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Adaptação de Cladóceros a ambientes heterogéneos contaminados por metais

Adaptation of Cladocerans in metal-polluted heterogeneous environments

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Barata Marti, Investigador Principal do Institute of Environmental Assessment
and Water Research Jordi Girona – Barcelona.

Esta tese é dedicada à memória do avô Américo e à memória da tia Virgínia, que muito contribuem para a minha busca diária em ser uma pessoa melhor.

o júri

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resumo

No presente trabalho o cladócero *Daphnia longispina* foi utilizado como organismo modelo para a avaliação dos efeitos ecológicos da adaptação a ambientes contaminados por metais. Foram amostradas populações naturais de *D. longispina* num local sujeito à contaminação por metais e num local próximo, de referência, ambos localizados no sistema aquático na área envolvente à mina abandonada de São Domingos. Várias linhagens clonais de ambas as populações foram mantidas em laboratório, sob condições controladas, para a execução dos testes. Um dos testes realizados permitiu estudar e quantificar as diferenças na tolerância letal entre as linhagens clonadas de ambas as populações e também avaliar os custos associados. Utilizando vinte linhagens clonais de *D. longispina* das duas populações verificou-se que apenas clones sensíveis ao cobre estavam presentes na população de referência e clones resistentes ao cobre estavam presentes na população do local contaminado. Os custos associados à tolerância foram ilustrados pela determinação de taxas alimentares mais baixas para a população tolerante quando comparadas com as da população de referência. Outro dos testes realizados permitiu comparar as respostas de clones de populações de ambos os locais – contaminado e referência – à exposição a concentrações sub-letais do metal cobre. A tolerância evidenciada anteriormente ao nível letal foi confirmada ao nível sub-letal, com o clone proveniente da população do local contaminado evidenciando uma maior tolerância ao cobre quando comparado com os restantes clones, para todos os parâmetros analisados (taxas alimentares, consumo de oxigénio, crescimento e reprodução). Os efeitos da aclimatação ao cobre ao longo de várias gerações foram também avaliados num clone de *D. longispina*. Os resultados evidenciaram a existência de uma adaptação fisiológica ao cobre ao longo das várias gerações que, no entanto, apenas aumentou marginalmente a tolerância a níveis de cobre letais. Para além disso, observou-se também uma grande variação nas respostas do clone de *D. longispina* estudado, não só entre concentrações de cobre mas também entre gerações. Os resultados obtidos nos vários estudos realizados com linhagens clonais de ambas as populações de *D. longispina* reforçam a importância de integrar a temática do desenvolvimento de tolerância à poluição aquando da avaliação dos riscos ambientais e ecológicos de compostos químicos, como os metais, no meio ambiente.

keywords

abstract

In the present study the cladoceran *Daphnia longispina* was used as a model organism to test the ecological side effects of adaptation to metal contaminated environments. *D. longispina* natural populations were sampled from a metal contaminated reservoir and from a nearby clean water reservoir, both belonging to the aquatic system surrounding the abandoned São Domingos cupric mine. Clonal lineages were established and maintained in the laboratory by means of asexual reproduction and were used for tests. The comparison of broad sense heritabilities and genetic correlations using up to twenty distinct clonal *D. longispina* lineages randomly obtained from the metal contaminated reservoir and the reference reservoir showed that only sensitive and resistant lineages to Cu were present in the reference and contaminated site, respectively. For Zn, however, both populations had a similar distribution pattern of sensitivities. Fitness costs of tolerance were illustrated by lower feeding rates of the tolerant population compared to the reference one. Another study assessing life-history responses to sublethal copper contamination in four *D. longispina* clones, two from a reference site and the other two from a historically copper-exposed site showed that tolerance manifested by *D. longispina* clones at lethal copper levels was also evident at sublethal concentrations, with the tolerant clone from impacted population showing higher tolerance to copper for all the parameters (feeding, oxygen consumption, growth and reproduction) compared to the rest of clones. The multigenerational effects of acclimation to copper were also evaluated by exposing a single clone of *D. longispina* originated from the reference population to copper over three consecutive generations. Results from the evaluation of its life-history performance illustrate that physiological adaptation to copper across several generations only increased marginally acute tolerance to copper. Besides that, a high variation in life-history traits was observed not only between copper treatments, but also among generations. For instance, generation had a significantly influence on the observed pattern of age at first reproduction and interact with copper in the observed variation of time and clutch size at first reproduction. Overall, the importance of studying long-term adaptation to metals in natural populations is highlighted in this study as the acquisition of genetically inherited tolerance could have associated ecological costs. The obtained results reinforce the need to integrate these issues when assessing risks posed by chemicals to the environment.

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Glossary

- **AMD** – Acid mine drainage
- **ASTM** – American Standard of Testing and Materials
- **CCME** – Council of Ministers of the Environment
- **CI** – Confidence interval
- **CRM** – Certified reference material
- **CSST** – Contaminated Sediment Standing Team
- **DOC** – Dissolved organic carbon
- **ICP-MS** – Inductively Coupled Plasma Mass Spectrometry
- **ITS-RFLP** – internal transcribed spacer (ITS) - restriction fragment length polymorphism (RFLP)
- **LC$_{50}$** – A statistically or graphically estimated concentration that is expected to be lethal to 50 % of a group of organisms under specified conditions.
- **LOI** – Loss on ignition
- **MAV** – Maximum admissible value
- **OCEE** – Optimal Concentration Range for Essential Elements
- **OECD** – Organization for Economic Co-operation and Development
- **PEC** (Consensus-based PEC) – consensus-based probable effect concentration according to Contaminated Sediment Standing Team
- **ROS** – Reactive oxygen species
- **SD** – Standard deviation
- **SE** – Standard error
- **SPM** – Suspended particulate matter
- **SQGV** – Sediment quality guideline value
- **USEPA** – United States Environmental Protection Agency
- **WQGV** – Water quality guideline value

CHAPTER 1

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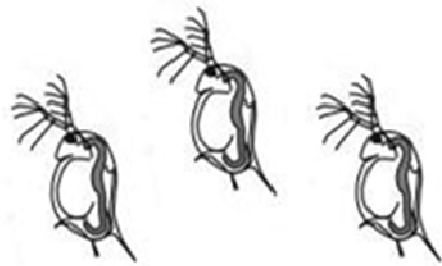
General Introduction and Conceptual Framework

Chapter

1

General Introduction and Conceptual Framework of the Study

1.1. Preamble

In the last few decades the scientific community and regulatory agencies have become increasingly aware of the long-term impacts of an ever increasing human population and its activities on climate, sustainability of ecosystems and biodiversity (Figure 1.1). These concerns have been amplified by successive reports on the depletion of the ozone layer, global climatic change, water and soil resources contamination and extinction of species. Regarding biodiversity, its maintenance is important for multiple reasons, including the formation of soils, the production of food, the purification of water, the breakdown or decomposition of waste products, the maintenance of the composition of gases in the atmosphere and other important functions that contribute to the fundamental stability of ecosystems (Spray and McGlothlin, 2003; Vituosek *et al.*, 1997). Recently the problem of biodiversity loss gained special attention (Downes, 1995; Spray and McGlothlin, 2003; Vituosek *et al.*, 1997). The Convention on Biodiversity was one of the international agreements on sustainable development at the Earth Summit, UN Conference on Environment and Development, Rio de Janeiro June 1992. It was signed by over 170 countries and it was implemented at the end of 1993 (Downes, 1995). In the Rio Convention, biodiversity was defined as "the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes in which they are part; this includes diversity within species, between species and of ecosystems" (Downes, 1995). So, three levels of

biological variability are associated with the definition of biodiversity, the genetic variation within species, species richness and ecosystems'.
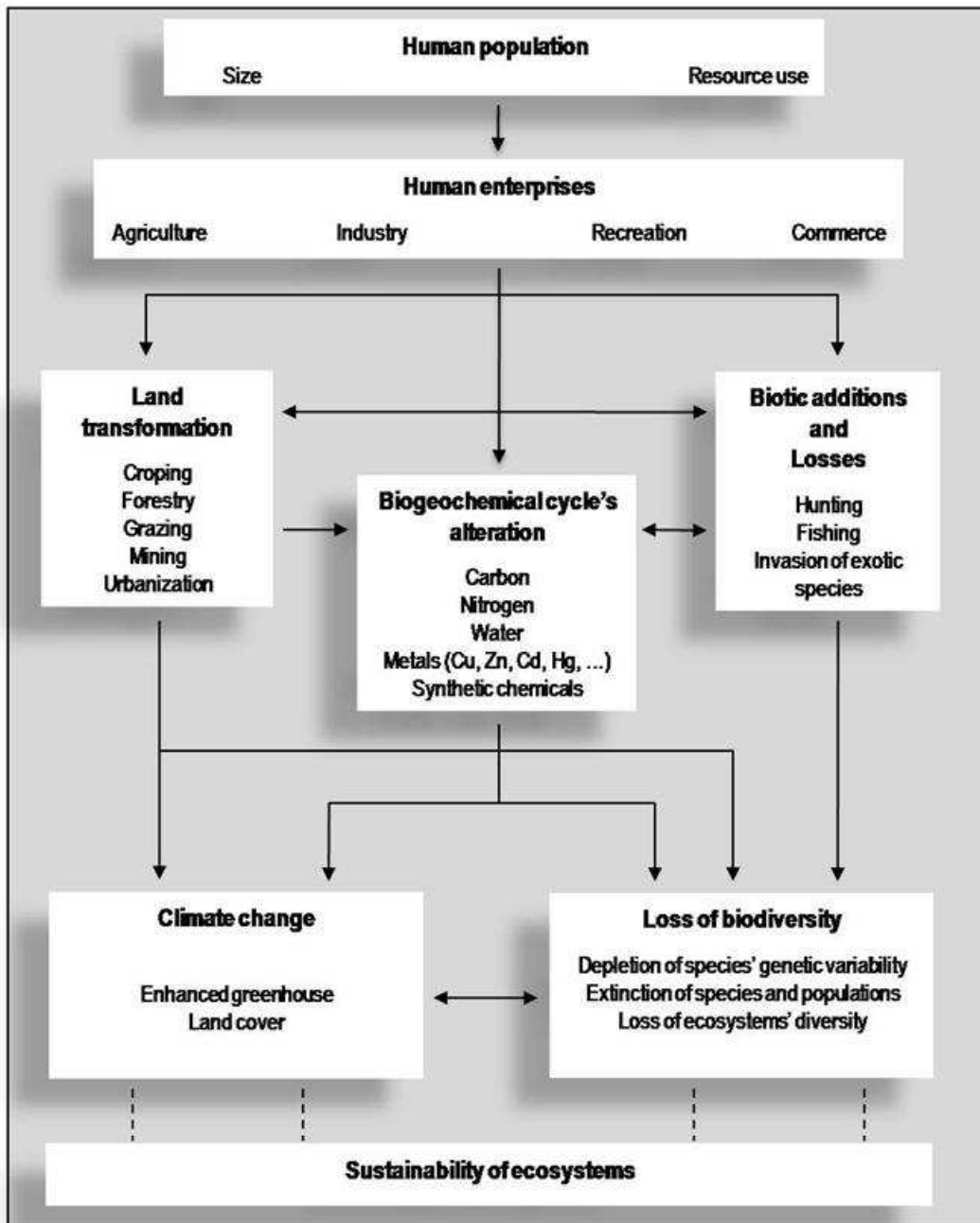
Figure 1.1. A conceptual model illustrating both direct and indirect effects of human activities' on the sustainability of ecosystems (Adapted from: Vitousek *et al*., 1997).

All the aforementioned levels are being threatened by human activities, in particular chemical contamination (Bickman *et al.*, 2000; Downes, 1995; Spray and McGlothlin, 2003; Van Straalen, 1999). Disruption of equilibrium at any of these levels has a direct relation to the decline of diversity, subsequent enhancement of vulnerability to environmental stress and extinction of species.

Most of the biodiversity debate that followed the Convention centered on ecosystems and species whereas genetic variability did not receive a similar attention (Bickman *et al.*, 2000; Van Straalen, 1999; Van Straalen and Timmermans, 2002). In fact, genetic variability in natural populations has been studied in other contexts like evolutionary and conservation biology but in the field of ecotoxicology there are only few studies that have addressed the effects of chemical contamination on population genetics (Bickman *et al.*, 2000; Hoffmann and Parsons, 1991; Medina *et al.*, 2007; Van Straalen and Timmermans, 2002). The emphasis of ecotoxicological research has remained on the species level and genetic variation has been studied mostly because it is a factor introducing variability on test results (Baird *et al.*, 1991; Barata and Baird, 1999). Standardization of species and test conditions are key factors in ecotoxicology as opposed to the variability evoked by the biodiversity discussion (Van Straalen, 1999). Only recently have ecotoxicologists started to explore the ecological consequences of genetic changes due to chemical pollution and its place in the Ecological Risk Assessment (Anderson *et al.* 1994; Belfiore and Anderson, 1998; Evendsen and Depledge, 1997; Fox, 1995; Medina *et al.*, 2007; Van Straalen, 1999).

It was shown that heterogeneous environments involving chemical stresses exert strong evolutionary pressures (Fox, 1995; Hansen and Johnson, 1999). Besides acting as selective or evolutionary forces, chemicals can also act as agents capable of disrupting chemically mediated communication and information exchange essential for effective behaviour, reproduction, development and maintenance (Fox, 1995; Hansen and Johnson, 1999). This work is a contribution to the increase of knowledge in the ecological consequences of adaptation to contaminated environments. Although survival of organisms in this type of environments is generally viewed as a positive ecological event, changes in genetic diversity or/and negative effects of adaptation can pose an effective threat to the communities and ecosystems.

1.2. Responses of populations to stressful environmental conditions

Microevolution is the term generally used to refer to changes that take place within species and populations within the lifespan of a human being. Microevolutionary changes can occur within introduced species to novel environments, in native species interacting with alien species or in species interacting with stressful environmental conditions (Ashley *et al*., 2003; Hoffmann and Parsons, 1991).

Stressful environmental conditions are those that limit organisms responses, related to fitness. Stressful responses are influenced by the organism's molecular and cellular makeup and by its physiological condition, contributing to the structures and processes characteristic of populations, communities and ecosystems (Forbes and Depledge, 1996; Hoffmann and Parsons, 1991; Koehn and Bayne, 1989; Sibly and Calow, 1989). All definitions of stress in an evolutionary context emphasize the reduction in fitness due to detrimental effects on survival and reproduction thereby endangering the existence of organisms and populations. According to Bijlsma and Loeschcke (2005), abiotic and biotic factors are responsible for the causation of stressful environmental conditions. Abiotic factors include fluctuations in environmental variables such as temperature, food and chemical components (naturally occurring or man-made). Biotic factors include competition, predation, parasitism and illnesses. Both abiotic and biotic stresses can act independently but in most cases there is interaction since organisms that have suboptimal stress due to abiotic factors are more susceptible to predators, competitors or parasites and the same for the other way around. So, stresses may have abiotic and biotic components, which in turn may be acute, chronic and/or seasonal and can affect organisms at the community, population and/or individual level (Bijlsma and Loeschcke, 2005; Parsons, 1996; Scott, 1995). Understanding the nature, interactions and consequences of these types of stresses from an ecological and evolutionary point of view is of extremely importance as the growing human population causes major changes in the biotic and abiotic environment (Bijlsma and Loeschcke, 2005).

One example of a stress is chemical pollution arising from human activities. Anthropogenic chemicals can act as selective agents altering gene frequencies, thus causing genetic divergence among and within populations and imposing directional selection (Hoffmann and Parsons, 1991; Lagisz and Laskowki, 2008). This process is called microevolution due to pollution (Klerks and Levinton, 1989; Medina *et al.*, 2007). Several works have highlighted the significance of the evolutionary consequences of population exposure to toxicants (Barata *et al.*, 2002; Depledge, 1994; Morgan *et al*., 2007). Populations can exhibit different levels of responses when challenged by a chemical stressor: i) They do not respond because they include organisms either resistant to the stressor or exposure is very brief or minimal; ii) They avoid stress due to the migration of individuals; iii) Alternatively, populations can be sensitive to pollution when exposure is too strong resulting in either local extinction or adaptation. The former can occur either accommodating the stress by behavioral or physiological adaptation or by selecting genotypes within the population resulting in progressive elimination of sensitive individuals and a shift in the population genetic structure (Fox, 1995; Hoffmann and Hercus, 2000).

