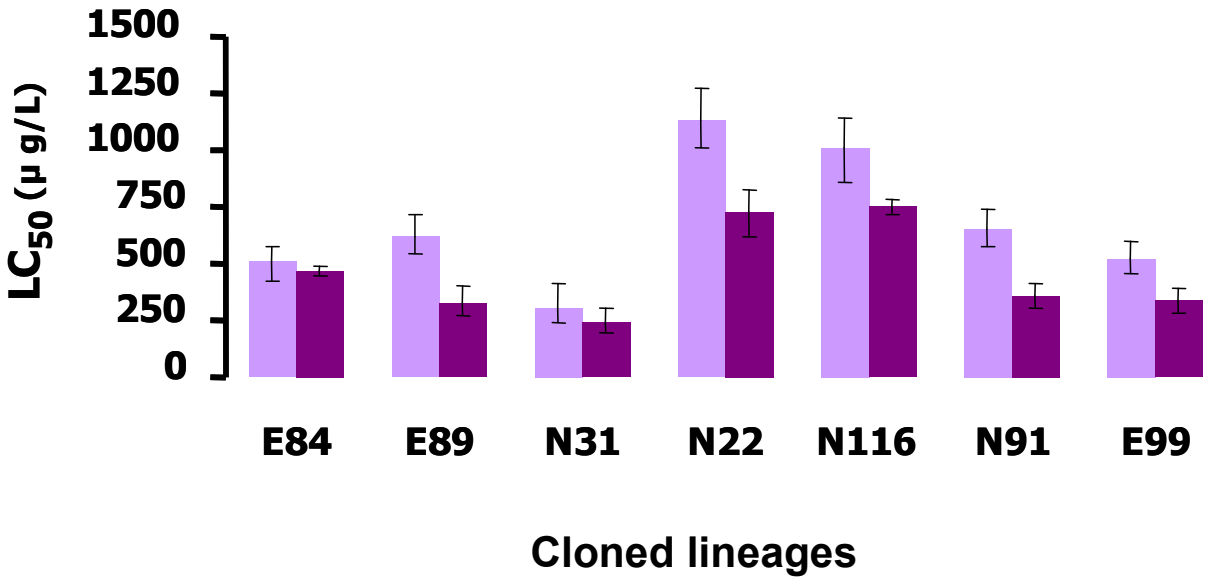

Fig. 2. 4. Values of the medial lethal concentration (LC_{50}) with the respective 95% confidence intervals (error bars), computed after 24 (light violet) and 48h (violet) of exposure, for the seven cloned lineages of *Daphnia longispina* exposed to chromium.

Multiple/co-tolerance to lethal toxicity of the tested metals

No significant correlations were detected between any pair of the tested metals ($r \leq 0.51$; $p \geq 0.30$), both after 24 and 48h of exposure (Table 2. 2.). However, clone E89 was the second most sensitive cloned lineage to all the four tested metals (Table 2. 2).

To allow a better comparison of the rank of sensitivities of cloned lineages for each metal, a summary representation is depicted in Table 2. 2.

Table 2. 2. Values of correlation coefficients calculated for the 24 and 48-h median lethal concentration values ($LC_{50,24h}$ and $LC_{50,48h}$) of the seven *Daphnia longispina* cloned lineages exposed to copper, zinc, cobalt, chromium.

	$LC_{50,24h}$	$LC_{50,48h}$
Cu/Zn	r = -0.01; p = 0.98	r = 0.45; p = 0.31
Cu/Co	r = -0.51; p = 0.24	r = -0.08; p = 0.86
Cu/Cr	r = 0.24; p = 0.61	r = 0.40; p = 0.37
Zn/Co	r = 0.46; p = 0.30	r = 0.03; p = 0.94
Zn/Cr	r = -0.34; p = 0.46	r = -0.22; p = 0.63
Co/Cr	r = 0.35; p = 0.44	r = 0.35; p = 0.44

Though no significant correlations were observed, an inversion in sensitivity to the tested metals was observed for some cloned lineages (Table 2. 2. and Table 2. 3.). For example, clone N116 was the most tolerant to chromium, but was the most sensitive to zinc and cobalt. Clones E99 and N91 were the most resistant to copper and zinc, while being one of the most sensitive to chromium and cobalt, respectively. Also, clone N31 was the second most resistant to zinc, while being the most sensitive to chromium (Table 2. 3.).

Table 2. 3. Representation of the sensitivity of the seven cloned lineages of *Daphnia longispina* to the four tested metals.

	E99	N91	N116	N22	N31	E89	E84
Copper							
Zinc							
Cobalt							
Chromium							

Rank of increasing sensitivity (from dark red-most tolerant to yellow-most sensitive):

Discussion

A number of works have reported the existence of multiple or co-tolerance among different metals in several species, both at lethal and sublethal levels. For example, Garcia-Toledo *et al.* (1985) observed that two species of fungi (*Rhizopus stolonifer* and *Cunninghamella blakesleeana*) tolerant to copper simultaneously acquired an increased tolerance to elevated levels of cadmium, cobalt, nickel, and lead. Münzinger and Monicelli (1992) observed that individuals of *Daphnia magna* that were chromium tolerant also showed a significantly higher tolerance to copper and nickel, for several monitored demographic parameters, comparatively to non chromium tolerant individuals. Also, several mechanisms have been described as responsible for tolerance to more than one metal. For example, plasmids are known to confer tolerance to several metals, namely, to mercury, zinc, cadmium, and lead (*e.g.* Weiss *et al*., 1978; El-Deeb, 2009). Hall *et al*. (1980), observed that the organic

material produced by the copper tolerant alga *Ectocarpus siliculosus* was able to bind copper more rapidly than the organic material produced by non-tolerant cells. Furthermore, he also observed that the organic material produced by the tolerant cells, could detoxify cobalt and zinc externally. Other authors, reported common uptake mechanisms for divalent cations like Cd^{2+}, Zn^{2+}, and Co^{2+} (Weiss *et al*., 1978; Tobin *et al*., 1984; Garnham *et al.*, 1992). For example, Bariaud and Mestre (1984) showed that the modifications of the membrane permeability to cadmium ions also explained the increased tolerance observed for cobalt and zinc in Cd-tolerant cells of *Euglena gracilis*.

In the presented study an association between lethal tolerance to more than one metal was not observed among the studied cloned lineages, suggesting the presence of different mechanisms responsible for the tolerance to each of the four metals (Cu, Zn, Co, and Cr) in the different clones. These results agree with other works that hypothesized that tolerance to high metal concentrations may be specific (Macnair, 1997). Actually, in the scientific literature, some authors also reported the inexistence of multiple or co-tolerance among several metals. Von Frenskell-Insam and Hutchinson (1993) observed that seeds of the plant *Deschampsia cespitosa* that were tolerance to nickel did not exhibited an increased tolerance to Zn, though seedlings selected for an elevated tolerance to Zn were also tolerant to Ni. Further, Adriaensen *et al*. (2005) observed that a copper resistant fungus (*Suillus luteus*) did not reveal co-tolerance to zinc: while Zn-tolerant isolates were very sensitive to copper, Cu-tolerant isolates were very sensitive to Zn. These authors discussed that the fact of both Cu and Zn being essential metal, does not necessarily involve similar mechanisms in the detoxification of these metals. Accordingly, other researchers showed that the genetic basis of metal tolerance in some bacteria and higher plants support the absence of co-tolerance for the pair Cu-Zn, since Cu tolerance and Zn tolerance may be under the control of different genes (Mergeay *et al*., 2003; Schat and Vooijs, 1997). Guo *et al*. (2008), after examining the contribution of metallothioneins (MT) on metal tolerance of *Arabidopsis*, observed that different types and different conjugations of MT are responsible for conferring tolerance to different metals in this species. Also, Tilstone and Macnair (1997), after studying several tolerance and non-tolerant populations of the plant *Mimulus guttatus* found that tolerance to many metals observed in this plant was due to multiple metal tolerance which was caused by independent genetic mechanisms for specific metals.