Adaptation corresponds to a process of changes in an organism to conform better to (new) environmental conditions, whereby the organism (or group of organisms) acquires characteristics (morphological, physiological or behavioural) that improve its survival and reproductive success in the particular environment (Bijlsma and Loeschcke, 2005; Hoffman and Parsons, 1991; Scott, 1995). Those changes can occur phenotypically (phenotypic adaptation) or genetically (genotypic adaptation). Phenotypic adaptation corresponds to changes within a genotype. In this case adaptation is the result of "phenotypic plasticity", the capability of an organism to express different phenotypes depending on the biotic or abiotic environment (Agrawal, 2001; Hoffman and Parsons, 1991). An example of this type of adaptation is the development of morphological changes in *Daphnia* in response to the presence of predators (Agrawal, 2001; Boersma *et al*., 1998; Tollrian and Dodson, 1999). Genetic adaptation corresponds to changes in the allele frequencies due to genetic drift or mutation and of the genetic material by amplification, alteration of its expression or by structural changes (Scott, 1995). It is an evolutionary mechanism which acts over several generations, by which genotypes with better constitutive or plastic responses toward

adverse environmental conditions have a higher fitness and hence increase their abundance in the population. To distinguish genetic adaptation from both physiological acclimation and maternal effects requires establishing that the characteristics involved in the divergence between populations are heritable from parents to offspring across generations (Klerks and Levinton, 1989; Lagisz and Laskowki, 2008). All adaptation processes are important from an evolutionary point of view since even the occurrence of physiological plasticity is genetically based. The possible occurrence of evolutionary adaptation depends on the standing genetic variation within populations (Bijlsma and Loeschcke, 2005). In fact, adaptation is the slowest form of response to an environmental stress, mainly because there is often insufficient variability for adaptive changes to occur and, when there is enough genetic variability, it may not be used (Hoffman and Hercus, 2000). The extent to which organisms can adapt can be limited not only by the availability of heritable variation but also by trade-offs between environments or traits (Hoffmann *et al.*, 1995).

1.3. The central role of genetic diversity

Genetic diversity of populations allows their adaptation to unpredictable environments and, by this way, is the basis for the stability of any ecosystem (Fox, 1995; Parsons, 2005). To maintain genetic diversity it is necessary to ensure the protection of both individuals (genetic variation) and processes of sex (mixis) and reproduction (Fox, 1995). Belfiore and Anderson (1998) define genetic variation as the level of genetic polymorphism ("variability") and the patterns of genotype frequencies that result from natural population processes and natural spatial and temporal fluctuations in environmental conditions. Natural genetic variation is distinguished, by the same authors, from genetic change, defined as significant alterations to genetic patterns due to the acute or chronic imposition of anthropogenic or exogenous forces. With the aim of simplification, hereinafter the term genetic variation will be used, either for natural genetic variation or for contaminant-induced genetic variation (according to Evendsen and Depledge, 1997). Heritable differences between individuals are due to

differences in their DNA. These differences are often expressed at the molecular, biochemical, physiological, morphological and life history levels and so genetic variation can be studied at these levels as well (Van Straalen and Timmermans, 2002).

According to Van Straalen and Timmermans (2002), there are four different ways in which pollutants can affect genetic variation (neutral and selectable genetic markers): by increasing mutation rates; by directional selection on tolerant genotypes; by causing bottleneck events; or by altering immigration (Figure 1.2). Selectable genetic markers are considered those traits that pose a fitness advantage or disadvantage in an environment where a specific toxicant is present. Such markers are directly responsive to the selective regime of the toxicant. Obvious examples are traits that are closely related to the mode of action of the toxicant, or to protective biochemical mechanisms, such as cholinesterases, cytochrome P450s and metallothioneins. Neutral markers are all the traits that are indifferent to the selection pressure of a specific chemical. Mutation and factors related to population size (genetic drift, gene flow) will affect both neutral and selectable markers whereas selection acts only on selectable markers. Neutral markers may still respond to selection if they are linked to selectable markers (Van Straalen, 1999; Van Straalen and Timmermans, 2002).

A reduction in the overall genetic variation of a population can occur through genetic drift and bottleneck events (which in turn increase inbreeding) after drastic reduction in population size and/or through directional selection (changes in the genetic composition of the population towards a higher mean tolerance) (Bickham *et al.,* 2000). As an example, Ward and Robinson (2005) found that cadmium selection caused a significant reduction of the genetic diversity of a combination of 8 laboratory cultures of *Daphnia magna* and an increase in average cadmium tolerance from 50 to 200 $\mu g\ L^{-1}$. Alternatively, an increase in the population's genetic variation can occur either by mutation, immigration and/or variable selection (Belfiore and Anderson, 1998; Van Straalen, 1999; Van Straalen and Timmermans, 2002).
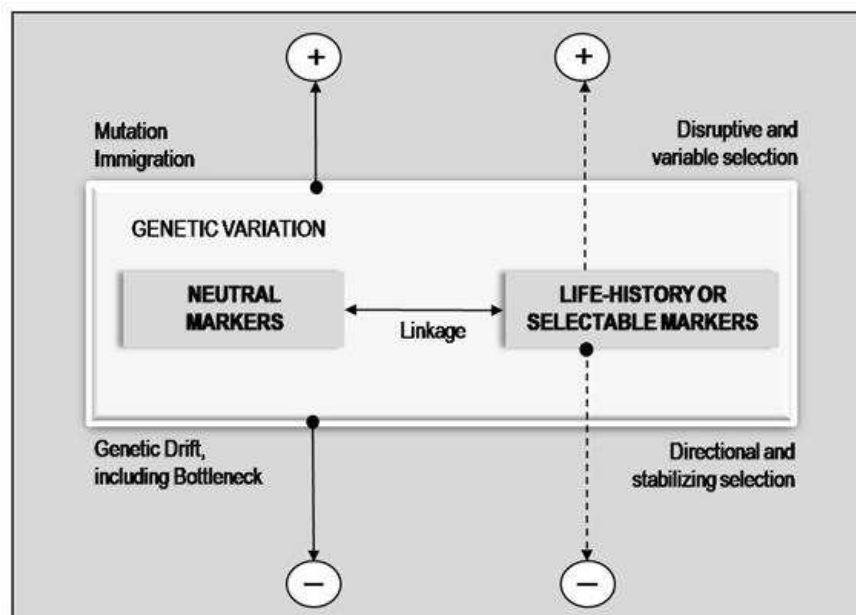
Figure 1.2. A conceptual framework for the effects of pollutants on genetic variation in natural populations. On both neutral and selectable markers, factors operate in a way that may increase (+) or decrease (-) genetic variation (Adapted from: Van Straalen and Timmermans, 2002).

Not always pollutants are responsible for changes in genetic variation in the impacted populations. For instance, Martins *et al.* (2009) found no reduction in genetic diversity in *Daphnia longispina* populations from a metal historically impacted site. As pointed out by Brendonck and De Meester (2003), immigration from nearby reference populations and/or the diversity in the subjacent ephippial gene bank can be high enough for the recovery of the diversity. Actually, in *Daphnia* and other cyclical parthenogenic species, the ephippial egg-bank is the main genetic diversity reservoir and can maintain the genetic diversity of a population over a broad range of conditions, buffering the effects of seasonal, environmental, competition or contaminant-induced local extinction of genotypes (Brendonck and De Meester, 2003). Other factors can be at the basis of unchanged genetic diversity. For instance, selection can occur at a limited number of genes and the reduction of the diversity in these genes can be not enough for a reduction in overall diversity (Cousyn *et al.*, 2001).

Changes in genetic variation may be related with changes in the number and frequency of alleles, thus with the level of heterozygosity (Fox, 1995). Heterozygosity

levels in natural populations tend to be positively correlated with fitness, especially for enzyme *loci* influencing metabolism and contributing to the amount of energy available for development and growth (Fox, 1995; Parsons, 2005). An example of this correlation is given by Koehn and Bayne (1989) that found that heterozygote mussels had lower energy requirements than homozygote ones, apparently because of greater efficiency in protein synthesis, which is energetically costly. In fact heterozygotes tend to have lower energy requirements than homozygotes (especially under extreme conditions) and thus they should have the potential to develop and reproduce under a wider range of environmental conditions (Fox, 1995; Kashian *et al.*, 2007; Parsons, 2005). Given the great variety of chemicals introduced into the environment, the risk of genetic erosion – the loss of genetic variation in a population due to directional selection, drift or inbreeding – is seen as a real risk, even with the compensatory mechanisms such as increased mutation rates (Van Straalen, 1999). In addition to the general loss of genetic diversity, chemical stress can elicit other changes in the population, changes that are correlated with resistance through pleiotropy or genetic linkage. The degree to which selection processes lead to negative-side effects is, however, unclear (Van Straalen, 1999).

1.4. Ecological consequences of genetic change. Costs of adaptation

Animals exposed to chemical stressors must mobilize defensive and repair processes if they are to survive. These processes are energy-demanding and can have negative fitness consequences (Morgan *et al.*, 2007). Theories on adaptive change predict that the development of resistance to chemical stress usually involves a cost; resistant genotypes would be at disadvantage in the absence of stress, being selected against (Harper *et al.*, 1997; Van Straalen and Hoffmann, 2000). Two hypotheses describe how a cost of metal resistance might be manifested: (a) the trade-off hypothesis – adaptive stress-responsive traits at the expense of resource expenditures on growth and reproduction reduces individual fitness in unpolluted environments; and (b) the metal requirement hypothesis – resistance based on less efficient metal bioavailability

can evoke micronutrient deficiency syndromes in nutrient-poor polluted environments (Harper *et al.*, 1997; Klerks and Levinton, 1993; Van Straalen and Hoffmann, 2000).

Several studies have shown that fitness costs of resistant genotypes are usually associated with altered physiological processes that enable resistant individuals to cope with toxic stress rather than due to increasing energy demands of detoxification. Thus the classical idea of trade-offs based on energy allocation is not well supported (Shirley and Sibly, 1999; Sibly and Calow, 1989; Van Straalen and Timmermans, 2002); trade-offs associated with resistance are more often due to negative pleiotropy or genetic linkage (Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002). The existence of costs associated with adaptation to pollution becomes important as these could affect the performance of the adapted population during the polluting event or could be observed as between-environment genetic trade-offs in unpolluted or recovered sites (i.e. cleaned). These costs will eventually change the physiology of organisms, thus the way how populations exploit their ecological niches and participate in the overall ecosystem functioning (Medina *et al.,* 2007). As an example, Semlitsch *et al*. (2000) were able to demonstrate that there was a fitness trade-off associated with chemical tolerance in gray tree frog (*Hyla versicolor*) tadpoles. Tadpole survival, in the absence of a chemical stressor, was negatively correlated with tolerance to the insecticide carbaryl. More complex scenarios involve ecosystem degradation leading to reductions in carrying capacity, thus resulting in smaller populations (Matson *et al*., 2006). For some populations, however, the cost of tolerance is negligible. Barata *et al*. (2002) studying up to 80 clones from different populations showed that resistance to cadmium was not costly in terms of fitness. Polluted habitats may comprise a spatially heterogeneous mosaic of contaminated and uncontaminated territories favouring highly plastic genotypes rather than adapted resistant genotypes. Plastic genotypes rather than highly tolerant ones to a specific environment may be favoured in habitats where the intensity of stress is temporally variable (Morgan *et al*., 2007).

A general feature of stressed systems is the increase in energy expenditure – energy balance shifts from maintenance and production to repair and recovery (Parsons, 2005). Fitness is defined as the distance of a given individual's thermodynamic parameters from its optimal value and can be assessed at the organismic level for a range of traits (fecundity, survival, longevity). Organisms tend to become increasingly

adapted to the resource status of their habitats and the correspondingly energetic efficiency of organisms in their habitats translates into an increase of fitness. In this way, a measure of fitness in a stressed world is energy efficiency (Parsons, 1996, 2005).

In a highly heterozygous population, there are likely to be certain genotypes that are more sensitive than others. This is especially true if the population is heterozygous at loci that are both critical to fitness and susceptible to toxicant-induced structural alterations (Anderson *et al.*, 1994; Belfiore and Anderson, 1998; Van Straalen and Timmermans, 2002). Exposure of a population to a chemical stress may lead to genetic selection of segments of the population capable of stress avoidance, stress detoxification or repair or compensation for injury. As selection pressure increases through repeated exposure, an increasing proportion of the population may become resistant to the chemical. The speed at which this process occurs depends upon the chemical and its distribution, the genetic makeup of organisms and the extent to which the entire population is exposed to the selection pressure (Fox, 1995). The result is a reduction in the total genetic variation within the population or a shift in genotypic frequencies (Anderson *et al.*, 1994; Belfiore and Anderson, 1998; Van Straalen and Timmermans, 2002). In addition to the general loss of genetic variability, there may also be changes correlated with resistance through pleiotropy or genetic linkage (Van Straalen and Timmermans, 2002).

The general loss of genetic variability can lead to: (i) inbreeding depression – a large population is reduced to a small size and individuals are forced to mate with close relatives and often experience reduced fecundity/viability of offspring; (ii) loss of evolutionary adaptability to novel environmental stresses due to reduced genetic variation in a small population; and (iii) loss of heterozygosity (Evendsen and Depledge, 1997; Fox, 1995; Van Straalen and Timmermans, 2002). If in a resistant but genetically homozygous population individuals with certain resistant genotypes have inferior growth rates or fecundity, or increased mortality rates, then this contaminant-induced-selection may create a population with decreased survival potential, hence susceptible to extinction (Fox, 1995). Besides the ecological impacts, pollutant-induced selection of resistant genotypes has also ecotoxicological implications as the levels of phenotypic heterogeneity in natural populations are often absent from populations of organisms of the laboratory. So, the risk of overestimating or underestimating the

susceptibility of the organisms exposed to the chemical under study is high (Evendsen and Depledge, 1997).

1.5. Adaptation to metals in organisms

Pollution of aquatic and terrestrial systems with metals is a problem affecting many areas worldwide. Metals are non degradable pollutants and tend to accumulate in water sediments and soil where they can persist for many years. Metal pollution has been responsible for the extinction of several species, whereas others became adapted, surviving and reproducing in those metal polluted environments (Groenendijk *et al.*, 2002; Postma *et al.*, 1995). Metal contamination represents a strong and stable directional selection pressure making metal adaptation often a very quick process (Posthuma and Van Straalen, 1993; Posthuma *et al.*, 1993). The actual level of metal adaptation is the residual effect of the dynamic interaction between the selective pressure of metal pollution and gene flow. For instance, if directional selection pressure in the metal-polluted zone is heavy the adapted population may be genetically differentiated from a population of non-adapted con-specifics even if there is no geographical barrier preventing gene-flow between them (Morgan *et al*., 2007). Alternatively, the adapted population may be genetically and phenotypically heterogeneous containing both tolerant and relatively sensitive genotypes if the selection pressure in the metal-polluted zone is low or moderate. The latter scenario, providing evidence of genetic erosion, was observed by Lopes *et al.* (2004) in field populations of *Daphnia longispina*, where an impacted population did not contain the most copper-sensitive lineages but the reference one did contain the most copper-tolerant lineages. Regarding gene flow, it can be responsible for creating unpredictable levels of adaptation. It can reduce the speed of adaptation to metals by the introduction of non-adapted organisms or, instead, it can increase the speed of adaptation by the introduction of essential new genes for the increase of tolerance (Groenendijk *et al.*, 2002). The presence of a population of reproducing individuals of a given species at a chronically polluted metal site does not justify the immediate inference that it is

adapted. Lagisz *et al.* (2005), for example, were unable to find any differences in metal uptake and excretion rates, or in respiratory rates, in F1-generation carabid beetles (*Pterostichus oblongopunctatus*) bred from parents originating from clean and metal-contaminated sites, respectively. One of the possible explanations is the fact that its individual members possess enough phenotypic plasticity to enable them to make the necessary biochemical or physiological adjustments within their individual life-histories to ensure survival at least until the successful completion of a minimum of one reproductive cycle. Alternatively, the population living at the chronically polluted metal site may be sustained by continuous recruitment of reproductively active, nonadapted, individuals from the adjacent "non-impacted" surroundings. Immigration of non-tolerant midges from sites located upstream, coupled with site differences in metal distribution, has been offered as an explanation for differential life-history patterns of chironomid populations living at cadmium-contaminated sites (Postma *et al.*, 1995b). The occurrence of gene flow was also the justification given in the work of Bengtsson *et. al.* (1992) for earthworm colonization of a copper-polluted site.

Genetic adaptation is usually demonstrated by comparing the response of lab-reared F1 generations of the assumed adapted and reference populations. Development of tolerance to metals through (genetic) adaptation has been documented for a diversity of taxa, from bacterial assemblages (Lehman *et al.*, 1999) to plants (Harper *et al.*, 1997; Jiménez-Ambriz *et al.*, 2007; Schat and Vooijs, 1997) and from terrestrial animals (Donker and Bogert, 1991; Posthuma *et al.*, 1992; Posthuma *et al.*, 1993; Timmermans *et al.*, 2005) to aquatic ones (Lopes *et al.*, 2004; Martinez and Levinton, 1996; Vidal and Horne, 2003; Wallace *et al.*, 1998; Ward and Robinson, 2005). Posthuma and Van Straalen (1993), Klerks and Weis (1987) and Medina *et al.* (2006) have provided reviews about terrestrial and aquatic studies, respectively, of tolerance to heavy metals in invertebrate species. A summary of the studies published since these reviews is listed in Table 1.1.

Table 1.1. Invertebrate species exhibiting genetically based heavy metal tolerance in laboratory-reared offspring from field populations

Group	Species	Metal	Evidence	Reference
Isopoda	*Porcellio scaber*	Cd	Sustained growth of F1 under high Cd exposure; decreased growth in the absence of Cd	Donker and Bogert (1991)
		Cu, Zn	Altered life histories, sustained growth under Cu/Zn exposure, early reproduction at a smaller size, elevated Zn excretion	Donker *et al.* (1993) Donker *et al.* (1996)
Oligochaeta	*Limnodrilus hoffmeisterii*	Cd, Co, Ni	Sustained growth of F1 population exposed to polluted sediment	Klerks and Levinton (1989) Martinez and Levinton (1996)
		Cd	Enhanced ability of population from a long-term polluted site to sequester Cd in insoluble intracellular, metal-rich, granules	Wallace *et al.* (1998)
	Tubifex tubifex	Hg	Increased survival in the presence of high Hg concentrations relative to reference population	Vidal and Horne (2003)
Collembola	*Isotoma notabilis*	Cu, Zn	Growth and reproduction of F1 populations from contaminated sites less affected by metals	Tranvik *et al.* (1993)
	Orchesella cincta	Cd	Sustained growth and survival in F1 population exposed to Cd, elevated Cd excretion through intestinal exfoliation	Posthuma *et al.* (1992) Posthuma *et al.* (1993) Timmermans *et al.* (2005) Roeloffs *et al.* (2009)
Gastropoda	*Biomphalaria glabrata*	Cd	Cd tolerance is reflected as a cost to parasite infection	Salice and Roesijadi (2002)
Diptera	*Drosophila melanogaster*	Cu, Cd	Duplication of Mtn gene confers resistance	Maroni *et al.* (1987) Maroni *et al.* (1995)
	Chironomus riparius	Cd	Altered life histories, increased Cd excretion, sustained growth under Cd exposure, Zn deficiency of F1 populations from impacted site	Postma *et al.* (1995a) Postma *et al.* (1996) Groenendijk *et al.* (1999) Groenendijk *et al.* (2002)
Cladocera	*Daphnia longispina*	Cu	Increased survival in the presence of high Cu concentrations relative to reference population	Lopes *et al.* (2004)
	Daphnia magna	Cd	Selection for Cd resistance resulted in a modification of the genetic struture of the population	Ward and Robinson (2005)
Bryozoa	*Bugula neritina*	Cu	Increased growth in the presence of high Cu concentrations and decreased growth in the absence of Cu, relative to reference population	Piola and Johnston (2006)

Tolerance of organisms to metals can be mediated by either physiological mechanisms of decreased response (target insensitivity to the toxicant) or mechanisms of decreased exposure (avoidance, reduced uptake, reduced body metal burdens, increased sequestration in non-bioreactive states) (Belfiore and Anderson, 1998; Klerks and Weis 1987; Morgan *et al*., 2007, Posthuma and Van Straalen, 1993; Taylor and Feyereisen, 1996). The mechanism of reducing body metal burdens through up-regulating excretory mechanisms is well-known in invertebrates (Callaghan and Denny, 2002; Groenendijk *et al*., 1999; Morgan *et al*., 2007; Posthuma *et al*., 1992). As an example, in the springtail *Orchesella cincta* the excretion of metals through exfoliation of the midgut epithelium at every moult is an important component of cadmium tolerance and adaptation (Posthuma *et al.*, 1993). However, increased sequestration in non-bioreactive states may be the most common strategy (Morgan *et al*., 2007). The distribution of metals over the various intracellular binding sites is determined: (1) by the metal affinity of the ligands; (2) by the number of binding sites; and (3) by the presence of competing metals (Posthuma and Van Straalen, 1993). Membrane-enclosed granules are one of the components capable of metal sequestration. It is known that worms of the oligochaete *Limnodrilus hoffmeisteri* originated from a long-term polluted site sequester cadmium in insoluble intracellular, metal-rich, granules (Wallace *et al.,* 1998); in contrast, worms from a clean reference site appeared to detoxify cadmium mainly by inducing metallothioneins. According to the study of Cain *et al.* (2006), the sequestration of cadmium in the caddisfly *Hydropsyche californica* occurs by metallothionein- like proteins in metal-exposed individuals compared with con-specifics from a clean site. The role of granules cannot be separated from the function of metallothioneins-like proteins. Metallothioneins are small proteins that aid in the metal homeostasis and detoxification. In addition they contribute to control of the cellular redox status (Viarengo *et al*., 2000). The diversity of metallothioneins systems in invertebrates is considerable, as is their metal-specificity. Often several isoforms are present which differ in their binding affinity to camium or copper and some of them are specifically induced only by cadmium, not by copper or zinc. Within the genus *Daphnia* a study from Shaw *et al.* (2007) revealed three metallothioneins genes in *Daphnia pulex* while a toxicogenomics study identified two metallothioneins in *Daphnia magna*, both inducible by cadmium and copper but not by zinc (Poynton *et al*., 2007).

1.5.1. The case of essential metals. Copper and Zinc

Essential metals are those required at structural and catalytic sites in proteins (e.g., iron, zinc, copper). Essential metals are an interesting subject of research as they are needed for normal metabolic function and, because of that, organisms have specialized cellular uptake carriers. For each essential metal, species have an optimal concentration range termed OCEE ("Optimal Concentration Range for Essential Elements"). The OCEE is determined by the natural bioavailable concentration of the essential metal in the species' natural habitat and the species' homeostatic capacity, which allows it to regulate the internal metal concentration (Muyssen *et al*., 2002). Although required for life, essential metals can be toxic if intracellular concentrations exceed the organism's requirements and its detoxification capability (Correia *et al.*, 2002).

Copper is the most commonly used metal for industrial purposes. Because of the widespread use of copper, various sources including industrial and domestic wastes, agricultural practices, copper mine drainage, copper-based pesticides and antifouling paints have contributed to a progressive increase in copper concentrations in aquatic and terrestrial environments (Akhtar *et al.*, 2005; Perales-Vega *et al.*, 2007; Suedel *et al.*, 1996). In organisms, copper is a micronutrient essential for respiration, growth and development (Long *et al.*, 2004). The Cu^+ and Cu^{2+} oxidation states permit this metal to participate in a variety of electron-transfer reactions, especially associated with oxidative enzymes and energy capture. Examples of copper proteins are haemocyanin, superoxide dismutase and cytochrome oxidase (Da Silva and Williams, 1993; Linder, 1991). However, copper ions can interact with oxygen and thus create highly destructive reactive oxygen species (ROS), such as superoxide (O^{2-}) and hydroxyl radical (OH), that damage biological macromolecules, including proteins, lipids and DNA.

Besides the production of ROS, copper may exert its toxicity by binding to various biological molecules, which impedes their functions, through a mechanism described by the biotic ligand model. Known ligands for copper include ion pumps, photosystem II, and gill membrane proteins (Akhtar *et al.*, 2005; Correia *et al.*, 2002). In aquatic systems copper bioavailability and speciation are dependent on the type and

concentration of organic and inorganic ligands present, the pH, hardness and dissolved organic carbon. In this way, numerous species of copper (free cupric ion, copper hydroxides, carbonate complexes, organic complexes and others) coexist simultaneously in the same system. Additionally, the sensitivity of indigenous species, organism age, temperature, alkalinity, and dissolved concentrations each contributes to the site-specific influences on copper toxicity to aquatic organisms (Long *et al.*, 2004; Meador, 1991; Suedel *et al.*, 1996).

Zinc, along with copper, is extremely used in human activities. Waste incineration, smelting, fossil fuel consumption, cement production and transportation-related activities constitute possible sources of this metal to both terrestrial and aquatic environments. In organisms, zinc is a micronutrient required as a cofactor for many enzymes such as Cu,Zn-superoxide dismutase, carbonic anhydrase, carboxypeptidase and several hydrogenases (Caffrey and Keating, 1997; Malik *et al.*, 1998; Vesela and Vijverberg, 2007). Zinc is also used in the body as a catalyst in metal biomolecules bound to amino acid side chains to form tetrahedral zinc metalloproteins and metal enzymes so it develops an important role in the stabilization of membranes and ribosomes (Malik *et al.*, 1998; Muyssen and Janssen, 2002, 2005).

1.6. An introduction to the studied model system

The freshwater area surrounding São Domingos mine was selected as a model system for the development of this PhD study.

1.6.1. General characteristics of São Domingos Mine

São Domingos (37° 38' 00'' N to 37° 40' 30'' N and 7° 19' 05 W to 7° 20' 05'' W) is a cupriferous pyrite mine located near the village with the same name, 17 km NE from the town of Mértola within Baixo Alentejo province, south of Portugal. Part of its area is integrated in the Guadiana Valley Natural Park, which is classified as a protected reserve since 1995 and nowadays covers a surface of nearly 80,000 ha (ICBN, 2008)

(Figure 1.3). With respect of geology, the area is included in the Iberian Pyrite Belt, one of the most important metallogenetic provinces of massive sulphides of the world (Barriga and Carvalho, 1983; Matos and Martins, 2006).

The modern exploitation in São Domingos mine started in the XIX century, with the extraction of Cu, Zn and Pb from the massive sulphide deposits. Pyrite ($FeS2$) was the sulphide mineral dominant, with chalcopyrite ($CuFeS2$), sphalerite (ZnS) and galena (PbS) being the most common accompanying sulphides (Álvarez-Valero et al., 2007; Pérez-López et al., 2008; Sáez et al., 1999). Associated with the mining works several facilities were built including water reservoirs, cementation tanks, a sulphur factory, a system of channels and ponds for acid water evaporation and a railway and harbour for ore transportation. The mine exploitation ended in 1966 leaving a legacy of attendant spoil tips, including waste rock piles, mine tailings and an unprotected open pit, which is flooded since then (De Vos et al., 2005; Matos and Martins, 2006; Quental et al., 2002) (Figure 1.4). Because iron sulphide, always as pyrite, was an ubiquitous component of all massive sulphide deposits, and because such pyrite was usually not recovered during mineral processing, this sulphide generally comprises the most environmentally-significant constituent of waste rock piles (Matos and Martins, 2006; Pérez-López et al., 2008; Quental et al., 2002). The mine tailings and sulphide-bearing waste rock piles left untreated are sources of environmental pollution, mostly by oxidation of pyrite and other sulphide minerals in a weathering environment, which gives rise to acidic waters that increase the mobility of base metals sulphides, namely Fe, Cu, Zn and Pb. These waters enriched with sulphate, metals and metalloids are known collectively as acid mine drainage (AMD) (Álvarez-Valero et al., 2007; Barriga and Carvalho, 1983; Costa and Duarte, 2005; Gadanho et al., 2006). The potential pollution and contamination dynamics of the waste dumps are continuous and the AMD is produced during the whole year. In warm periods, part of the AMD-pollutants is retained by precipitation of evaporitic salts that are further re-dissolved in the rainy periods (Álvarez-Valero et al., 2007; Costa and Duarte, 2005; Pérez-López et al., 2008). The AMD is responsible for contamination of surrounding freshwater system, groundwater resources and soils. Besides AMD dispersion, other environmental problems are associated with mining activity, such as air dispersion of waste material and landscape disruption.

1.6.2. São Domingos aquatic system characterization

São Domingos mine is located in the hydrographic basin of Guadiana River, the 4th largest river basin in the Iberian Peninsula, with a total surface of 66800 Km^2, 11580 Km^2 (17%) of which are in Portuguese territory. The climate in the river basin is considered to be semiarid mesothermic (according to the Thornthwaite classification), characterised by hot dry summers and temperate and rainy winters. The annual average air temperature is 17 ºC, reaching its maximum value (26ºC) in July and August. Annual precipitation is 560 mm with the highest rate in November and the lowest in July. Average wind velocity is below 10 km/h. Annual average humidity is about 65% and water deficit is reported from May to October, a period during which water level in small rivers and streams is reduced almost until dryness (MAOT, 2000).

In São Domingos the drainage of the mining area occurs along several kilometres until the Mosteirão stream, a Chança River tributary.
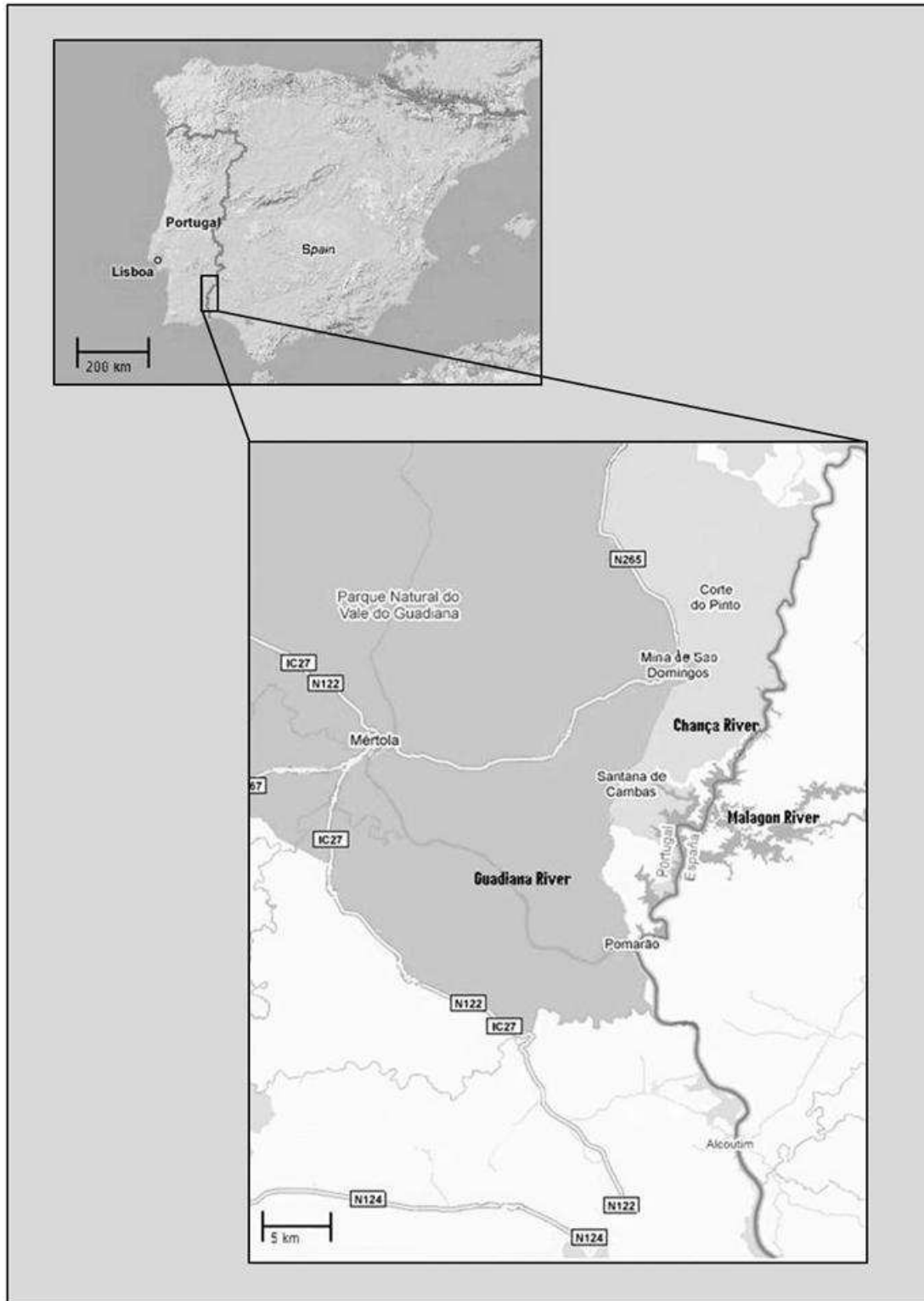
Figure 1.3. Geographic situation of São Domingos mine (Alentejo, Portugal) (source: maps.google.com). A detailed map of the study area is presented in Figure 1.4.

The Chança River has its headwaters in the Aracena Mountains in Spain. The river flows for 96 km, until its confluence with Guadiana River in Pomarão (Figure 1.3). Upstream the confluence, a large dam was built in 1985 creating a new reservoir – the Chança Reservoir – with a maximum volume of 3.9×10^8 m^3, a maximum waters depth of 63 m and a surface area of 19.4×10^6 m^2. This reservoir is used to supply water for municipal and agriculture uses (Quental *et al.*, 2002). The Chança reservoir is a dynamic system and fluctuations in terms of metal contents depend on both AMD flow and composition, which in turn are dependent on pluviometry (Álvarez-Valero *et al.*, 2007; De Bisthoven *et al.*, 2004). In fact the raining events influence the water level in the channels and reservoirs along the excavated valley where the acid drainage flows down towards the Mosteirão stream. Before entering into the Chança river reservoir the mine effluent is slightly diluted with water coming from the Mosteirão stream. The resulting reduction in acidity causes the precipitation of metals, originating yellow to reddishbrown contaminated sediments. These so-called ''ochre precipitates'' consist of Fe-phases precipitated from the Fe dissolved in AMD and coming from pyrite oxidation in the mine sites (España *et al.*, 2005; Fukushi *et al.*, 2003). Most of these precipitates are unstable and can be either transported as colloids downstream during high flow conditions or, on the other hand, can be transformed to more stable mineral forms, and originate cemented and more permanent chemical sediments (España *et al.*, 2005).