Though no multiple or co-tolerance among Cu, Zn, Co, and Cr was observed in the present study, some lineages reveal to be tolerant to one metal and sensitive to others. For example, N116, that is tolerant to copper, proved to be sensitive to zinc and cobalt (Table 2),

and clone N31 showed to be sensitive to chromium but tolerant to zinc. These results make clear implications for ecological risk assessment, as it suggests that a population genetically eroded may be at a higher risk if exposed to future lethal pulses of different contaminants. For example, let us consider a hypothetical population composed by the seven cloned lineages of *D. longispina* here studied. If this population experiences exposure to a strong pulse of zinc contamination, most probably the most sensitive cloned lineages to this metal (N116, N22, E89, and E84) will die and disappear from the initial population. If, in the future, this genetically eroded population is exposed to a pulse of chromium cloned lineages E99 and N31, surviving to the pulse of zinc, will probably die, because they are sensitive to this second metal. Thus, from an initial population with seven clones, only one clone (N91) would remain in the genetically eroded population after being exposed to two pulses of different metals. Through the occurrence of genetic erosion (elimination of sensitive genotypes to each chemical), the number of clones in the population would be reduced drastically increasing the future potential risk of population extinction. These results also highlight that laboratory assays carried out with single clones may not allow accurate predictions of the responses of natural populations.

Finally, though not being the main objective of the present work, it was observed that, in general, the sensitivity of the cloned lineages of *D. longispina* to the tested metals followed the same order of decreasing sensitivity: copper, chromium, zinc and cobalt. This result is inline with data published for other cladoceran species exposed to the same four metals. For example, Baudouin and Scoppa (1974) observed the same order of sensitivity to lethal levels of copper, chromium, zinc, and cobalt, for *Daphnia hyalina.* Other tests performed with copper showed that this metal is several times more toxic (about a hundred) than cadmium, zinc and aluminium (Fargasova, 2001). These differences between sensitivities to metals may be due to the fact that invertebrates may accumulate different metal concentrations in their body structures (Eisler, 1981). Rainbow (2002) points out that, the fate of each metal depends, not only on their chemical characteristics, but also depends on physiological particularities of the invertebrate, as well if the metal is needed for essential metabolic functions, being stored or excreted. Although Cu and Zn are essential metals, lower invertebrates demonstrate varying degrees of copper and zinc regulation (Amiard *et al*., 1987). It was also verified that the range of responses to copper was the widest, though the coefficient of variation was only significantly different from that of cobalt, during the 24h period of exposure. This higher variability of responses to copper was expected as cloned lineages were selected accordingly to a wide range of lethal sensitivities to this metal. However, and in agreement with the

inexistence of multiple/co-tolerance between the four metals, the same wide range of responses was not observed for the other three tested metals. As the results suggest indenpendency in lethal tolerance to the four metals, it could be expected that the responses to zinc, cobalt and chromium could exhibit a lower variability, accordingly to the essenciality hypothesis, which states that responses to non-essential metals are more variable than responses to essential metals. For example, Barata *et al.* (1998), after studying the genetic variability of *D. magna* responses to essential and non-essential metals, found that the hypothesis of a higher variability in responses to non-essential metals than in responses to essential metals was corroborated for cadmium and zinc, although copper and uranium did not support this hypothesis. However, the results obtained in the present study did not support the essentiality hypothesis, since the variability of responses to copper was high (CV of 77%).

Conclusion

In conclusion, no multiple or co-tolerance was observed among the four tested metals, arguing against the hypothesis of a relevant generalised tolerant mechanism. Furthermore, though any significant correlation was reported for any of the pairs of tested metals, an inverse relationship in tolerance to metals was observed for some cloned lineages: the most sensitive to one metal being the most tolerant to another metal. These results highlight the possibility of scenarios of great risk of extinction in genetically eroded populations due to inverse tolerance between chemicals.

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Chapter Three

Is a higher resistance to copper correlated with a faster recovery in daphnids?

Abstract

Natural populations are commonly exposed to sequential pulses of contaminants. If the input of a pulse of lethal levels of a contaminant causes the disappearance of the most sensitive genotypes from a population (genetic erosion), then, the ability of the impacted population to withstand a further pulse of the contaminant will largely depend upon recovery rate of the remaining organisms. This study aimed at evaluating if a genetically determined tolerance is positively related with a faster recover capacity, which would guarantee a double advantage to the tolerant genotypes. To achieve this goal, four cloned lineages of the cladocera *Daphnia longispina* O.F. Müller, exhibiting different sensitivities to lethal levels of copper, were exposed to sequential pulses of this metal, to evaluate specific recovery rates and their relation to tolerance. For each cloned lineage, the intensity of pulses corresponded to the respective concentration of copper causing 30% of mortality after 24h of exposure ($LC_{30,24h}$). Obtained results showed no association in tolerance to copper and a faster physiological recovery. Tolerant clones were not able to recover after being exposed to a first pulse of copper, and organisms continued to die even after being transferred to ASTM hardwater. The sensitive cloned lineage showed the capacity to recover after being exposed to the first pulse of copper, however, after being exposed to the second pulse of copper all organisms died. The reduction of the recovery period from 72h to 24h lead to an increased mortality in cloned lineage E99, as all organisms died after the second pulse of copper. In addition, it was observed that neonates (6 to 24h old) were more sensitive to copper than older organisms (96h-old) for cloned lineages N91 and E99 (the most tolerant clone), while the opposite was observed for cloned lineages N116 and E89 (the most sensitive clone).

The obtained results suggest that standard lethal assays sub-estimate toxicity (as endpoints are computed solely based in mortality occurring during the exposure to the toxicant), as even during the recovery period a high mortality continued to occur for some cloned lineages. Furthermore, the lack of a positive correlation between tolerance and a faster recovery highlights the increased risk at which impacted populations are subject after being exposed to a pulse of lethal levels of a contaminant.

Keywords: Recovery rate *Daphnia longispina* Metals Sequential pulses

Introduction

Industrial releases, superficial and underground run-off events, fuel and agrochemical spills are often causes of concern for ecotoxicologists (Bearr et al., 2006; Zhao and Newman, 2006). These kind of incidents, although frequent, are not constant in concentration and time, so it is rare for communities, aquatic or/and terrestrial, to be exposed to constant and continuous concentrations of contaminants (pesticides, metals, insecticides). Therefore, most commonly, organisms experience fluctuations to contamination exposure that vary with intensity, frequency and duration (Hoang and Klaine., 2008; Diamond *et al.,* 2006; Stephens *et al*., 1988, 1992; White and Dubrovsky, 1994). These arguments reveal the importance of toxicity assays simulating more ecologically relevant scenarios of exposure to contaminants. Actually, a number of studies have shown that the impact of pulsed exposure of aquatic organisms to pollutants can be quite different from continuous exposure. For example, Roberts *et al*. (2008) demonstrated the extreme importance of the evaluation of pulsed exposure for very small periods of stress, since they registered decreasing abundances of 50% in invertebrates within five minutes after a single pulse of storm water stress. As well, Ingersoll and Winner (1982) reported that *Daphnia pulex* suffered a higher mortality rate when exposed to pulsed doses of copper than when exposed to copper continuously. In spite of these examples, some researchers have argued that pulsed exposure to pollution should be less toxic to aquatic organisms if sufficient time is allowed between exposures for recovery to take place (Mancini, 1983; Wang and Hanson, 1985).

Furthermore, in an ecotoxicological point of view the extension of the pulses is intimately related with the time between them and therefore, plays an important role in the response of organisms (Diamond *et al*, 2006; Bearr *et al*., 2006). Fathead minnow and water flea survival was severely affected, after being exposed to multiple pulses of copper and zinc for 24 h, when time to recover was not sufficient to allow the elimination of the toxic compounds rapidly enough to prevent internal accumulation (Diamond *et al*., 2006).