Contamination of Mosteirão and Chança surface waters and sediments by metals (especially As, Cd, Cu, Mn, Ni, Pb, Co, and Zn) is documented in early investigations conducted in the area (Canteiro, 1994; De Bisthoven *et al.*, 2004; Lopes *et al.*, 1999; MAOT, 2001; Moreira-Santos *et al.*, 2004; Pereira *et al.*, 2000). Besides metals no other significant contamination sources are known (e.g., pesticides, industrial discharges or urban runoffs) in the Chança reservoir, as agriculture is scarce and rudimentary, industrial activity is inexistent, and the demographic density of the area is among the lowest in Europe (MAOT, 2001; Pereira *et al.*, 1999; Pereira *et al.*, 2004).
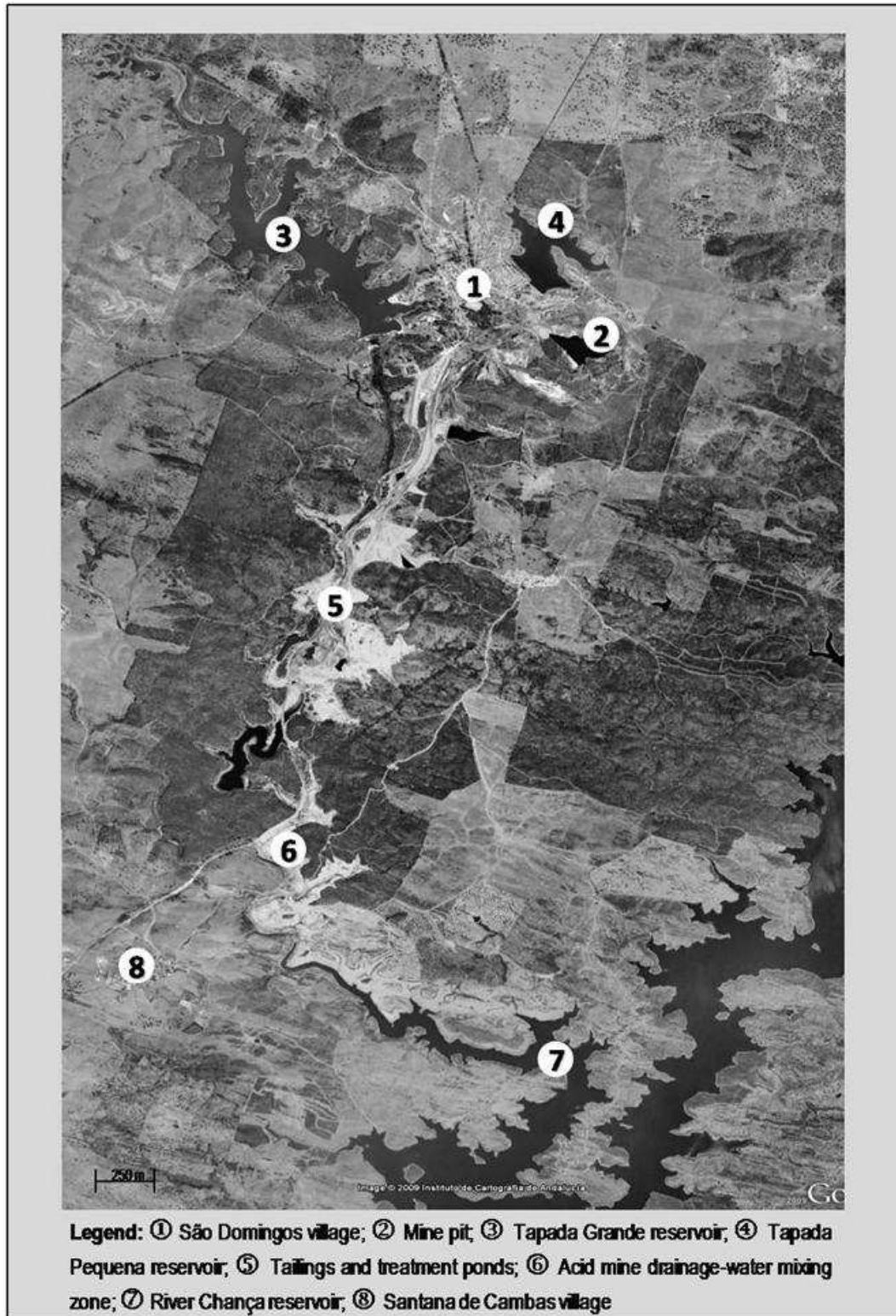
Figure 1.4. Detailed map of the study area (source: maps.google.com).

Tapada Grande is a freshwater shallow reservoir that is also included in the São Domingos aquatic system, along with the Tapada Pequena reservoir. Both lakes were built by the mine company to provide water to mining activities. Nowadays, Tapada Grande is highly demanded for recreational activities so its waters are monitored by the government and sampling is made every month to verify the quality according to specific legislation (50th article of DL nº 236/98) (Pereira *et al.*, 2000; MAOT, 2001; Quental *et al.*, 2002). In a study for the implementation of Water Framework Directive, Tapada Grande was pre-classified as a "reference reservoir" based on water quality data and information regarding pressures (Ferreira *et al.*, 2009). Figure 1.4 shows the detailed location of the study area, which includes the two sampling cladoceran sites – Tapada Grande and Chança reservoirs – and the mine drainage basin.

For this study, Tapada Grande reservoir was chosen as the reference site and the Chança reservoir was selected as the metal impacted site. Additional points along the mine drainage basin were also sampled for characterization of season fluctuations in the São Domingos mine effluent. Chança reservoir is ideal for the study of adaptations occurring in cladoceran field populations exposed to historical metal stress due to the fact that the source of contamination is identified and isolated and a reference site free of metal mining pollution, like Tapada Grande reservoir, is present in its surroundings.

1.7. An introduction to the studied model organism

The model organism chosen for the development of the work was a cladoceran species from the *Daphnia longispina* species complex. Cladocerans (''water fleas'') are small-sized crustaceans belonging to the class Branchiopoda. They are primarily-freshwater organisms occurring more abundantly in both temporary and permanent stagnant waters where they represent an important component of the microcrustacean zooplankton (Benzie, 2005; Ebert, 2005; Forró *et al.*, 2008; Schwenk *et al.*, 1998). Most of the species live as filter-feeders and occupy in this way a central role in the food chain, feeding on algae and detritus and being in turn consumed by planktivorous fish and invertebrate predators (Tessier *et al.*, 2000). The trunk and appendages of most

cladocerans are enclosed in a bivalved carapace, antennules are uniramous whereas antennae are biramous, natatory, with 2–4 segments per branch. Four to six pairs of trunk limbs are either mostly similar in shape or modified individually for various functions (Forró *et al.*, 2008). Some cladocerans reproduce by obligate parthenogenesis but most of species reproduce by cyclical parthenogenesis and populations are mostly dominated by females. Sexual dimorphism is normally rather distinct, with males distinguished from females mainly by their smaller size, larger antennules and modified post-abdomen (Ebert, 2005).

Within the cladocerans *Daphnia* is possibly one of the most studied taxa (Korovchinsky, 1997). This genus is well distributed in temperate regions (Benzie, 2005) and is included in the Daphniidae family, within the order Anomopoda (Benzie, 2005; Ebert, 2005). During the last decades this genus has been studied not only for its key role as a primary consumer in the food chain of freshwater ponds and lakes (Boersma *et al.*, 1996; Carpenter and Kitchell, 1996; Kasprzak *et al.*, 1999; Sterner *et al.*, 1993) but also for its physiology (Campbell *et al.*, 2004; Glover and Wood, 2005), behavior (e.g., vertical migration) (De Meester, 1994; Loose and Dawidowicz, 1994; Ringelberg, 1999), phenotypic plasticity (e.g., cyclomorphosis and predator-induced defense) (Hanazato *et al.*, 2001; Laforsch and Tollrian, 2004; Parejko and Dodson, 1991; Stabell *et al.*, 2003), host–parasite interactions (Ebert, 1995) and ageing (Dudycha, 2003). *Daphnia* is an important model organism for ecology, evolutionary biology (De Meester, 1996; Dudycha and Tessier, 1999; Hairston *et al.*, 1999; Lynch, 1983), ecotoxicology (Baird *et al.*, 1991; Barata *et al.*, 2006; Klüttgen *et al.*, 1996) and, more recently, for genomics (Watanabe *et al.*, 2007).

Daphnia possess several attributes that make them valuable as model organisms such as its short generation time, easy handling in the laboratory and cyclic parthenogenetic reproduction. Generation times vary between one to two weeks in culture at 20ºC (USEPA, 2002), which rivals that of most other model eukaryotes and makes it possible to track response throughout their ontogeny (Ebert, 2005). They have a suitable size for laboratory manipulation and handling. They are easily maintained in culture, in relatively simple defined media (Elendt and Bias, 1990; USEPA, 2002) and fed simple diets that include usually controlled concentrations of algae. Another practical attribute that make some of the *Daphnia* species good model systems for

experimental investigation is the cyclic parthenogenetic reproduction (De Meester, 1997; Ebert, 2005).

Cyclic parthenogenetic reproduction corresponds to the alternation of asexual and sexual reproduction and is schematically represented in Figure 1.5. During the parthenogenetic (asexual) cycle, each female produces a clutch of parthenogenetic diploid eggs after every adult molt that develop directly into female daphnids. The environment determines sex in *Daphnia* and the transition to sexual reproduction is initiated when parthenogenetic females begin to produce broods of diploid males. Females then switch from parthenogenetic to sexual egg production. In fact, sexual reproduction can be triggered by a complex set of biotic and abiotic stimuli, such as an increase in competition, a reduction in food availability or changes in temperature and day length (Benzie *et al*., 2005; Ebert *et al*., 2005). The sexual cycle initiates when females produce two haploid resting eggs. Resting egg production follows the asexual production of diploid males, which are needed to fertilize the haploid eggs. The brood chamber is modified to form a protective shell – the ephippium. Following mating and fertilisation of meiotically produced eggs, they are released into the ephippium, a protective structure modified from the carapace. Diapausing eggs can survive freezing and desiccation and hatch only when favourable environmental conditions return. They function both as a dispersal as well as a dormant life-history stage deposited in the sediments of many freshwater bodies (Benzie *et al*., 2005; Ebert *et al*., 2005; Weider *et al*., 1997).

In natural systems cyclic parthenogenesis can lead to a wide scope of population structures, ranging from almost monoclonal to genetically highly diverse ones. In addition, sexual reproduction in aquatic cyclic parthenogens is associated with the production of dormant stages, which both enhance potential gene flow among populations as well as impact local evolutionary rates through the formation of dormant egg banks (De Meester, 1996, 1997; Ebert, 2005; Thielsch *et al*., 2009).
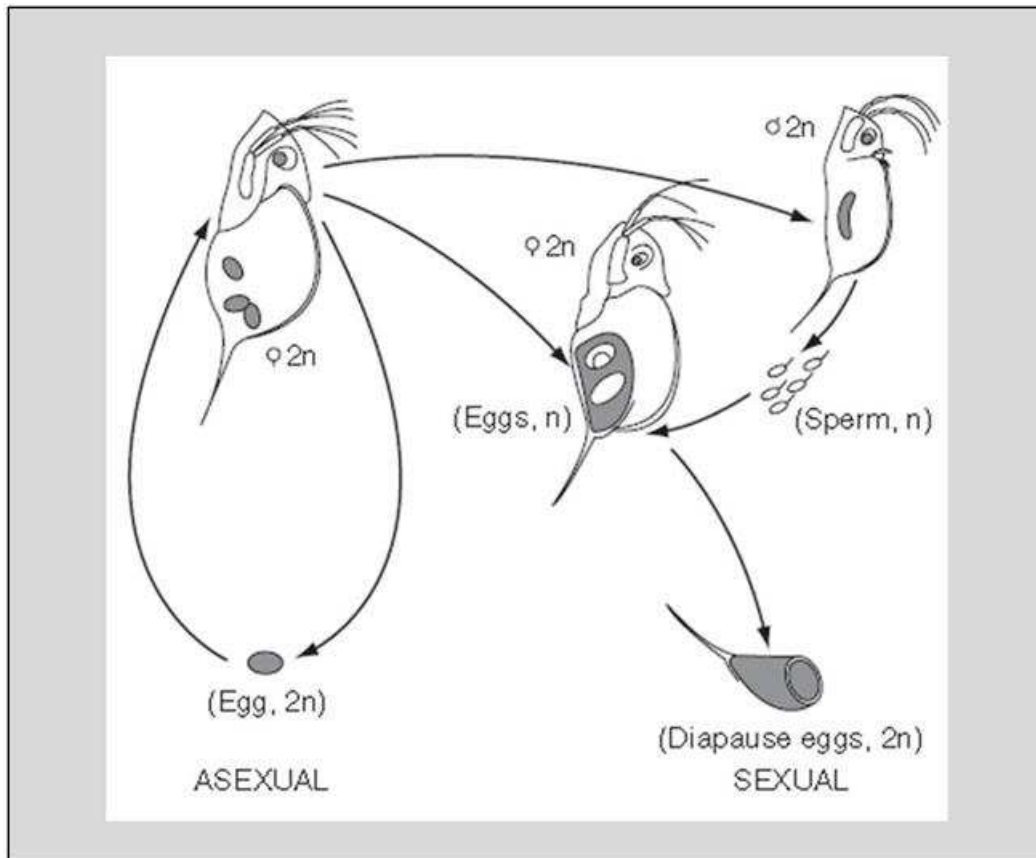
Figure 1.5. Life cycle of a cyclic parthenogenetic *Daphnia* (Adapted from: Mort *et al.*, 1991).

In recent years an increasing number of researchers has recognised the advantages offered by the cyclic parthenogenetic reproduction of *Daphnia* for the study of the interplay between genetic polymorphism and phenotypic plasticity in determining the variability in ecologically relevant traits observed in natural populations, and a series of advances in ecological genetic studies in this genus has been promoted (De Meester, 1997; Ebert, 2005; Forró *et al.*, 2008). Within- and between-clone comparisons can demonstrate genetic variation for various traits within and between populations, thus helping to reconstruct the evolutionary history of a population. Examples of traits include age and size at maturity, size at birth, aging, reaction norms for life history traits, vertical migration, phototactic behavior, fish escape behavior, production of defense spines and helmets, resistance against parasites, immune response, competitive ability, growth and feeding rate.

Daphnia longispina (O. F. Müller, 1776) is a small *Daphnia* species with a maximum body size of 2.5 mm. *D. longispina* is distributed in Europe, Asia and Africa, where it can be found in temperate freshwater bodies such as small lakes, ponds, slow flowing waters and sometimes in deep lakes (Benzie, 2005). Its body is transparent (in larger lakes) or yellowish (in small ponds), often with bright blue or red oil globules (Scourfield and Harding, 1966) (Figure 1.6).

This species presents a strong seasonal and geographic variation in morphology, including form and length of spina, carapax and head shape (Benzie, 2005). The high morphological variation and interspecific hybridisation within *D. longispina* species complex (*D. longispina*, *D. hyalina*, *D. galeata*, *D. cucullata* and *D. rosea*) has long been a cause of confusion in the species delineation (Giessler *et al.*, 1999; Schwenk *et al.*, 1998; Skage *et al.*, 2008). In fact, the name *D. longispina* initially included a wide range of genetically distinctive forms. Now only few of these forms are recognised as having specific status (Benzie, 2005).

Legend:
① *Daphnia longispina* adult female: with three eggs in the brood chamber
② *D. longispina* adult female carrying a resting egg. The ephippium is not yet fully developed as its wall is still transparent and the two eggs are visible. These eggs are haploid and require fertilisation.
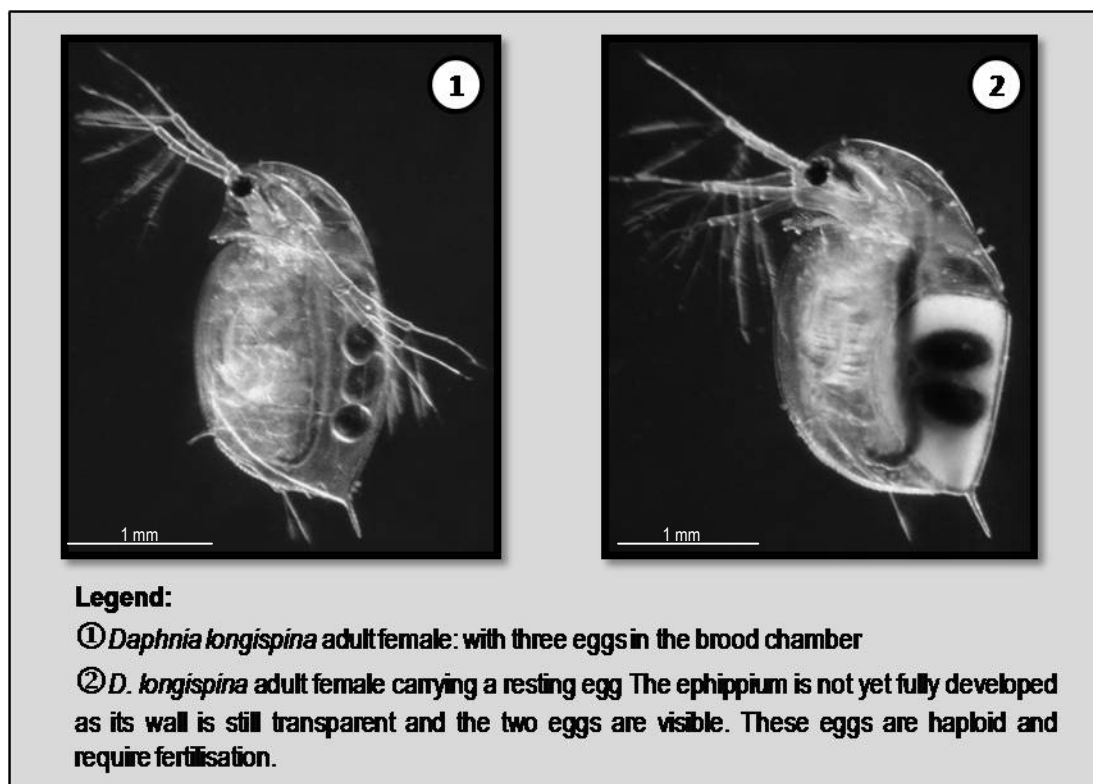
Figure 1.6. *Daphnia longispina* adult female.

In Portugal, species belonging to the *D. longispina* complex (parental species and interspecific hybrids) are well represented in lakes and reservoirs from north to south of Portuguese territory (Abrantes *et al*. 2006; Castro, 2007; Geraldes e Boavida, 2004). The actual taxonomic position of *D. longispina* in Portugal is unclear due to pronounced phenotypic plasticity and hybridisation between species. In early investigations conducted in the area, *D. longispina* was identified as one of the zooplanktonic species present in Tapada Grande and Chança reservoirs along with the cladoceran *Ceriodaphnia pulchella* and the copepod *Copidodiaptomus numidicus* (Lopes *et al.*, 1999; Pereira *et al.*, 2000). The clones sampled in the present study were analysed by ITS-RFLP techniques (Billiones *et al.*, 2004) confirming that these *D. longispina* clones were in fact *D. galeata* x *longispin*a interspecific hybrids.

1.8. Research objectives and thesis outline

The research presented in this thesis aims to improve the understanding of ecological side effects of long-term adaptation to metals in natural populations of a cladoceran species – *Daphnia longispina*. To address this aim, four separate chapters, following the general introduction, are organized focusing on different issues. An overview of the different chapters and their content is given below:

In Chapter 2, a seasonal characterization of the field sites was done along with the chemical and ecotoxicological characterization of water and sediment. The toxicity of field water and sediment using the cladoceran *D. magna* and the midge *Chironomus riparius* was determined, as well as the abiotic parameters correlated with toxicity.

In Chapter 3 the genetic consequences of adaptation to copper and zinc were assessed comparing two populations of *Daphnia longispina*, one located in the water reservoir contaminated by the acid mine drainage and the other located in a nearby clean water reservoir (both sites characterized in Chapter 2). Genetic analysis included changes in tolerance, genetic variation in tolerant and fitness related traits and genetic by environmental trade-offs (costs of tolerance).

In Chapter 4 an exhaustive life-table response study was conducted on two clones per population (a sensitive and tolerant one) to determine how adaptation affected the life history strategies on *D. longispina*.

The effects of copper across generations were then studied in one of the copper sensitive clones to assess possible variations in the tolerance to copper with a continuous exposure to the stressor (Chapter 5).

Finally, in Chapter 6, the implications of this work for understanding the effects of long-term exposure to metals on field cladoceran populations are discussed while some possible directions for future research are also referred.

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CHAPTER 2

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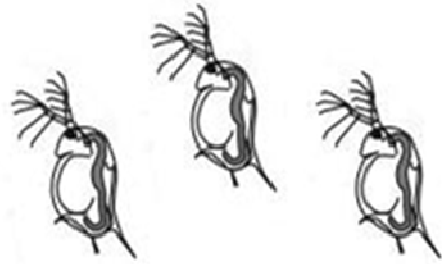
**The São Domingos mine aquatic surrounding system
– a metal-polluted heterogeneous environment**
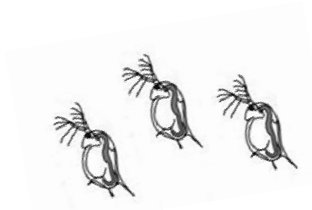
Chapter

2

The São Domingos mine aquatic system – a metal-polluted heterogeneous environment

Abstract

The aquatic system surrounding São Domingos mine, including the Chança Reservoir, has been exposed to acid mine drainage (AMD) in the last decades. This study aims to evaluate metal exposure levels in both water and sediments and its toxicity to the cladoceran *Daphnia magna* and the midge *Chironomus riparius*. In order to evaluate seasonal fluctuations in the water/sediment chemistry and toxicity, sampling was performed in two distinct periods – September 2006 (representing the dry period) and March 2007 (representing the wet period). Seven stations were selected for this study: five impacted sites along the mine pollution gradient and two reference sites. The results confirmed the seasonal contamination of both environmental compartments (water and sediments) by metals, including copper and zinc, and the presence of high concentrations of sulphates in the water-column. Bioassays showed that water samples significantly affected the *D. magna* survival which could be associated with high levels of dissolved metals and low pH values. Whole-sediment tests performed in *D. magna* and *C. riparius* were acute toxic in September whereas sub-lethal effects were observed in March. Results obtained illustrate seasonal differences in metal contamination and allowed characterizing optimal cladoceran sampling periods and one of the studied sites, located in the Chança Reservoir, as a metal polluted heterogeneous environment.

2.1. Introduction

São Domingos mine (Southern Portugal) is one of the many massive sulphide deposits of the Iberian Pyrite Belt (De Vos *et al.*, 2005; Quental *et al.*, 2002). The mine is closed at present, but the sulphide-bearing waste rock piles and metal enriched tailings are important sources of chemical contamination to the environment, being able to affect extensively mined areas (De Vos *et al.*, 2005; Matos and Martins, 2006; Quental *et al.*, 2002; Reimann and Caritat, 1998). The occurrence of these mine waste materials (in general, fragmented and finely-ground materials) enhances and promotes the development of a number of chemical reactions in a weathering environment, such as oxidation of pyrite (iron sulphide) and other sulphide minerals. This complex process involves chemical, biological and electrochemical reactions and the rate at which occurs depend on several factors such as pH, morphology of pyrite, presence or absence of bacteria and/or clay minerals and hydrology (Evangelou, 1998; Reimann and Caritat, 1998). The oxidation of pyrite and sulphide minerals in the presence of oxygen and water produces acid waters characterized by low pH values (1.5 to 3.5) and large concentrations of sulphate (usually higher than 3 g l^{-1}) and dissolved metals (e.g. iron, copper, zinc, lead) and metalloids, being the ions and their concentrations variable depending on the composition of the minerals lixiviated (Álvarez-Valero *et al.*, 2007; Costa and Duarte, 2005; Evangelou, 1998). Those acid waters are collectively known as acid mine drainage (AMD) (Álvarez-Valero *et al.*, 2007; Costa and Duarte, 2005; Evangelou, 1998; Silva *et al.*, 2005). AMD has been recognized as a major environmental pollution problem over the past three decades (Silva *et al.*, 2005) and is considered the main pollution source of natural watercourses in mining environments of the Iberian Pyrite Belt (Álvarez-Valero *et al.*, 2007). In the particular case of São Domingos mine, AMD is continuously produced during the entire year but the AMD contamination potential of the surrounding aquatic system depends on its flow and composition, which in turn are dependent on pluviometry (Álvarez-Valero *et al.*, 2007;

Janssens de Bisthoven *et al*., 2004). During warm periods, one part of the AMD-metals is retained in surface sediments after precipitation, but in rainy periods the retained metals are resuspended and re-dissolved becoming bioavailable (Álvarez-Valero *et al*., 2007; Pérez-López *et al*., 2008). Those rainy periods increase the flow of AMD towards nearby fluvial courses, such as the Mosteirão stream and its affluent Chança River, causing its partial pollution (Álvarez-Valero *et al*., 2007). The potential for AMD to contaminate surface waters, ground waters and stream sediments is influenced not only by hydrologic and climate conditions but also by the stream chemistry, especially pH and dissolved organic carbon (DOC) concentration (Niyogi *et al*., 1999; Álvarez-Valero *et al*., 2007). Decreased pH, increased concentrations of dissolved metals and a high amount of metal precipitation caused by AMD runoff into aquatic systems can have severe detrimental effects on aquatic ecosystems such as the loss of sensitive species, reductions in abundance and diversity of aquatic communities and loss of ecosystem integrity, through effects on growth, reproduction, and behavior of individual organisms (Clements, 1994; Gray, 1998; Hazen *et al*., 2002; Starnes and Gasper 1995). Studies of effects of acid mine drainage on plankton (Levings *et al*., 2005; Monteiro *et al*., 1995; Oliveira, 1985), benthic macroinvertebrates (Cherry *et al.*, 2001; Clements, 1994; Clements and Kiffney 1995; Gray, 1998; Nelson and Roline, 1996; Winterbourn and McDiffet, 1996) and fish (Gray, 1998; Rutherford and Mellow, 1994) generally revealed reduced diversity and abundance in impacted areas. Despite the large number of field studies of AMD impacts on biota, only few studies have assessed toxicity of acid mine drainage water column and/or sediments. Vinyard (1996) observed chronic water column toxicity to the cladoceran *Ceriodaphnia dubia* with acidic samples collected from an actively mined area, and Soucek and colleagues (2000) found water and sediment samples collected from an AMD impacted watershed to be acutely toxic to *Daphnia magna*. Pereira and coworkers (2000), working on the same aquatic system focused here, assessed the toxicity of both São Domingos mine effluent and Chança Reservoir water samples using *D. magna* and *C. dubia* in water-column and sediment laboratory bioassays. In these studies, however, the toxicity of AMD and metal contaminated sediment were only partially assessed. The objectives of the present study were: (1) to perform the overall chemical characterization of both water and sediment along seven defined points in the aquatic system surrounding São Domingos mine; (2)

to evaluate the toxicity of AMD water and sediment using the cladoceran *D. magna* and the midge *Chironomus riparius*; (3) to determine which abiotic parameters were correlated with toxicity; (4) to assess seasonal fluctuations (dry period versus wet period) in the water/sediment chemistry and toxicity. The above mentioned tasks were in part aimed to characterize the station selected for cladoceran sampling as a metal polluted heterogeneous environment.

2.2. Material and Methods

2.2.1. Study area and sampling stations

The cupriferous pyrite mine of São Domingos (37º 38' 00'' N to 37º 40' 30'' N and 7º 19' 05 W to 7º 20' 05'' W) is located 17 km NE from the town of Mértola within Baixo Alentejo province, south of Portugal. Seven stations in the aquatic system surrounding S. Domingos mine were selected for this study: five impacted sites (A, C, D, DJ and E) and two reference sites (DM and F). Figure 2.1 shows a scheme with the relative location of the studied sampling stations. Stations A (37º39'54.3''W; 7º 30'10.6''N) and C (37º38'34.2''W; 7º30'49.9''N) were located in the mine drainage basin before confluence with the Mosteirão tributary, which was used as settlement ponds when the mine was still active; station D (37º38'1''W; 7º30'57.7''N) was situated at the confluence of the mine drainage with the Mosteirão tributary; station DJ (37º37'57.7''W; 7º30'59.7''N) was located downstream the confluence of Mosteirão stream with the mine effluent; station E (37º37'30.8''W; 7º30'51.5''N) was located in the River Chança reservoir; station DM (37º37'56.4''W; 7º 31'12.4''N) was a point located in the Mosteirão tributary upstream its confluence with the mine effluent; station F (37º 40' 31'' N, 7º 30'42'' W), located in an independent semiartificial lagoon (Tapada Grande) with no history of contamination by metals and far away from the effluent discharge. The semiarid mesothermic climate characteristic of the region can be divided in two distinct periods, a wet period from November to March and a dry period from April to October (Abreu *et al*., 2008). In this study, in order to assess temporal

variations, the samples were collected in two distinct periods – September 2006 (representing the dry period) and March 2007 (representing the wet period).
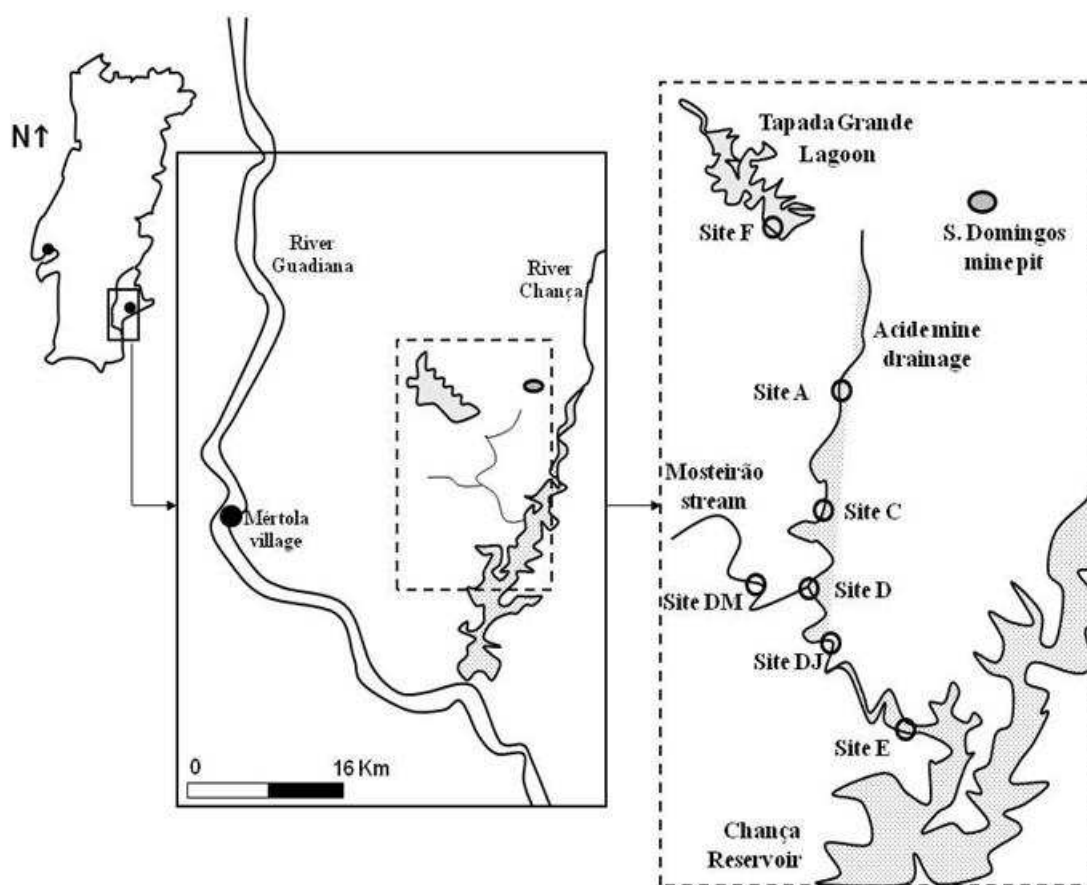
Figure 2.1. Schematic map of the study area with the sampling stations.

For a detailed climatic characterization of the region between sampling events, rainfall data between July 2006 and July 2007 are shown in Figure 2.2.
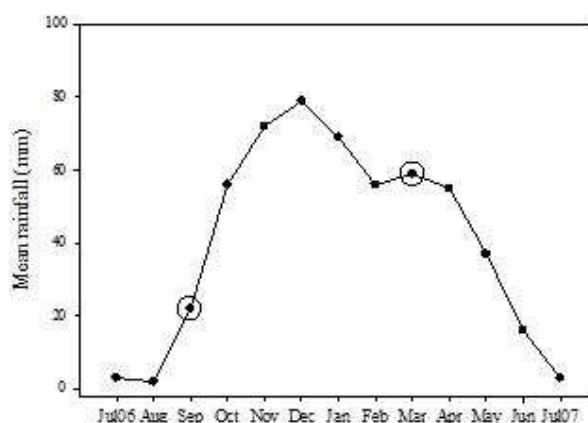
Figure 2.2. Rainfall data (mm) recorded in Guadiana basin area (meteorological station of Serpa) during the study periods. ○: sampling events (Source: INAG, 2007).

2.2.2. Collection of water and sediment samples

Water samples were collected for environmental parameters analyses and for bioassays, according to USEPA guidelines (USEPA, 2002). Subsurface water samples were collected by hand submerging precleaned 1.5 l polyethylene bottles approximately 10-15 cm beneath the water surface. Simultaneously to sample collection, parameters such as water temperature, conductivity, pH, and dissolved oxygen were determined *in situ* by using portable water testing meters from WTW (WTW, Weilheim, Germany). Water samples were kept refrigerated during its transport to the laboratory.