The magnitude (concentration in which the stressor is present), frequency (number of pulses), duration and recovery period (time between pulses) have been the main factors considered in pulsed exposure tests by many authors (*e. g.* Bearr *et al*., 2006; Diamond *et al.,* 2006) but diversity among populations as been underestimated. However, this is an important parameter to take into account, when evaluating the effects that sequential pulses of a contaminant may have on natural populations. Natural populations hold a large number of genetically different individuals, which also exhibit different sensitivities to chemicals, so,

when a toxicant enters the environment it may lead to the genetic erosion of the initial populations by eliminating the most sensitive individuals (e.g. Belfiore and Anderson, 2001; Van Straalen and Timmermans, 2002; Morgan *et al.*, 2007; Bourret *et al.*, 2008; Nowak *et al.*, 2009; Ungherese *et al*., 2010). If the remaining tolerant genotypes are also the ones that exhibit a faster physiological recovery from the pulse, then they will present a double advantage relatively to the sensitive ones; as if another pulse of contamination occurs in a short time they will be the ones coping better with this new event of contamination.

In the presented study, the main objectives were to: 1) evaluate the recovery capacity of different lineages of the cladoceram *Daphnia longispina* after being exposed to a pulse of lethal concentrations of copper, and 2) identify if an association exist between copper tolerance and faster physiological recovery in cloned lineages of *D. longispina*. To address these objectives, four cloned lineages of the cladocerans *Daphnia longispina* O.F. Müller exhibiting different genetically determined sensitivities to lethal levels of copper were studied. Three scenarios of association, between lethal sensitivity to copper and physiological recovery, could occur, and having different impacts in a population: (i) if a positive correlation occurs (Fig. 1. 1.), it is expected that, in the first pulse of copper contamination, the most sensitive individuals will die, remaining the most resistant ones, which are also the ones that will recover more rapidly. In the second exposure period, in addition to the sensitive organisms, the ones that take more time to recover will also die, corresponding to the same sensitive individuals. In this case, the most resistant ones present a double vantage (are the most resistant and recover faster) and will be able to resist the two exposure periods. (ii) if no correlation occurs (Fig. 1. 1.), it can happen that the most sensitive or the most tolerant clone has very slow recovery rates or vice-versa. In this particular case, after the first copper exposure, the most sensitive organisms will die and in a second pulse the ones whose recover takes more time. (iii) if a negative correlation occurs (Fig. 1. 1.); the most tolerant individuals will exhibit a longer recovery rate while the most sensitive ones will recover faster. The first pulse will affect the most sensitive individuals while the second pulse will affect the most tolerant ones, which show slower recoveries. This is the worst case scenario because it can lead to the extinction of almost all the initial population.

Material and Methods

Test organisms

Four cloned lineages of *Daphnia longispina* O.F. Müller, exhibiting different genetically determined lethal tolerance to copper, were selected to perform this study (E99, N91, N116, and E89). *Daphnia* is a genus commonly used in ecotoxicological studies because it reproduces by cyclic parthenogenesis, which allows maintaining in the laboratory exactly the same clone for several generations, in addition these selected clones of *D. longispina* have long been studied by some of the authors of the presented work Lopes *et al*. (2004).

The four selected cloned lineages were isolated from two natural populations (Lopes *et al*., 2004); one inhabiting a metal impacted and another a reference site. Cultures, of each cloned lineage, were maintained in laboratory, for more than 1000 generations, under controlled conditions of temperature (19 to 21ºC) and photoperiod (14:10 h L:D) in ASTM hardwater (American Society for Testing and Materials (ASTM, 2002), with vitamins and organic additive Marinure 25 (Baird *et al*. 1989); medium being changed every other day and fed daily with the green algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (formerly known *Selenastrum capricornutum* Printz) ($1.5*10^5$ cells/mL/d). All lineages were cultured by asexual reproduction being selected neonates from third, fourth or fifth brood to maintain the cultures and perform recovery assays.

Tested chemical

The stock solution of copper was acquired by adding copper sulphate (supplied by Sigma-Aldrich) to nanopure water (80 mg/L Cu). The $LC_{30,24h}$, of each cloned lineage, was attained by diluting this stock solution in ASTM without the addition of vitamins. Values were rounded to the first decimal number

Ten neonates with ages between 6 to 24 hours, and from the third or fourth brood, were introduced in 70 ml vessels containing 50 ml of test solution with six replicates per each treatment. Control vessels contained 50 ml of ASTM. The medium was changed and individuals fed accordingly to the described treatments (Fig. 3.1.). During the assays, individuals were fed only during the recovery periods (between pulses) and as well the medium was changed. This procedure was also followed for the control vessels, so that a variability on laboratorial parameters could not happen, and allowing optimal conditions for

individuals affected by exposure. The green algae *P. subcapitata* ($1.5*10^5$ cells/mL/d) and algae extract *Ascophyllum nodosum* constitute the diet of the cladocerans.

The medium ASTM was prepared according to American Society for Testing and Materials (2002) except without the addition of vitamins. Mortality was registered at 24, 96, 120 and 144 hours, considering that an individual was death when it remained immobile during 15 s after gentle prodding. Conductivity (µS/cm; LF 330/SET, best nr. 300 204), pH (pH 330/SET-2, best nr. 100 788) and dissolved oxygen (mg/L; OXI 330/SET, best nr. 200 232) parameters were registered at the beginning and end of each assay.

Pulsed exposures

In pulsed assays, each cloned lineage was exposed to a single chemical concentration of copper, which corresponded to the lethal concentration causing 30% of mortality after 24h of exposure ($LC_{30,24h}$). This values was computed based on data gathered in the previous work (please see chapter 2). The selection of this concentration is related to the fact that the objective was not to induce death but only exposed the organism to a dose that could induce alterations in their body homeostasis so that recovery could occur. Four treatments with at least six replicates were defined.

In treatment A, consisting of the control treatment, neonates (< 24 h old) were exposed to ASTM hardwater during the test duration. In treatment B (Fig. 3. 1.), organisms were exposed to a copper pulse during the first 24 h of assay, being transferred afterwards to ASTM until the end of the assay. In C treatment, (Fig. 3. 1.), neonates where exposed to copper in the first 24h of assay, afterwards were transferred to ASTM for a period of 72 hours, after this recovery period were exposed again for 24 h to copper, and then, until the end of the assay, transferred to ASTM. Finally, in treatment D (Fig. 3. 1.), neonates were maintained in ASTM, until 96 hours, being then transferred to a copper solution for 24h and recovering in the last 24 hours period. During the recovery periods, maintenance in ASTM, organisms were fed with *P. subcapitata* ($1.5*10^5$ cells/mL/d) and algae extract (*A. nodosum*) to promote a optimal conditions for physiological recovery, while during chemical exposure conditions none of these factors were added. This assay was carried out twice for each cloned lineage.

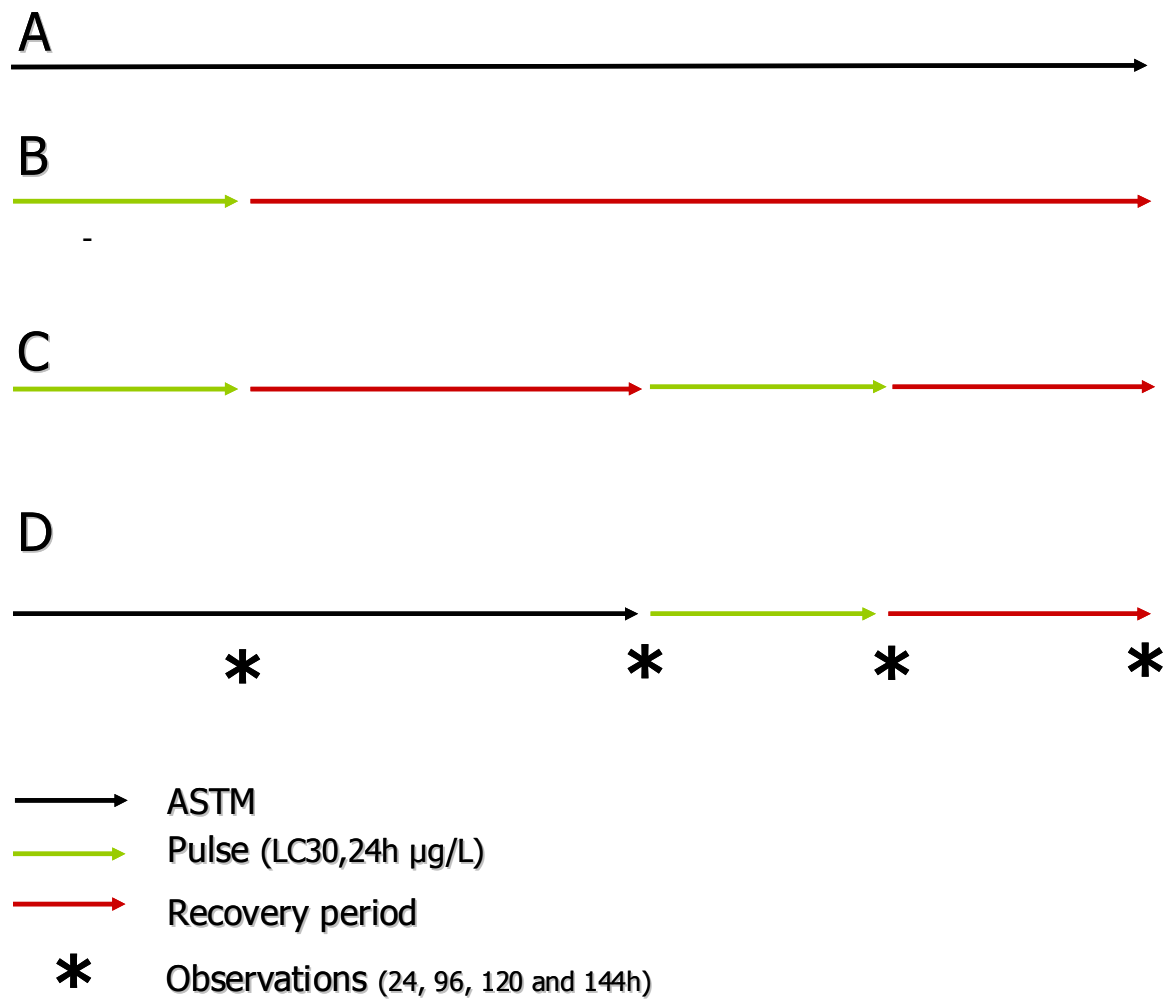

Fig. 3. 1. Schematic representation of the 1st experimental design showing the copper and ASTM exposure periods in each of the four treatments. Light green arrows represent exposures of 24 hours to the $LC_{30,24h}$ for copper, and black arrows represent exposure to ASTM. Red arrows represent the period of recovery that followed periods of exposure.

To evaluate if a shorter recovery period would influence the sensitivity to a second pulse of contamination, an additional recovery assay was carried out with lineages E99, N116, and E89. The same four treatments were carried out, however the duration of the first recovery period was shortened to 24h (Fig. 3. 2.).

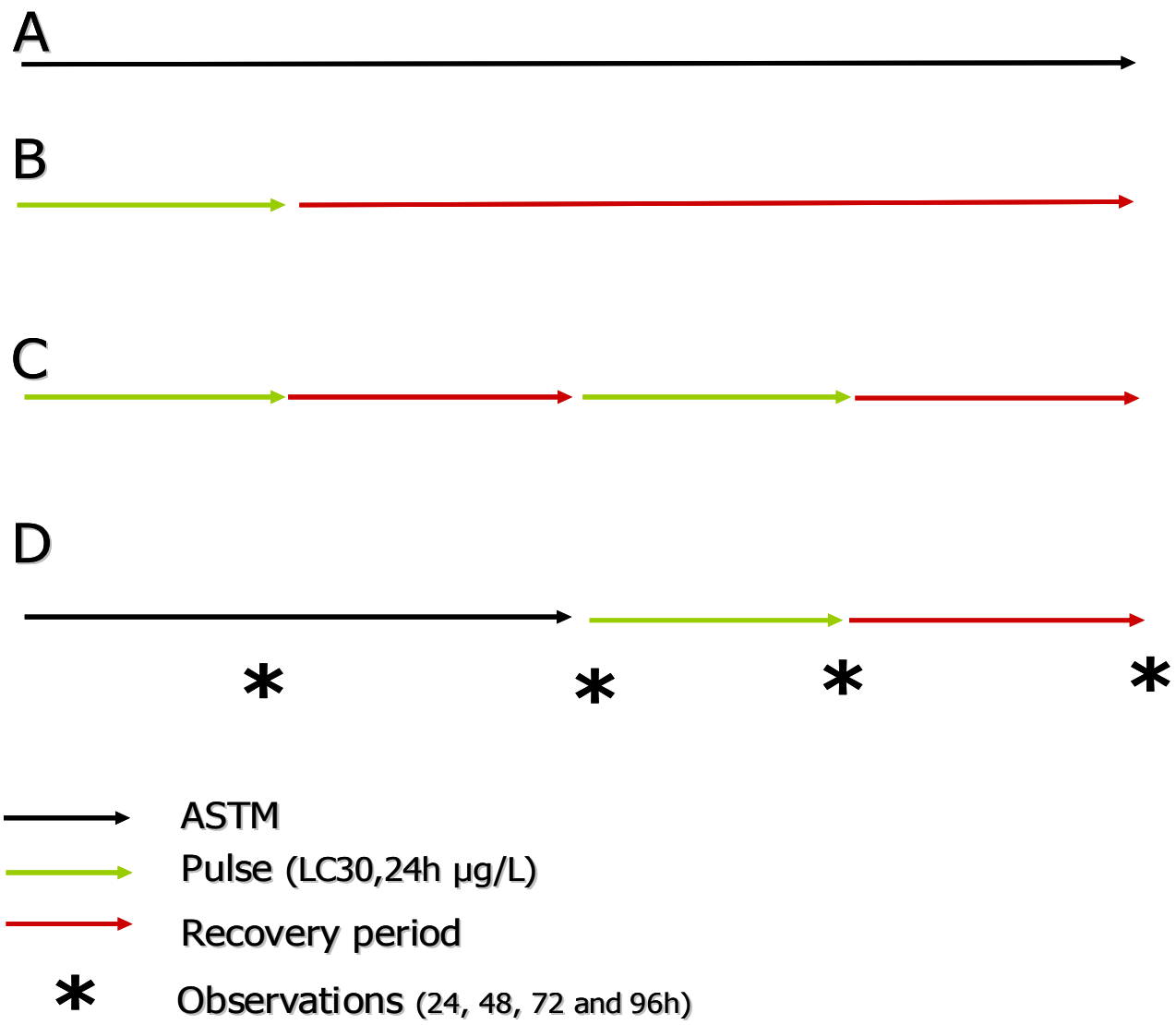

Fig. 3. 2. Schematic representation of the 2nd experimental design showing the copper and ASTM exposure periods in each of the four treatments. Light green arrows represent exposures of 24 hours to the LC30,24h for copper, and black arrows represent exposure to ASTM. Red arrows represent the period of recovery that followed periods of exposure.

Data analysis

Lethal concentrations, with the respective 95% confidence limits, causing 30% of mortality (LC30) after 24 and 48h of exposure were calculated for each cloned lineage using the software Priprobit (Sakuma, 1998).

Comparisons of mortality, during pulsed exposures to copper, were made through analysis of variance, after transforming data with the Ascombe arcsin of the square root (Zar,

1996). To compare data obtained in the first experimental design (Fig. 3. 2) a three- factorial analysis of variance (ANOVA) was carried out by considering cloned lineages and observation period as fixed factors and repetition (the two assays that were carried out) as a random factor, followed by the multiple comparison Tukey test. The data obtained in the second experimental assay was compared using a two factor ANOVA where cloned lineages and observation periods were considered as fixed factors (Zar, 1996). These analyses were carried out in the software Minitab 15.

Finally, to compare sensitivities between individuals with different ages, the percentage of mortality observed in treatment B (after exposing for 24h neonates 6 to 24h old) was divided by the percentage of mortality observed in treatment D (after exposing for 24h juveniles 96h old).