Surface sediments were collected according to USEPA recommendations (USEPA, 2001) for both environmental parameters analyses and bioassays. In each station sediment was collected to polyethylene bags from the top 5 cm with a plastic scoop. Sediment redox potential measurements were performed *in situ* with a WTW 330i pH-meter (WTW, Weilheim, Germany) equipped with a redox electrode Mettler Toledo Pt 4808-S7 (Mettler-Toledo, Columbus, Ohio, USA). Sediment samples were kept refrigerated during its transport to the laboratory.

2.2.3. Analytical methods

On arrival to the laboratory, water samples for bioassays were filtered through a 60 μm mesh size net, following the recommendations of USEPA guidelines for assays with cladocerans (USEPA, 2002), and stored at 4ºC until the assays were performed (maximum storage time: one week). Alkalinity and hardness were measured immediately by titration in non-filtered water samples as described in APHA (1995). Water samples for chemical analysis were filtered through acetate cellulose filters (0.45 μm porosity) for nutrient analysis and through pre-weight nitrocellulose filters (0.45 μm porosity) for both metal analysis (dissolved and particulate fractions) and determination of suspended particulate matter (SPM). Filtrates (particulate fractions) were dried at <50ºC until constant weight, weighted and stored in dark. Dissolved fractions for metal analysis were acidified with nitric acid to pH around two to reduce adsorption phenomena and stored in polyethylene bottles. Filters were treated in Teflon lined digestion bombs, with a mixture of nitric and fluoric acids (6:3 by vol., AA-grade) and digestion was performed in a microwave oven. The obtained residues were diluted to 25 ml with high purity water (MilliQ, Millipore) and used to determine metal amounts in the particulate fraction. Metal analyses in both dissolved and particulate fractions were performed with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (APHA, 1995). Nutrient analyzes – ammonia-nitrogen (NH_3-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), sulphates (SO_4^{2-}) and reactive phosphorus (PO_4^{3-}) – were performed by spectrophotometric methods using a DR/2000 Portable Spectrophotometer from Hach (Hach, Loveland, Colorado, USA).

In the laboratory, sediment samples were homogenised and visually checked for visible indigenous fauna and large debris (leaves, etc.), which were removed with forceps. Sediments for bioassays were stored at 4ºC in the dark for a maximum period of two weeks. Sediments for chemical analysis were air dried for 3 days and oven dried at 50ºC for 1 day. Organic matter content was estimated as weight loss on ignition (LOI) after 4 h at 500 ºC (Williams, 1985). After the drying step, sediments were disaggregated and dry sieved using a 63-μm sieve and analysed for metals. Samples of 0.5 grams were treated in Teflon lined digestion bombs, with a mixture of nitric and fluoric acids (6:3 by vol., AA-grade) and digestion was performed in a microwave oven. The obtained residues were diluted to 25 ml with high purity water (MilliQ, Millipore)

and used to determine metal amounts in the sediment fraction <63μm by ICP-MS (APHA, 1995).

Concentrations of metals in dissolved and particulate fractions were expressed in mg l^{-1} and μg l^{-1}, respectively. Concentrations of the metals in sediments were calculated on a dry weight basis and expressed as mg Kg^{-1}. Each sample was analysed at least in duplicate. The obtained detection limits for analysed samples were: silver (Ag): 0.02; aluminium (Al): 1; arsenic (As): 0.2; cadmium (Cd): 0.01; chromium (Cr): 0.1; copper (Cu): 0.5; iron (Fe): 20; manganese (Mn): 5; nickel (Ni): 0.5; lead (Pb): 0.1; selenium (Se): 0.1; silicon (Si): 50; tin (Sn): 1; zinc (Zn): 5, all in μg l^{-1} corresponding to μg of metal per g of sediment. Analytical accuracy was determined by using blanks and certified reference material (CRM) of the Community Bureau of Reference (European Union, Brussels, Belgium). Recoveries were within 10% of the certified values.

2.2.4. Test organisms

Experiments were carried out with two organisms – the planktonic water flea *Daphnia magna* Straus (Cladocera, Crustacea) and the benthic larvae *Chironomus riparius* Meigen (Chironomidae, Diptera).

2.2.4.1. *Daphnia magna*

Monoclonal cultures of *D. magna* (clone F *sensu* Baird *et al*. 1991) had been successfully maintained in laboratory conditions for more than 4 years under controlled conditions of temperature (20±1°C) and photoperiod (16 h light - 8 h dark). Cultures were maintained in 1 l glass beakers with 800 ml of laboratory artificial water. This artificial water consisted in ASTM hard water (ASTM, 2002) enriched with a standard organic extract Marinure 25 (Glenside, Stirling, UK; Baird *et al.*, 1989) and added to the culture medium at a concentration of 6 ml l^{-1}. This standard organic additive is added to the culture medium to provide essential microelements to daphnids. Culture medium was renewed three times a week and daphnids were daily fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak at a concentration of 3×10^5 cells

ml^{-1}. About 25 daphnids per beaker were kept and neonates from the fifth or sixth broods were used to replace the old cultures.

2.2.4.2. *Chironomus riparius*

C. riparius larvae had been successfully maintained in laboratory conditions for more than five years at controlled conditions of temperature ($20\pm2°C$) and photoperiod (16 h light - 8 h dark). Organisms were reared in an enclosed transparent acrylic unit (dimensions $120\times60\times40$ cm) containing all the structures necessary to complete the whole life cycle of the chironomids. At the start of a new culture approximately 100 newborn larvae were transferred to plastic beakers (10×20 cm) containing a 2 cm layer sediment and ASTM water in a proportion of sediment:water of 1:4. A natural sediment from the unpolluted Bestança River (Douro, Portugal) washed several times with distilled water and sized by a mesh of 1 mm was used in cultures. A suspension of ground TetraMin® (Tetrawerke, Germany) (0.5 mg larvae^{-1} day^{-1}) was added as the food source and each beaker was gently aerated. After one week the beakers were renewed and 60 larvae were then introduced in each beaker with fresh medium, sand and food until emergence occurred. Adults were fed on a sucrose solution in paper, placed inside the culture unit. Freshly laid egg masses were transferred onto glass crystallization dishes with ASTM water and the new born larvae were then used to start a new culture approximately 2-3 days after egg deposition.

2.2.5. Bioassays

2.2.5.1. *Acute (48-hour) test with Daphnia magna*

The 48-hour acute (immobilization) test with *D. magna* followed the procedures established in ASTM (2002). Tests were initiated with neonates (<24 h-old) released from the 3rd to 5th brood females coming from the bulk group cultures. A static design was employed, using 20 daphnids (randomly divided into four groups of five organisms) per control (ASTM water) and per effluent: ASTM water dilution (%). Test vessels (four per treatment) consisted of glass beakers containing 100ml of test water.

Tests were run under the same temperature and photoperiod regimes as described for rearing procedures. A total of seven test concentrations were tested: 0% (only ASTM – control), 6.25%, 12.5%, 25%, 50%, 75% and 100% (only site water).

2.2.5.2. *Whole sediment toxicity test with Daphnia magna*

The 7-day whole sediment test with *D. magna* followed the procedures established in ASTM (2000). Tests were initiated with 5-day old organisms. A semi-static design was employed, with the medium being renewed every day. Test vessels consisted of 50 ml glass beakers containing 20 ml of ASTM plus 5 g of sediment. Ten replicates (1 daphnid/replicate) were performed per treatment (sediment). A control treatment with ASTM and reference laboratory sediment (sediment used in chironomid laboratory cultures) was also included. Tests were run under the same temperature and photoperiod regimes as described for rearing procedures. Dead organisms and the number of produced neonates (fertility) were monitored every day. At the end of the test, organisms were measured (from the top of the head to the base of the tail spine) in a microscope MS5 (Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer (accuracy of 0.01 mm). Temperature, conductivity and pH were determined in test vessels at the beginning and at the end of tests. Dissolved oxygen was also measured at the same time in order to fulfill test criteria.

2.2.5.3. *Whole-sediment toxicity test with Chironomus riparius*

The 10-d whole sediment test with *C.riparius* with the renewal of the overlying water followed the general procedures dictated by ASTM (2000). Test vessels (ten replicates per treatment) consisted of 250 ml glass beakers containing 150 ml of ASTM water and 80 g of sediment. A control treatment was done with the sediment used in chironomid laboratory cultures. Eight first-instar larvae per replicate were used. Medium renewal was done every two days taking off half of the volume without disturbing the sediment. Tetramin (from TetraWerck) was the sole source of food added; the ration was set at 0.2 mg per larvae per day in order to provide enough nutrition for the midges. At Day 10 the remaining larvae were collected for body length

measures in a stereomicroscope MS5 (Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. Growth (body length increase) of larvae was calculated by subtracting the average initial length (obtained from a pool of 30 larvae) from each individual final length. After measurements, larvae were transferred individually to pre-weighted foil cups, dried at 60ºC for 48 hours and weighted on a microbalance (Mettler UMT2). Biomass was quantified as dry weight per larvae. Temperature, conductivity and pH were determined in test vessels at the beginning and at the end of tests. Dissolved oxygen was also measured at the same time in order to fulfill test criteria.

2.2.6. Data analysis

Pearson's correlation was used to determined relationships between physical-chemical variables. Concentrations of metals in surface water were compared with the maximum admissible values (MAV) defined by the Portuguese legislation for surface waters (MA, 1998) and also with the USEPA criteria for protection of aquatic life and human health (WQGV – water quality guideline value; USEPA, 2006). Since no Portuguese legislation exists regarding sediment, concentrations of metals in sediment were compared with the sediment quality guideline values (SQGV) fixed by Canadian Council of Ministers of the Environment (CCME) to protect benthic-dwelling species (CCME, 2001) and with Consensus-Based Probable Effect Concentrations (Consensus-Based PEC) defined by the Contaminated Sediment Standing Team (CSST, 2003). The number of immobilized daphnids from each acute immobilization test was plotted against the test concentrations, and a 48-h LC_{50} with a 95% confidence interval (CI) was calculated by the standard probit procedure (Finney, 1971). Fisher's exact test was used to determine the sites where survival of *D. magna* in the whole-sediment tests was significantly reduced. One-way analyses of variance (ANOVA) were conducted to test for existence of significant differences in fertility/growth responses of *D. magna* or *C. riparius*. Whenever null hypothesis was rejected, post hoc multiple comparison test (Tukey's test) were used to determine which groups of individuals differed from each other (Zar, 1996). Whenever heteroscedasticity or nonnormality of data was pronounced, a nonparametric analysis of variance was employed (Kruskal-Wallis test),

followed by nonparametric multiple comparison testing (Zar, 1996). To make two-sample comparisons, the Student's t test and the nonparametric Mann-Whitney's U-test were used. All statistical analyses were performed with the SigmaStat statistical software (SPSS, 1995).

2.3. Results

2.3.1. Physical and chemical characterization

The physicochemical properties of water and surface sediments from São Domingos sampling sites are presented in Tables 2.1 and 2.2. With the exception of station DM (pH 6.6-8.1) and F (pH 7.4-8.3), waters from all sites were acidic, with pH values ranging from 2.7 to 5.2 (Table 2.1). The pH values remained relatively constant between seasons, the main differences being registered for station E, with a pH variation of 2. Conductivity values were always higher in September than in March for all study sites and all sites presented high conductivity values (above 1000 μS cm^{-1}), with the exception of stations DM and F. Dissolved oxygen concentrations were high (above 8.5 mg l^{-1}) in all sampled waters in both sampling events. Alkalinity values were generally low to moderate (<100 mg l^{-1}). Hardness ranged from 89.7 to 119 mg l^{-1} for stations DM and F. Hardness values were not obtained for the AMD impacted stations (A, C, D, DJ, E) due to interference in the titration method. Organic matter in sediments was in general low for all sampled stations (< 3 %). With the exception of station F, all sites had sediment redox potentials between 375 and 575 mV.

Table 2.1. Physicochemical water (pH, conductivity, dissolved oxygen, temperature) and sediment (redox potential and organic matter) parameters for the sampling sites.

Site	September						March					
	pH	C	DO	T	Eh	OM	pH	C	DO	T	Eh	OM
A	2.7	3200	8.9	27.2	508	2.9	2.9	2490	9.3	19.8	470	2.7
C	2.7	3250	8.7	23.9	450	2.4	2.8	1424	8.5	20.0	480	2.9
D	2.8	4620	9.5	27.3	500	2.3	2.9	1273	9.6	20.7	450	2.1
DJ	2.9	3180	9.3	24.1	575	2.9	3.1	1080	9.8	18.0	490	2.7
E	3.1	2480	8.7	23.5	515	2.4	5.2	1027	9.5	18.4	485	2.0
DM	6.6	980	8.9	24.7	464	2.0	8.1	468	9.1	17.3	378	2.4
F	7.4	309	8.6	23.8	20	2.2	8.3	219	9.6	16.5	30	2.4

Abbreviations: C –conductivity (μS cm^{-1}); DO – dissolved oxygen (mg l^{-1}); T – Temperature (ºC); E$_h$- redox potential (mV); OM – organic matter (%)

Nutrients in water samples showed their highest values in September, especially for sulphate ions (Table 2.2). In most samples Station F (reference) presented the lowest nutrient concentrations in both sampling months, with nitrates being an exception. Although nitrates were usually higher in station F, values were below environmental benchmarks (MA, 1998). The highest sulphate concentrations were found in stations A and C (between 666.7 and 3616.7 mg l^{-1}), whereas the lowest ones were found in stations F and DM (between 0.30 and 290.7 mg l^{-1}). Values for suspended particulate matter (SPM) in surface waters are also presented in Table 2.2. Values of SPM changed between seasons, with the highest and the lowest values being registered in the station D, 15.3 mg l^{-1} (September) and 0.28 mg l^{-1} (March), respectively.

Trace metal concentrations on surface waters and sediments are shown in Figures 2.3 and 2.4 (and in Appendix.1, Appendix.2 and Appendix.3). In general, the highest dissolved metal concentrations in the water compartment were recorded in September (Figure 2.3). The exception was observed for lead (Figure 2.3), which was present in higher concentrations in March. DM and F were the stations presenting the lowest metal concentrations in both seasons, whereas A, C and D were the ones with the highest metal levels.

Table 2.2. Average nutrient concentrations and respective standard deviations (in brackets) for the sampling sites (mg l^{-1}, n=3). Suspended particulate matter (mg l^{-1}) is also represented.

Nutrients		A	C	D	DJ	E	DM	F
NH$_3$-N	September	0.15 (0.026)	0.27 (0.086)	0.77 (0.069)	0.46 (0.010)	0.19 (0.060)	0.24 (0.017)	0.01 (0.009)
	March	0.90 (0.065)	0.50 (0.046)	0.46 (0.080)	0.38 (0.021)	0.17 (0.037)	0.01 (0.009)	0.13 (0.010)
NO$_3$-N	September	0.28 (0.031)	0.12 (0.033)	0.44 (0.090)	0.54 (0.079)	0.23 (0.016)	0.88 (0.015)	0.66 (0.011)
	March	0.32 (0.015)	0.33 (0.012)	0.29 (0.015)	0.25 (0.014)	0.22 (0.044)	0.86 (0.067)	0.71 (0.067)
NO$_2$-N	September	0.03 (0.022)	0.43 (0.049)	0.36 (0.023)	0.30 (0.021)	0.25 (0.083)	0.15 (0.069)	0.08 (0.050)
	March	0.08 (0.007)	0.13 (0.024)	0.02 (0.002)	0.02 (0.011)	0.02 (0.002)	0.06 (0.016)	0.04 (0.002)
PO$_4^{3-}$	September	0.28 (0.014)	0.34 (0.02)	0.23 (0.037)	0.18 (0.010)	0.14 (0.03)	0.12 (0.040)	0.01 (0.00)
	March	0.01 (0.006)	0.02 (0.012)	0.02 (0.006)	0.02 (0.012)	0.01 (0.0)	0.01 (0.00)	0.01 (0.006)
SO$_4^{2-}$	September	2733.3 (837.7)	3616.7 (76.4)	616.0 (169.7)	1263.3 (155.0)	1141.3 (6.6)	290.7 (44.1)	31.0 (6.6)
	March	1766.7 (146.5)	666.7 (28.9)	591.7 (52.0)	583.3 (80.4)	425.0 (0.00)	0.30 (0.6)	25.7 (3.5)
SPM	September	1.65	3.14	15.3	0.52	1.20	2.19	1.62
	March	0.37	6.23	0.28	2.92	1.18	0.42	8.72

Abbreviations: NH$_3$-N – ammonia-nitrogen; NO$_3$-N – nitrate nitrogen; NO$_2$-N – nitrite nitrogen; SO$_4^{2-}$ – sulphate; PO$_4^{3-}$ – reactive phosphorus; SPM – suspended particulate matter

Surface waters were in general highly contaminated with arsenic (As) and sulphide-related metals namely copper (Cu), cadmium (Cd), zinc (Zn), lead (Pb), and iron (Fe) (Figure 2.3). Cu, Zn and Fe were the metals showing the higher levels: 11 mg l^{-1} (station A, September), 60 mg l^{-1} (station D, September) and 88 mg l^{-1} (station C, September) respectively. Station DJ and E, in the Mosteirão stream and Chança Reservoir respectively, presented concentrations of Cu, Zn, Cd, and Fe (dissolved fraction) far above the regulatory values defined by the Portuguese legislation for surface waters (MAV – "maximum admissible value; MA, 1998) in both seasons. Moreover, the dissolved concentrations of Cu, Zn, Cd and Fe also exceeded the USEPA criteria for

protection of aquatic life and human health (WQGV – water quality guideline value; USEPA, 2006).