Results

During the pulsed exposure assays, physico-chemical parameters measured showed small variations: dissolved oxygen was always above 7.2 mg/L (well above 2mg/L, the limit value referred in the OECD guideline for acute toxicity assays with *Daphnia* sp.), pH values were close to neutrality (the lowest value measured was 7.71), and conductivity values ranged from 537 (in ASTM hardwater-control) to 578 µS/cm (N116 cloned lineage). E89 cloned lineage exbhited the highest pH variaton (7.71-8.5). The highest variation of dissolved oxygen registered was in E89 (ranging between 9.7 at the beginning and 8.2 at the end of the test).

In Table 3. 1. are shown the LC_{30} computed after 24h for each cloned lineage of *D. longispina* exposed to a gradient of copper concentrations (please see Chapter 2 for more details on these cumulative mortality assays). Cloned lineage E89 exhibited the highest sensitivity to copper, followed by N116, N91, and E99.

Table 3. 1. Values of lethal concentration causing 30% of mortality (LC_{30}), with the respective 95% confidence intervals (error bars), computed after 24 h of exposure, for the four cloned lineages of *Daphnia longispina* exposed to copper.

Cloned lineages	$LC_{30,24h}$ (95% CI)
E89	22.7 (11.0.1-29.5)
N116	179 (156-203)
N91	205 (154-245)
E99	259 (229-308)

Lethal responses to copper pulsed exposure

1st experimental design

No mortality was observed for any cloned lineage exposed to the treatment A (please see Fig. 3. 1.).

Cloned lineage **E89** (the most sensitive to lethal levels of copper) exhibited a percentage of mortality between 20 and 30% after being exposed to the first pulse of copper (Fig. 3. 3.). Following their transfer to ASTM hardwater (treatments B and C), a mortality of 7 and 14% (respectively for each test repetition) was observed in treatment C (72h period of recovery in ASTM), but mortality continued to occur in organisms exposed to ASTM until the end of the assay (17 and 15% of mortality at 144h; treatment B). These small percentages of mortality, during the recovery period, suggest that the elimination of the chemical stress allowed the organisms to recover. However, all organisms died after being exposed to the second pulse of copper, both in treatment C and D. Furthermore, the ratio between mortality observed in the first pulse (treatment B; 24h of exposure of neonates - 6 to 24h old) and mortality observed in the second pulse of copper (treatment D; 24h of exposure of juveniles – 96h old) was inferior to 0.2, reporting a higher sensitivity to copper of juveniles comparatively to neonates (Fig. 3. 3.).

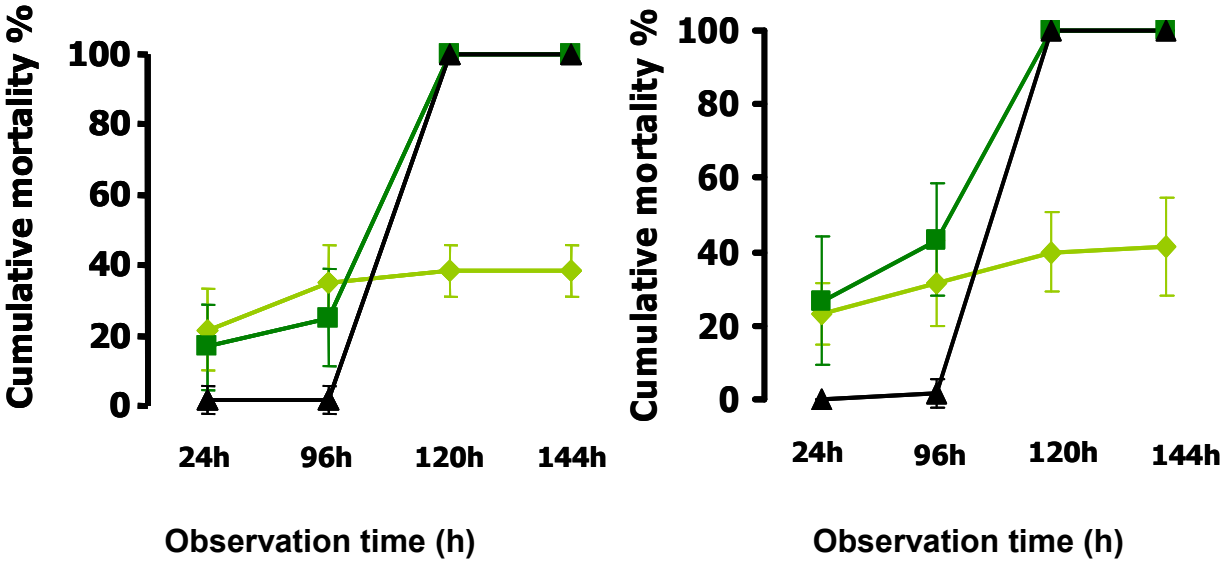

Fig. 3. 3. Cumulative mortality curves representing the response of a sensitive cloned lineage of *Daphnia longispina* (E89), exposed to pulses of copper ($LC_{30,24h}$), with a 72 h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 96-120h). In D (black), organisms suffer a copper pulse from 96 to 120h. The two figures represent two repetitions of the assay.

Cloned lineage **N116** (tolerant to lethal levels of copper) exhibited a percentage of mortality between 20 and 30% after being exposed to the first pulse of copper (Fig. 3. 4.). Following their transfer to ASTM hardwater (treatments B and C), a high mortality continued to occur both in treatment C (65 and 45%; 72h period of recovery in ASTM) and B (68 and 52%; exposure to ASTM until the end of the assay), suggesting that after being exposed to a pulse of copper organisms were not able to recover. Furthermore, the ratio between mortality observed in the first pulse (treatment B; 24h of exposure of neonates - 6 to 24h old) and mortality observed in the second pulse of copper (treatment D; 24h of exposure of juveniles – 96h old) was inferior to 0.8, reporting a higher sensitivity to copper of juveniles comparatively to neonates (Fig. 3. 4.).

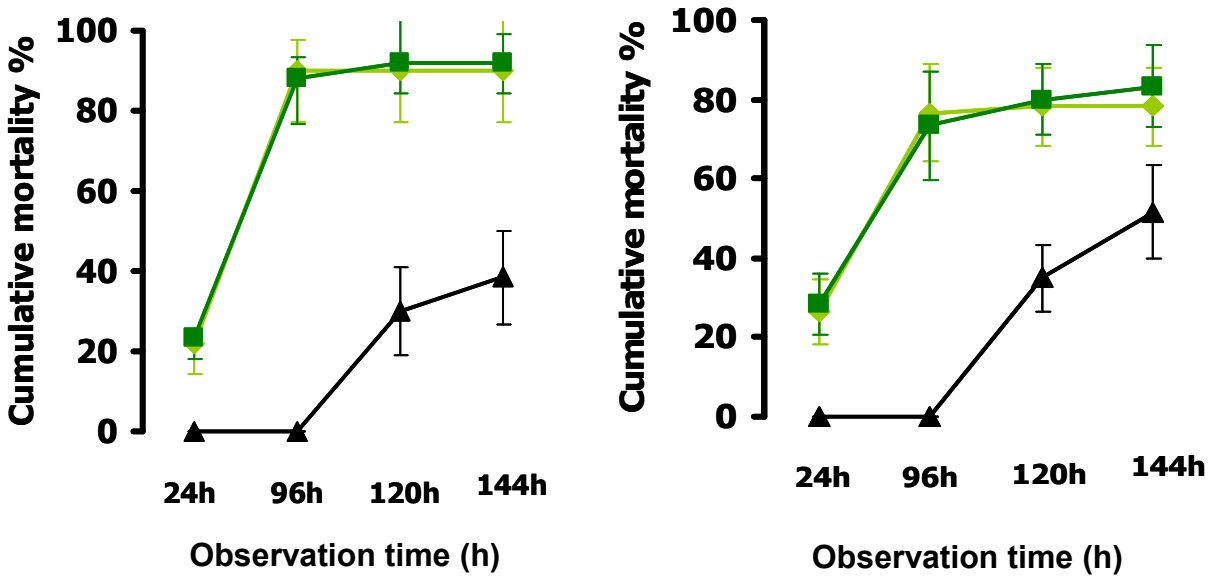

Fig. 3. 4. Cumulative mortality curves representing the response of a tolerant lineage of *Daphnia longispina* (N116), exposed to pulses of copper ($LC_{30,24h}$), with a 72h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 96-120h). In D (black), organisms suffer a copper pulse from 96 to 120h. The two figures represent two repetitions of the assay.

Cloned lineage **N91** (tolerant to lethal levels of copper) exhibited a percentage of mortality between approximately of 30% after being exposed to the first pulse of copper (Fig. 3. 5.). Following their transfer to ASTM hardwater (treatments B and C), as for cloned lineage N116, high mortality continued to occur both in treatment C (60 and 57%; 72h period

of recovery in ASTM) and B (67 and 62%; exposure to ASTM until the end of the assay), suggesting that after being exposed to a pulse of copper organisms were not able to recover. Furthermore, the ratio between mortality observed in the first pulse (treatment B; 24h of exposure of neonates - 6 to 24h old) and mortality observed in the second pulse of copper (treatment D; 24h of exposure of juveniles – 96h old) was higher than 1.9, reporting a higher sensitivity to copper of neonates comparatively to juveniles (Fig. 3. 5.).

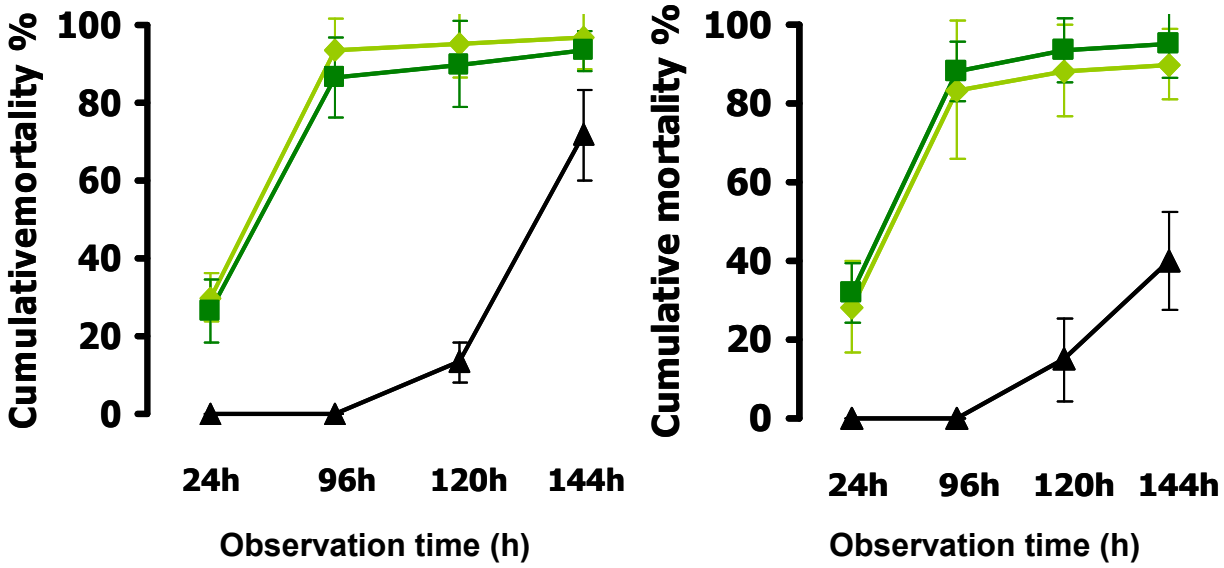

Fig. 3. 5. Cumulative mortality curves representing the response of a tolerant cloned lineage of *Daphnia longispina* (N91), exposed to pulses of copper ($LC_{30,24h}$), with a 72 h period of recovery between pulses.. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 96-120h). In D (black), organisms suffer a copper pulse from 96 to 120h. The two figures represent two repetitions of the assay.

Finally, cloned lineage **E99** (the most tolerant to lethal levels of copper) exhibited a percentage of mortality between approximately of 30% after being exposed to the first pulse of copper (Fig. 3. 6.). Following their transfer to ASTM hardwater (treatments B and C), as for cloned lineage N116, mortality continued to occur both in treatment C (30 and 34%; 72h period of recovery in ASTM) and B (50 and 44%; exposure to ASTM until the end of the assay), suggesting that after being exposed to a pulse of copper organisms were not able to recover. Furthermore, the ratio between mortality observed in the first pulse (treatment B; 24h

of exposure of neonates - 6 to 24h old) and mortality observed in the second pulse of copper (treatment D; 24h of exposure of juveniles – 96h old) was on average 1.2, reporting a higher sensitivity to copper of neonates comparatively to juveniles (Fig. 3. 6.).

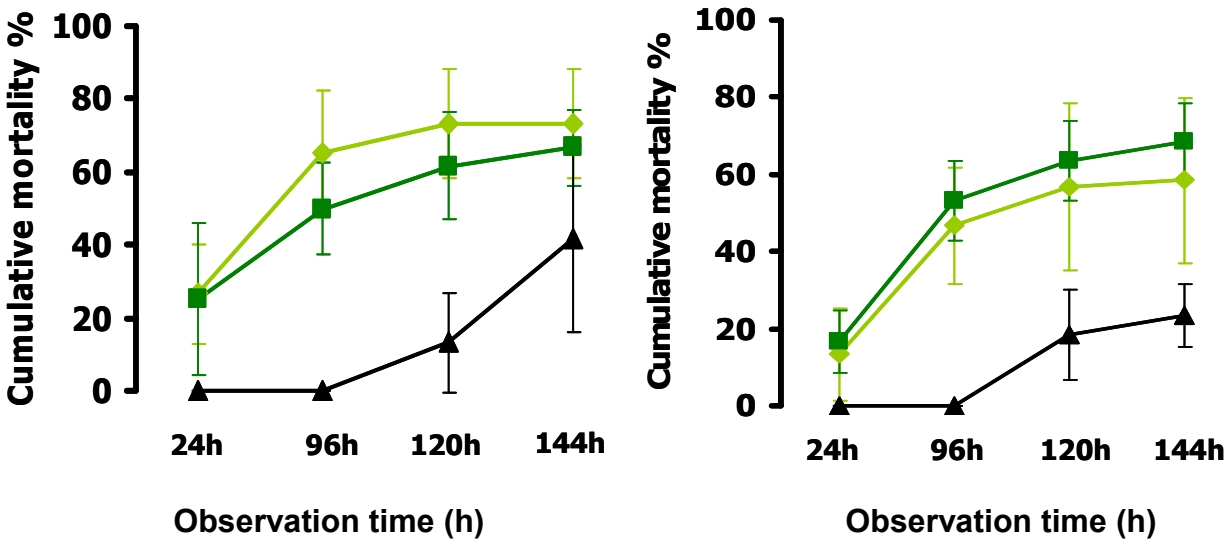

Fig. 3. 6. Cumulative mortality curves representing the response of a tolerant cloned lineage of *Daphnia longispina* (E99), exposed to pulses of copper ($LC_{30,24h}$), with a 72h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 96-120h). In D (black), organisms suffer a copper pulse from 96 to 120h. The two figures represent two repetitions of the assay.

Overall, significant differences in cumulative mortalities existed between clones, observation periods, and repetitions ($F \geq 2.29$; $p \leq 10^{-3}$). With cloned lineages E99, N116, and N91, registering a higher mortality during the 72 h recovery period comparatively to the sensitive cloned lineage E89 ($p \leq 0.01$).

2nd experimental design

In this second experimental design, the reduction of the 1st recovery period from 72h to 24h did not alter the pattern of responses of each cloned lineage. No mortality was observed for each of the four tested clones when exposed to the treatment A (please see Fig. 3. 1.).