Stream sediments (fraction < 63 μm) were seriously contaminated with As and sulphide-related metals (Cu, Pb, Zn, Cd, Fe) (Figure 2.4). Although concentrations in sediments were less variable between seasons when compared with metal levels in surface waters, some degree of variation was found. Arsenic and lead were the metals found in sediments in higher concentrations (5714 mg Kg^{-1} of As in station DJ and 7322 mg Kg^{-1} of Pb in station C, both in September). All stations (except F) presented concentrations of metals Cu and Zn in sediments above the SQGV defined by CCME for the protection of aquatic life (CCME, 2001).

Correlation analysis between abiotic parameters (C, pH and concentrations of some metals in both dissolved and sediment phases) revealed several significant ($p <$ 0.05) relationships (Table 2.3). Water column pH was significantly negatively correlated with conductivity, dissolved Fe and Zn, with absolute correlation values $\geq$ 0.80. Although no significant, negative correlations were also found between pH and dissolved Cu in both seasons. Conductivity was significantly ($p < 0.05$) positively correlated with dissolved Fe, Al, Mn, Cu and Zn. In sediments, Fe was significantly ($P < 0.05$) positively correlated with Cu and Zn.
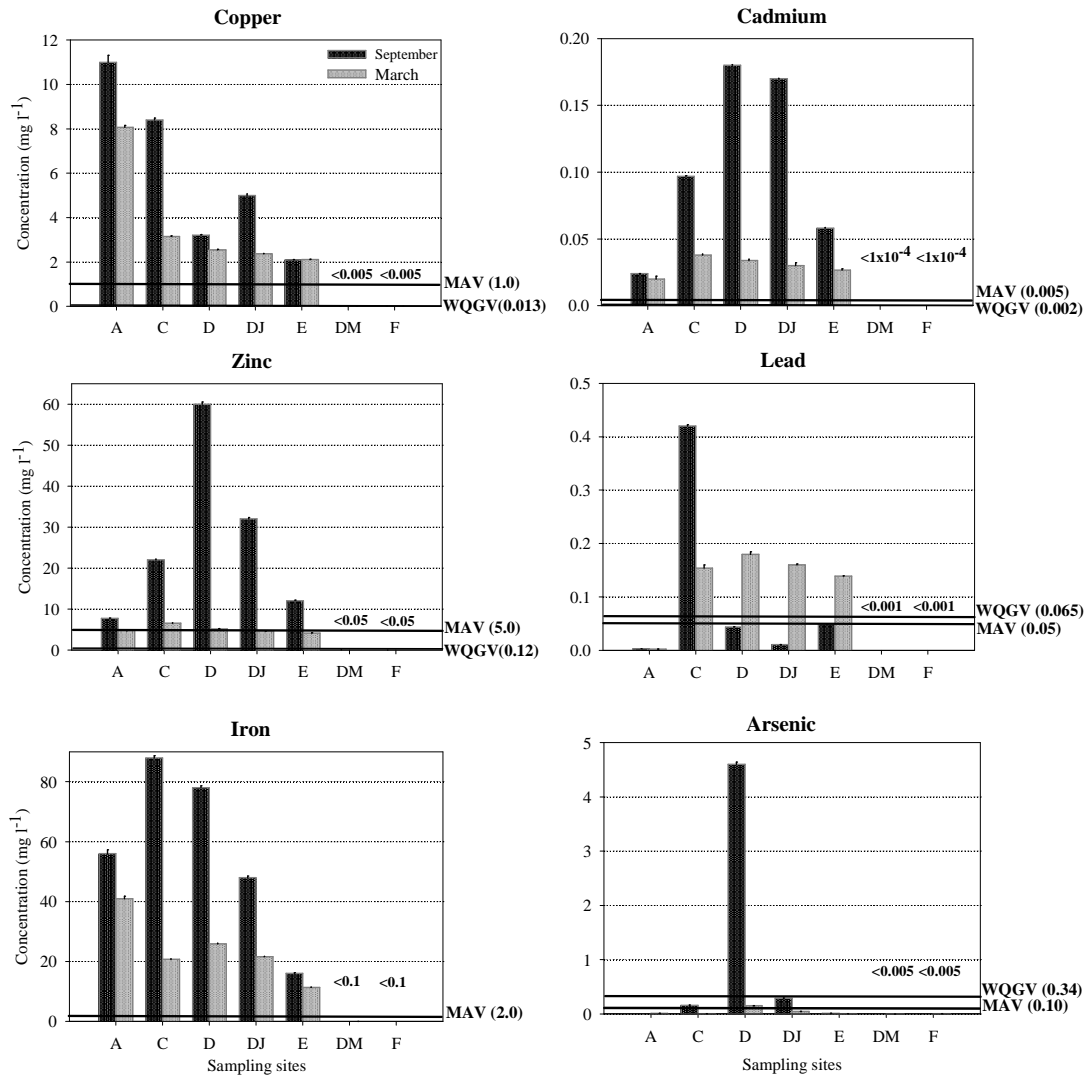
Figure 2.3. Average concentrations (mg l^{-1}) ± SD (standard deviation) of dissolved metals (copper, cadmium, zinc, lead, iron and arsenic) in surface water across sampling sites in São Domingos' aquatic surrounding system MAV – maximum admissible value (mg l^{-1}) in surface waters according to the Portuguese legislation; WQGV – water quality guideline value (mg l^{-1}) fixed by USEPA for protection of aquatic life and human health. No USEPA guideline is available for iron.
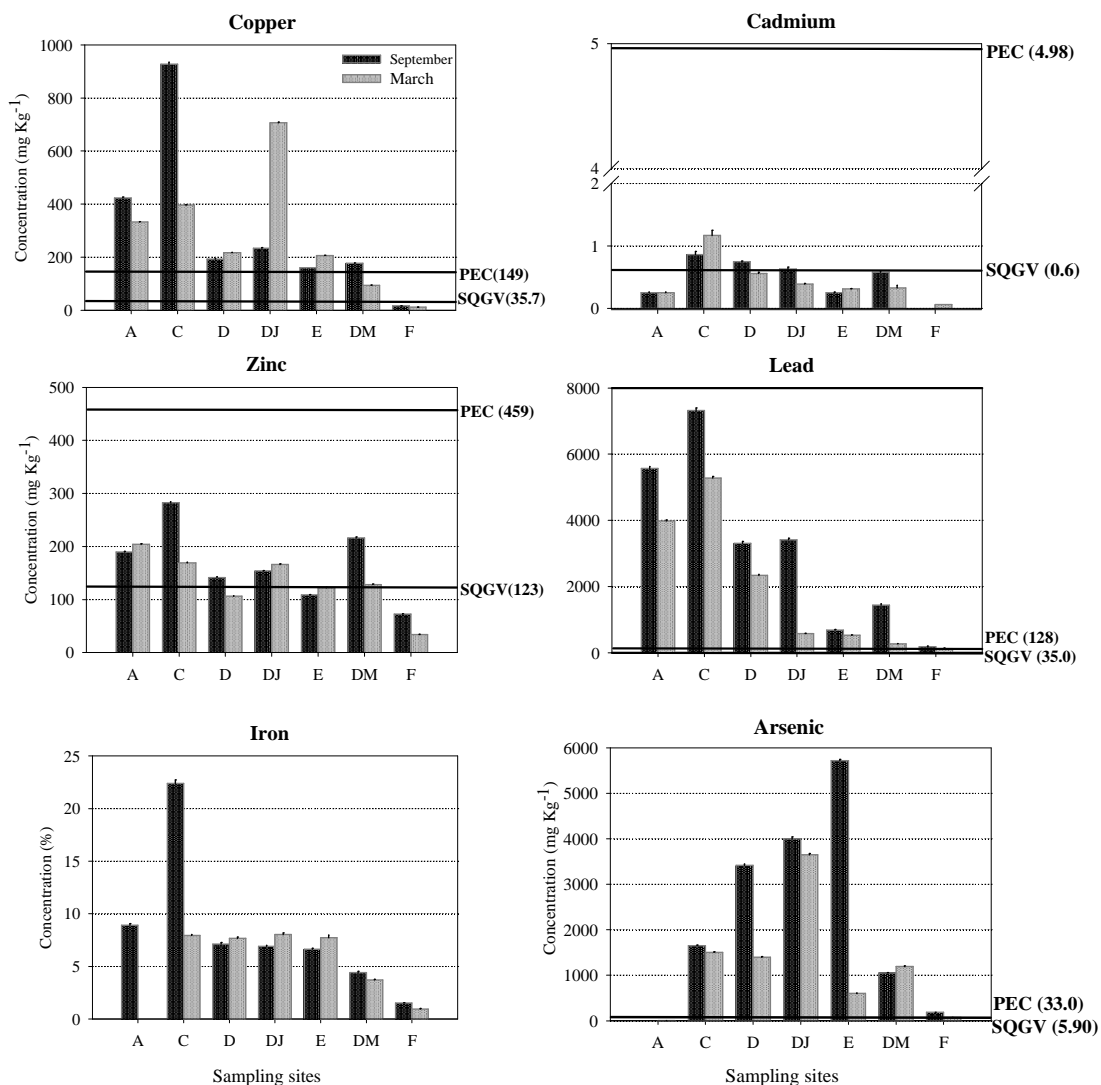
Figure 2.4. Average concentrations (mg Kg⁻¹ dry weight) ± SD (standard deviation) of metals (copper, cadmium, zinc, lead, iron and arsenic) in sediments across sampling sites in São Domingos' aquatic surrounding system. PEC – consensus-based probable concentration (mg Kg-1 dry weight) according to CSST; SQGV – sediment quality guideline value (mg Kg⁻¹ dry weight) according to CCME. Note: no guidelines are available for iron.

Table 2.3. Pearson correlation coefficients for water chemistry data from seven sampling stations. Data for both sampling events are shown. Significant correlations ($p < 0.05$) are shown in boldface. Note that a negative sign before a correlation value indicates a negative correlation, while no sign indicates a positive correlation. (n=7)

SEPTEMBER											
	EC	Fe_{di}	Al_{di}	Mn_{di}	Cu_{di}	Zn_{di}	Fe_{sd}	Al_{sd}	Mn_{sd}	Cu_{sd}	Zn_{sd}
pH	**-0.91**	**-0.80**	-0.73	-0.73	-0.73	-0.59	-0.60	0.092	0.63	-0.54	-0.33
EC	—	**0.88**	**0.94**	**0.87**	0.59	**0.85**	0.48	-0.16	-0.55	0.41	0.28
Fe_{di}	—	—	**0.79**	0.64	0.73	0.71	**0.76**	-0.57	-0.58	0.72	0.53
Al_{di}	—	—	—	**0.91**	0.44	**0.92**	0.22	-0.18	-0.54	0.16	0.06
Mn_{di}	—	—	—	—	0.25	**0.92**	0.13	0.073	-0.51	0.02	-0.06
Cu_{di}	—	—	—	—	—	0.15	0.167	-0.57	-0.53	0.73	0.55
Zn_{di}	—	—	—	—	—	—	0.21	-0.09	-0.43	0.10	0.01
Fe_{sd}	—	—	—	—	—	—	—	-0.55	-0.32	**0.98**	**0.80**
Al_{sd}	—	—	—	—	—	—	—	—	0.38	-0.62	-0.43
Mn_{sd}	—	—	—	—	—	—	—	—	—	-0.26	0.23
Cu_{sd}	—	—	—	—	—	—	—	—	—	—	**0.86**
MARCH											
	C	Fe_{di}	Al_{di}	Mn_{di}	Cu_{di}	Zn_{di}	Fe_{sd}	Al_{sd}	Mn_{sd}	Cu_{sd}	Zn_{sd}
pH	**-0.82**	**-0.91**	-0.71	**-0.78**	-0.74	**-0.95**	**-0.92**	-0.16	**0.77**	-0.74	-0.72
EC	—	**0.96**	**0.98**	**0.99**	**0.98**	0.72	**0.86**	0.34	-0.61	-0.44	**0.81**
Fe_{di}	—	—	**0.92**	**0.94**	**0.93**	0.77	**0.86**	0.16	-0.71	-0.56	0.74
Al_{di}	—	—	—	**0.99**	**1.00**	0.59	0.75	0.28	-0.58	0.37	0.75
Mn_{di}	—	—	—	—	**1.00**	0.68	**0.82**	0.30	-0.64	0.43	**0.77**
Cu_{di}	—	—	—	—	—	0.62	**0.78**	0.28	-0.60	0.40	**0.76**
Zn_{di}	—	—	—	—	—	—	**0.89**	0.22	**-0.78**	0.66	0.65
Fe_{sd}	—	—	—	—	—	—	—	0.52	-0.61	0.68	**0.84**
Al_{sd}	—	—	—	—	—	—	—	—	0.19	0.18	0.60
Mn_{sd}	—	—	—	—	—	—	—	—	—	-0.43	-0.25
Cu_{sd}	—	—	—	—	—	—	—	—	—	—	0.69

Abbreviations: C- Conductivity; M_{di} – dissolved metal; M_{sd} –metal levels in sediment

2.3.2. Water-column bioassays

Water samples collected in September and March from the stations A, C, D, DJ and E affected significantly the survival of *D. magna*, killing all test organisms within 48-hours (Table 2.4). LC_{50}s ranged from 3.70% (CI_{95}: 1.65 – 7.75%) (Station C - September) to 38.8% (CI_{95}: 28.7 – 50.5%) (Station E – March). Water samples from the remaining two sites were not acutely toxic to *D. magna*.

Table 2.4. Percent survival of *D. magna* at the end of the 48-h exposure (in the treatment corresponding to 100% test water) and 48-h LC_{50} values (%) + 95% confidence intervals (CI).

Site	Survival (%) September	48-h LC_{50} (%) September	Survival (%) March	48-h LC_{50} (%) March
A	0	4.38 (1.47-6.70)	0	20.3 (17.0-26.4)
C	0	3.70 (1.65-7.75)	0	10.1 (5.80-14.4)
D	0	7.87 (3.08-11.9)	0	7.97 (1.80-11.0)
DJ	0	4.40 (0.86-7.4)	0	4.40 (0.87-6.28)
E	0	10.8 (7.92-14.2)	0	38.8 (28.7-50.5)
DM	100	100% survival	100	100% survival
F	100	100% survival	100	100% survival

By comparing the 48-h LC_{50} values, the waters from stations A, C and E collected in September were more toxic than those of March. Waters from stations D and DJ in both sampling periods had the same toxicity (i.e. similar 48-h LC_{50} values).

2.3.3. Sediment bioassays

2.3.3.1. Daphnia magna *bioassay*

Percent survival of *D. magna* in the different sediment samples along with the values of physical-chemical parameters at the end of 7-day whole-sediment tests is presented in Table 2.5. With the exception of sediments from reference (F) and control (CTR) sites all sediment samples exhibited marked changes in conductivity and pH values compared to initial values. Fisher's exact test revealed significant acute toxicity in the sediment bioassay of stations A, C, D, DJ and E in September, where no surviving organisms were registered at the end of the bioassay. In March, Fisher's exact test revealed significant lethal toxicity only at stations A, C and DJ. Differences in survival between seasons were found in sediments from sites A, D and E, with 60%, 90% and 70% of survival, respectively, in March versus total mortality for the same sediment samples in September. pH and copper were the water parameters that significantly correlated with sediment *D. magna* survival in September and March, respectively (correlations of 0.90, 0.89, respectively; $p < 0.05$; $n = 7$). Significant differences in fertility between sediment samples were also recorded in September (ANOVA: $F_{2, 28} = 277.3$; $p < 0.001$) and in March (ANOVA: $F_{5, 47} = 145.0$; $p < 0.001$) (Figure 2.5). In both sampling periods the number of eggs produced by daphnia females exposed to contaminated sediment was always lower than those of lab control and reference site. Regarding body length, significant differences were also found between sediment samples in September (ANOVA: $F_{2, 28} = 30.75$; $p < 0.001$) and March (Kruskal-Wallis: $H = 30.29$; $n = 49$; $p < 0.001$). No significant differences in body length were found between daphnids from DM and F sediment samples (Tukey test: $p > 0.05$) in September whereas in the test with March sediments, daphnids from sediment samples A, D, E and DM were smaller than control and reference daphnids (Dunn's method: $P < 0.05$). Comparisons between sampling periods (September and March) revealed significant differences in fertility for station DM (t test: $t(16) = 5.55$; $p \leq 0.001$) whereas no differences were found for F (t test: $t(18) = -0.760$; $p = 0.457$) nor control (t test: $t(18) = -0.962$; $p = 0.349$). Regarding daphnids' length at the end of test, no differences were found between seasons (September/March) at sediments from sites DM, F and control (t test: $t(18) = -0.273$; $p = 0.788$; Mann-Whitney U test: $U = 26.0$ $n=10$ $p = 0.074$; t test: $t(18) = -0.681$; $p = 0.504$, respectively).