Cloned lineage **E89**, as in the first experimental design, was able to recover after exposure to the first pulse of copper, as demonstrated by the low mortality observed in

treatment C (2%; at the 96h observation period), and treatment B (22%; at the 144h observation period) (Fig. 3. 7.). Also, all organisms died after being exposed to the second pulse of copper, both in treatment C and D, and juveniles (48h old) showed to be more sensitive to copper than neonates (6 to 24h old) (the ratio between mortality observed in the first pulse and mortality observed in the second pulse of copper: 0.2) (Fig. 3. 7).

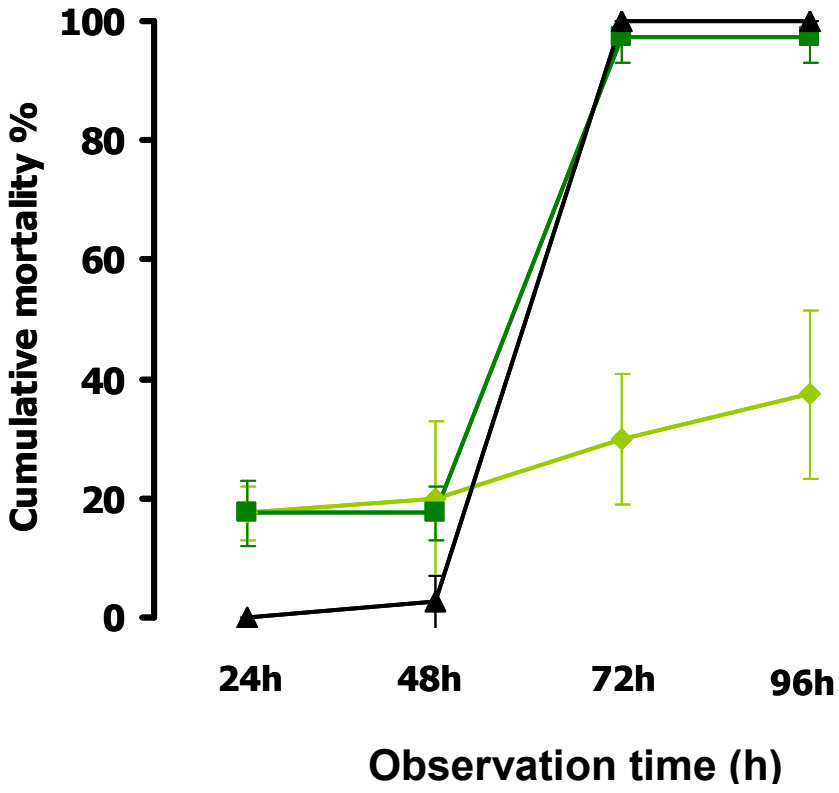

Fig. 3. 7. Cumulative mortality curves representing the response of a sensitive cloned lineage of *Daphnia longispina* (E89), exposed to pulses of copper ($LC_{30,24h}$), with a 24h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 48 to 72h). In D (black), organisms suffer a copper pulse from 48 to 72h.

Cloned lineage **N116**, as in the first experimental design, was not able to recover after exposure to the first pulse of copper, as demonstrated by the high mortality occurring during the recovery periods in treatment C (39%; at the 96h observation period), and treatment B (59%; at the 144h observation period) (Fig. 3. 8.). Furthermore, neonates (6 to 24h old) revealed to be more sensitive to copper than juveniles (48h old) (the ratio between mortality observed in the first pulse and mortality observed in the second pulse of copper: 2.0) (Fig. 3. 8.).

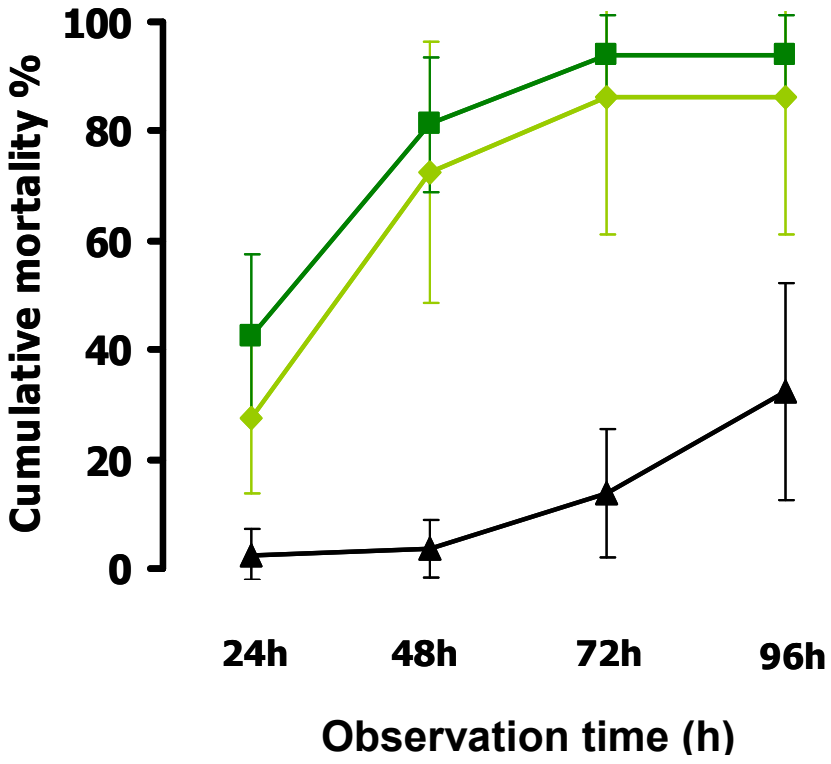

Fig. 3. 8. Cumulative mortality curves representing the response of a tolerant cloned lineage of *Daphnia longispina* (N116), exposed to pulses exposure of copper ($LC_{30,24h}$), with a 24h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 48 to 72h). In D (black), organisms suffer a copper pulse from 48 to 72h.

Cloned lineage **E99**, was not able to recover after exposure to the first pulse of copper, as demonstrated by the high mortality occurring during the recovery periods in treatment C (39%; at the 48h observation period), and treatment B (59%; at the 96 observation period)

(Fig. 3. 7.). A higher mortality was registered in the second pulse of copper after a recovery period of 24h, comparatively with the mortality that occurred in the 1st experimental design, when organisms were exposed to a second pulse of copper after being allowed to recover for a period of 72h (please see Fig. 3. 6.). Furthermore, neonates (24h old) revealed to be more sensitive to copper than juveniles (48h old) (the ratio between mortality observed in the first pulse and mortality observed in the second pulse of copper: 2.0) (Fig. 3. 9).

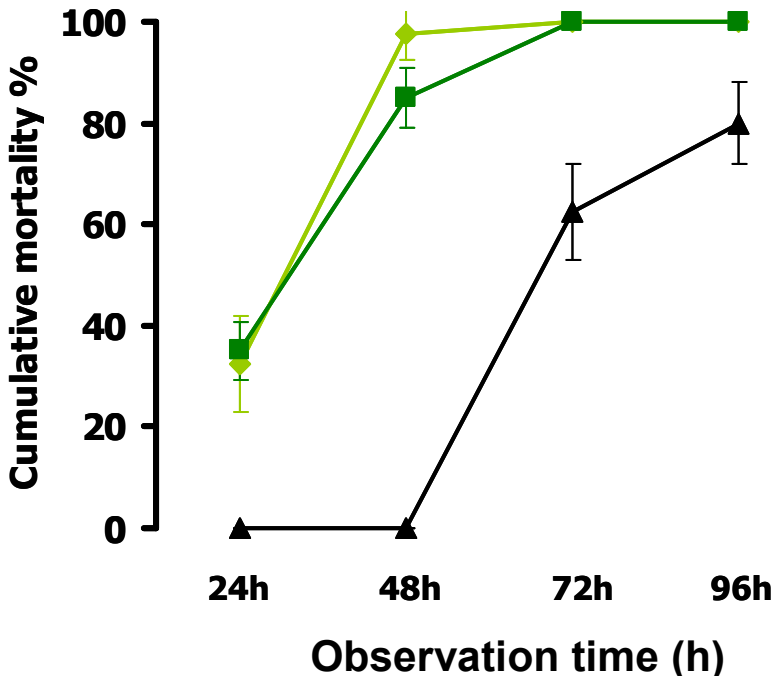

Fig. 3. 9. Cumulative mortality curves representing the response of a tolerant cloned lineage of *Daphnia longispina* (E99), exposed to pulses of ($LC_{30,24h}$), with a 24h period of recovery between pulses. In treatment B (light green) pulse exposure occurred in the first 24h. In C (dark green), two pulses were applied (0-24h and 48 to 72h). In D (black), organisms suffer a copper pulse from 48 to 72h.

Significant differences were detected between clones and observation periods ($F \geq 28.1$; $p = 10^{-3}$). Again, clone E89 exhibited a lower mortality during the recovery period (from 24 to 48h of exposure in treatment C) comparatively to cloned lineages N116 and E99 ($p = 10^{-2}$).

Discussion

The results obtained in this study showed that, in all cloned lineages that were tested, mortality continues to occur, at different intensities for each clone, even after removing the copper pulse. This latent mortality occurring during recovery periods (in the absence of the chemical pulse) has been reported by other authors with different species. For example, Zhao and Newman (2006) observed that the amphipod *Hyalella azteca* continue to die after being removed from a pulse exposure to copper. Also, Diamond *et al.* (2006) reported that 24h pulses of zinc caused continued effects in *D. magna* for several days after removing the pulse, suggesting a slow uptake and/or depuration rate for this metal.

In fact, the latent mortality, occurring during the recovery periods (in the absence of the chemical pulse), may be associated with several detoxification mechanisms that are involved in the reduction of metal uptake, increased metal exportation, and metal sequestration inside the cells (*e.g.* Dameron and Harrinson, 1998). Some hypotheses have been discussed in order to understand why organisms continue to die even after the elimination of the chemical stress (in the present study, treatments B and C). For example, if mechanisms of metal excretion acts more slowly than the rate at which metal uptake occurs, most probably the organisms will need longer recovery periods to return to the basal level and depurate the metal (Fargasová, 2001; Zhao and Newman, 2006; Hoang and Klaine, 2008). Actually, Bearr et al. (2006) reported that when the metal is accumulated faster than it is expelled, permanent effects can be are triggered in the organisms. The results obtained within this work did not allowed to identify different recovery rates for each clone, but it was possible to identify that among the four cloned lineages of *D. longispina*, only the most sensitive one to lethal levels of copper (E89) was able to recover after exposure to a pulse of 24h of this metal. Though some mortality occurred during the recovery period, this mortality was significantly lower than that occurring in the other three cloned lineages (N116, N91, and E99), therefore, it is suggested that these clones most probably exhibit differences in the referred recovery/detoxification mechanisms. However in a situation of a sequential pulse, this clone would not exhibit any advantage over the other three clones, as all organisms died after being exposed to a second pulse of copper, while this did not occurred in the tolerant cloned lineages. Actually, though the individuals of these clones continued to die during the recovery period, some of those individuals were able to cope with the second input of copper. However, mortality was always higher for individuals that already experienced a pulse of

copper (treatment C), than for those that only experienced the second pulse (treatment D), thus, denoting, their incapacity to return to their basal level within a recovery period of 72h. Furthermore, the results obtained for cloned lineage E99 showed that the duration of the recovery period influenced mortality in the second pulse of copper, since a higher mortality occur in individuals allowed to recover for only 24h than in those allowed to recover for 72h. These results are inline with other published works, Guan and Wanghe (2004) found that some trace metals could stay in *D. magna*, hold in clean water after exposure to cadmium, for several days still causing effects. Also, Diamond *et al*. (2006) observed that *D. magna* mortality decreased as copper pulses were more spaced in time. Furthermore, some other authors have suggested that mortality in the recovery period or in a posterior pulsed exposure, may probably be associated with an overloading of metalloproteins or with exceeding the critical body burden causing organisms to continue to die or to not being able to trigger a sufficient powerful response to eliminate a second dose (accumulation) (Diamond *et al*., 2006; Naddy and Klaine, 2001).

Food supplies were also taken in account as they are considered an important factor for the individual's recuperation from contamination. Under toxicant stress and producing proteins for detoxification, the nourishment is important to replace energy reserves in organisms. Mangas-Ramírez *et al*. (2004), verified that algae had an important effect on *Moina macrocopa* recovery after being exposed to sub-lethal concentrations of cadmium and parathion pulses. Although, as shown by the results above, mortality can still occur even when food was available, meaning that, though important, its role is limited. In fact, inorganic elements, like metals, can be integrated in essential metabolic processes in cells, be excreted, or stored (Klerks and Bartholomew, 1991). But if the stress source is not removed or the internal body burden is achieved, organisms began to dispend more energy reserves and toxicants can access by error to certain biomolecules and exert a toxic effect on the organism independently on the quantity of food available. For example, Mangas-Ramírez *et al*. (2004), detected that food levels only had significant effect at 25 and 50% of median lethal concentrations of the tested toxicants.

Finally, although the results here obtained showed that the most sensitive cloned lineage was able to recover after the first pulse of copper, any of the individuals from the sensitive lineage, survive to the second pulse of copper (treatments C and D, respectively), suggesting that older individuals (48h to 96h old) were more sensitive that younger ones (24h old): as a similar percentage of mortality occurred in treatment C and B. This different sensitivity between individuals with different ages was also observed for the other three

cloned lineages tested. For clones N116 and E99 younger individuals were also less sensitive to copper than juveniles 48h old. Generally, toxicity assays are based in the premise that younger organisms are more sensitive contrasting with results attained, and, previous studies have already demonstrated that higher sensitivity to toxicants in older organisms is possible. As an example, Hoang and Klaine (2006) found out that *D. magna* organisms with 4-d-old were more sensitive to a single 12-h pulse of Cu than younger organisms.

Conclusion

The results obtained in this work revealed that organisms exhibiting different lethal sensitivities to copper also exhibit different recover mechanisms to copper pulsed exposures, however a pattern of responses could not be set between sensitive and tolerant clones. Further experiments must be carried out with other sensitive cloned lineages to infer about possible consistency in mechanisms of recovery/detoxification between lineages with different sensitivities. Furthermore, the capacity to recover, of the sensitive clone, after a first pulse of copper did not conferred an advantage in a situation of a second pulse of copper since all organisms died. The results also point out that long periods within inputs of chemical contamination are essential to the resilience of populations, since the most tolerant cloned lineage here tested revealed that a shorter recovery time between pulses induced a higher mortality of individuals

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Main Conclusions

In this work no association was observed between genetically determined lethal tolerance to copper and other metals or a faster recovery rate. Nevertheless, it is interesting to notice that the most sensitive cloned lineage (E89) always exhibited a different response from the other tested clones (it was the second most sensitive clone to the four tested metals and was the one exhibiting recovery after exposure to a first pulse of copper), indicating that extreme genotypes may present specific mechanisms for the here studied lethal responses. Furthermore, though the inexistence of correlations between the studied responses, it was observed that negative correlations may exist in some clones between lethal tolerances to different metals. Thus, suggesting that, depending on the genetic composition and diversity of a population, the input of successive different chemicals may seriously compromise its resilience.

The obtained results in the third chapter indicate that current approaches used in ecological risk assessment may underestimate the toxic effects caused by exposure to contaminants. Pulsed exposures may represent true risk to aquatic biota more accurately than standard toxicity assays involving continuous exposure to a chemical concentration, since, constant concentration exposures to toxicants in the environment are unusual and they have been revealing to exert more toxic effects than constant and exposure. Furthermore, more knowledge on possible associations between sensitivity to chemicals and recovery responses should be generated in order to understand the real risks posed to populations that suffer episodic exposure to contaminants. In the present work, the studied tolerant lineages exhibit a high mortality during the recovery period, while the sensitive one showed some capacity to recover after the copper pulse. However, the tolerant cloned lineages were more able to cope with a second pulse of copper than the sensitive one, in which all organisms died. If a general association exists between tolerance and these different recovery responses, then, the survival of a population may be compromised under lethal episodic exposure scenarios of lethal levels of a contaminant.