Table 2.5. *Daphnia magna* survival at the end of 7-day whole-sediment tests. Temperature (T), pH and conductivity (C) measures (mean ± SD) at the end of the test (7th day) are also presented. Values in boldface indicate a significant reduction in survival (Fisher exact test).

(Range of physical-chemical parameters measured in the beginning of tests: T 19.7-20.0 °C; pH 6.0 -8.1; EC 519 - 625 µS cm-1).

site	September 2006 Survival (%)	T (°C)	pH	C (µS cm-1)	site	March 2007 Survival (%)	T (°C)	pH	C (µS cm-1)
A	**0**	19.7 (0.1)	3.2 (0.006)	1057 (28.2)	A	**60**	19.9 (0.2)	3.3 (0.03)	815.5 (16.6)
C	**0**	19.7 (0.1)	3.0 (0.005)	1084 (21.6)	C	**0**	20.0 (0.2)	3.9 (0.02)	560.5 (1.92)
D	**0**	19.8 (0.1)	3.4 (0.07)	926.0 (41.8)	D	90	19.8 (0.2)	4.5 (0.1)	521.2 (7.93)
DJ	**0**	19.6 (0.1)	4.2 (0.05)	697.5 (11.3)	DJ	**0**	19.9 (0.2)	4.8 (0.2)	573.8 (32.0)
E	**0**	19.7 (0.1)	5.7 (0.3)	590.0 (2.94)	E	70	19.9 (0.3)	6.5 (0.02)	507.2 (4.20)
DM	100	19.5 (0.1)	7.8 (0.008)	617.0 (8.52)	DM	100	19.8 (0.3)	7.7 (0.02)	515.0 (2.40)
F	100	19.8 (0.1)	7.7 (0.02)	608.0 (2.58)	F	100	19.9 (0.3)	7.5 (0.03)	534.0 (4.20)
CTR	100	19.9 (0.1)	7.9 (0.09)	605.3 (1.50)	Ctr	100	19.7 (0.2)	7.8 (0.05)	520.0 (2.10)
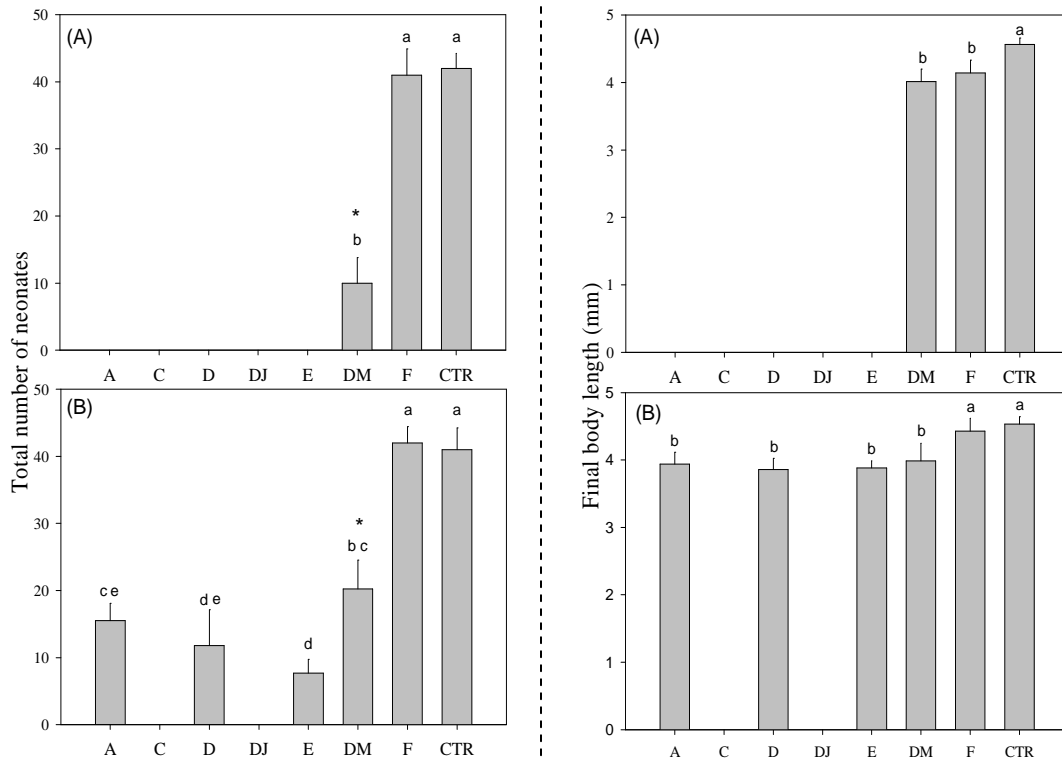
Figure 2.5. Total number of neonates (±SD) and final *D. magna* body length (mm) at the end of a chronic 7-d whole-sediment laboratory test with *D. magna,* conducted in September (A) and March (B). Bars that do not share a letter are significantly different at $p < 0.05$. Bars between panels (site comparisons) that share an asterisk (*) are significantly different at $p < 0.05$.

2.3.3.2. Chironomus riparius *bioassay*

Mean survival of *C. riparius* in the different sediment samples along with the values of physical-chemical parameters at the end of 10-day whole-sediment tests is presented in Table 2.6. With the exception of DM, reference (treatment with sediment from station F) and control (CTR - treatment with laboratory control sediment), all sediment samples exhibited marked changes in pH values compared to the range of values at the beginning of tests. More than 90% of survival was obtained in sediments from sites DM, F and Ctr with sediments from both seasons and in sediment E from

March. Total mortality in sediments from September and March was found in site A (Table 2.6).

Table 2.6. *Chironomus riparius* mean survival (±SD) at the end of 10-day whole-sediment tests. Temperature (T), pH and conductivity (C) measures (mean ± SD) at the end of the test (10th day) are also shown. Survival means followed by the same letter in boldface are not significantly different ($p \geq 0.05$). *(Range of physical-chemical parameters measured in the beginning of tests: T 19.7-21.0 ºC; pH 7.6-8.0; EC. 514- 612 µS cm⁻¹)*

	September 2006					March 2007			
site	Survival (%)	T (ºC)	pH	C (µS cm⁻¹)	site	Survival (%)	T (ºC)	pH	C (µS cm⁻¹)
A	0**a** (0)	19.8 (0.2)	3.5 (0.003)	1023 (38.5)	A	0**a** (0)	19.9 (0.2)	3.5 (0.02)	860.2 (23.6)
C	0**a** (0)	19.7 (0.1)	3.2 (0.004)	1105 (14.7)	C	38**a** (7.45)	20.2 (0.2)	3.7 (0.01)	590.1 (12.9)
D	0**a** (0)	19.6 (0.1)	3.4 (0.007)	945.0 (23.6)	D	41**a** (7.69)	20.1 (0.2)	4.9 (0.2)	589.1 (17.9)
DJ	16**a** (3.75)	19.8 (0.1)	4.5 (0.006)	701.6 (22.3)	DJ	25**a** (7.45)	20.6 (0.2)	4.7 (0.1)	600.8 (41.0)
E	55**b** (5.95)	19.9 (0.1)	5.8 (0.03)	578.0 (29.4)	E	100**b** (0)	20.5 (0.3)	6.0 (0.03)	567.5 (14.6)
DM	100**c** (0)	19.8 (0.1)	7.9 (0.002)	598.0 (7.51)	DM	98**b** (1.67)	19.9 (0.3)	7.9 (0.01)	565.0 (2.70)
F	100**c** (0)	19.8 (0.1)	7.9 (0.01)	597.0 (6.49)	F	100**b** (0)	19.8 (0.3)	7.8 (0.01)	550.5 (8.50)
Ctr	100**c** (0)	19.9 (0.2)	8.0 (0.004)	601.7 (1.34)	Ctr	96.2**b** (2.67)	20.2 (0.2)	7.9 (0.01)	569.0 (7.90)

Significant differences in survival between sites were recorded in September (Kruskal-Wallis: $H = 77.04$; $p \leq 0.001$) and in March (Kruskal-Wallis: $H = 70.13$; $p \leq 0.001$). In September survival in site E was significantly lower than in reference sediments (Tukey

test: $p < 0.05$). In March no significant differences in survival were found between both sediments (Tukey test: $p > 0.05$). Comparisons between sampling periods (September and March) revealed significant differences in survival for sediments collected in sites E, C and D (t tests and Mann-Whitney U test: $p < 0.05$), with 100%, 38% and 41% survival in March, respectively, versus 55% survival (E) and total mortality (C, D) in September. There were significant differences in the growth (body length increase) of *C. riparius* larvae between sediments from September ($F_{4, 294} = 563.0$; $p < 0.001$) and March ($H = 225.5$; $n = 374$; $p < 0.001$) (Figure 2.6). With exception of reference sediments (F) and those of DM site, all the other sites differ significantly in the *C. riparius* growth in September (Tukey test: $p < 0.05$) and in March (Dunn's method: $p < 0.05$). There were also significant differences in the biomass (body dry weight) of chironomid larvae across sediment samples in September ($H = 129.6$; $n = 296$; $p < 0.001$) and March ($H = 193.1$; $n = 374$; $p < 0.001$). Control (Ctr) and reference (F) sediments did not differ significantly in larvae biomass in September and March (Dunn's test: $p < 0.05$). Comparisons between sampling periods (September and March) revealed significant differences in body length and body weight for sediments from sites DJ, E, DM and F (t tests: $p < 0.05$).

Significant correlations (ρ) were found between chironomid survival and the abiotic parameters pH ($\rho = 0.996$; $p < 0.001$; $n = 7$), Pb in sediment ($\rho = - 0.819$; $p < 0.05$; $n = 7$) and As in sediment ($\rho = - 0.760$; $p < 0.05$; $n = 7$) in September. In March, chironomid survival correlated significantly with pH ($r = +0.898$; $p = 0.006$; $n = 7$), Zn in sediment ($\rho = - 0.755$; $p < 0.05$; $n = 7$) and Fe in sediment ($\rho = - 0.760$; $p < 0.05$; $n = 7$). Regarding body length and body dry weight, both were positively correlated with pH in September (body length: $\rho = +0.985$; $p < 0.05$; $n = 4$; body dry weight: $\rho = +0.987$; $n = 4$; $p < 0.05$) and in March (body length: $\rho = +0.912$; $n = 6$; $p < 0.05$; body dry weight: $\rho = +0.923$; $n = 4$; $p = 0.009$).
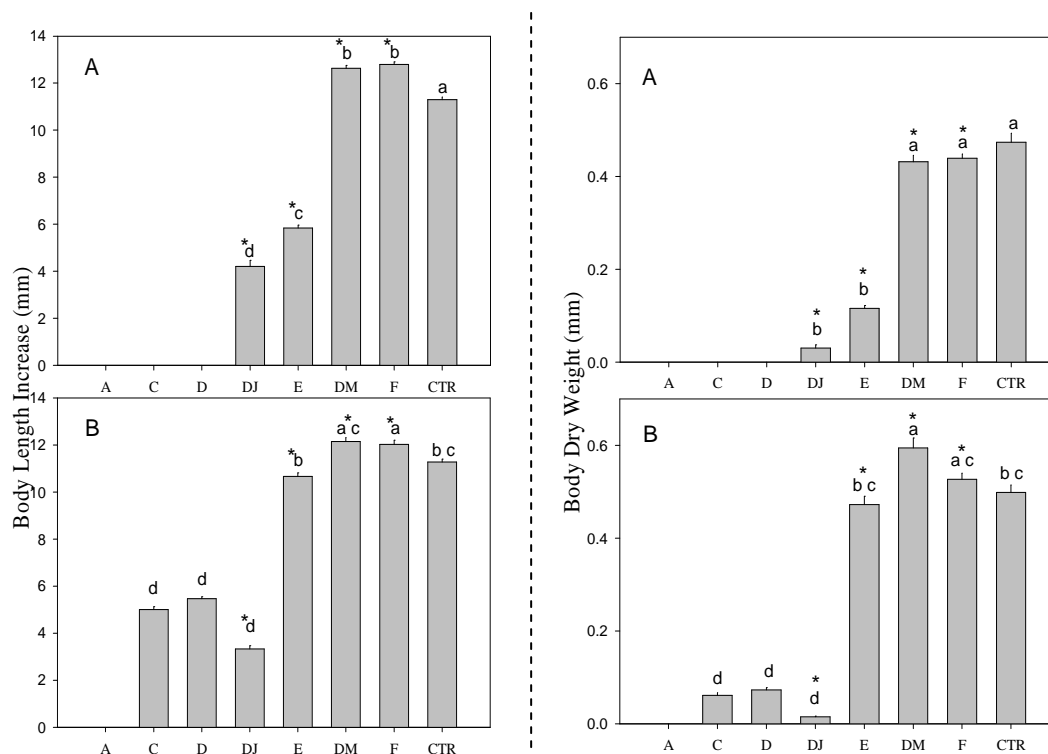
Figure 2.6. Body length increase (±SE) and body dry weight (±SE) at the end of a chronic 10-d whole-sediment laboratory test with *Chironomus riparius*, conducted in September (A) and March (B). Bars that do not share a letter are significantly different at p < 0.05. Bars between panels (site comparisons) that share an asterisk (*) are significantly different at $p < 0.05$.

2.4. Discussion

2.4.1. Chemical characterization of water and sediments

Stations located upstream the confluence with Mosteirão tributary (A and C), the one at the confluence (D) and the other downstream the confluence in the Mosteirão stream (DJ) were the most impacted sites, presenting the lowest values of pH and the highest values of conductivity, sulphate ions and dissolved metals. Site E showed an intermediate degree of contamination whereas DM and F were stations with the lowest levels of conductivity, sulphate ions and dissolved metals and with pH close to neutrality. The characteristics of stations A, C, D and DJ are consistent with those

expected in mining areas impacted with highly porous waste deposits and tailings, such as São Domingos mine. In fact, continuous oxidation/dissolution of pyrite and other sulphide minerals in a weathering environment produces an acid mine drainage rich in hydrogen ions, sulphate ions and dissolved metals (Bryan *et al*., 2006; Costa and Duarte, 2005; De Vos *et al.*, 2005; Reimann and Caritat, 1998). The oxidation of most sulphides can follow several routes depending on factors such as the pH, availability of potential oxidants (e.g. ferric ion) and occurrence of acidophilic microorganisms, the latter contributing to a great increase in the rate of acid generation (Costa and Duarte, 2005). The type of metals occurring in the acid drainage is dependent on the nature of sulphide minerals (Bryan *et al*., 2006; Costa and Duarte, 2005). Iron, copper and zinc and, to a lesser extent, cadmium, lead and arsenic, were the metals found in highest quantities dissolved in the water column, which is a common feature of mine waters draining massive sulphide deposits (España *et al*., 2005). Aluminum and manganese were also elements found in high quantities in the dissolved fraction. The geochemical study of Bryan *et al.* (2006) have also identified copper, zinc and iron as the most readily mobilized metals in São Domingos mine wastes. The higher conductivity levels and dissolved metals recorded in September as compared to March were related with the lack of precipitation during the summer period. In fact, when the flow increases due to precipitation events generally a decrease in concentration of dissolved metals is observed due to the dilution effect of the less concentrated surface runoff (Langmuir, 1997). The exception was observed for lead, which was present in higher concentrations in March. The same behaviour for lead was found by Cánovas and co-workers (2005) who observed lead concentration to be increased with water flow during floods, contrary to other elements. The negative correlations with pH observed for iron, zinc and copper are caused by their good solubility at low pH environments (Gerhardt *et al*., 2004; Stumm and Morgan, 1981). In fact, lower pH values increase the competition between metal and hydrogen ions for binding sites and may also dissolve metal-carbonate complexes, releasing free metal ions into the water column (Stumm and Morgan, 1981). Conductivity was significantly positively correlated with dissolved iron, aluminium, manganese, copper and zinc, which was expected since conductivity is directly related with the concentration of ions in solution.

Concentrations of metals in sediments were determined in the fraction below 63 μm since this appears to be the most useful fraction for contaminant assessments, helping to minimize the "dilution" effect of larger particles with low element concentrations (Burton, 1991). In fact, this fraction consists of finely grained minerals like quartz, carbonates, feldspars, clays and organic matter, which are often coated with iron and manganese (hydro)-oxides. The resulting coating, due to its chemical nature and high surface area, serves as an active sorption site for metals (Burton, 1991; Yu *et al*., 2001). Again, stations A, C, D and DJ were the most impacted sites in terms of sediment contamination, with arsenic being the exception, with its highest level in station E (September sampling). Station E and DM showed an intermediate level of sediment contamination whereas F showed the lowest metal concentrations in sediments. In general, sediments were highly contaminated with arsenic and lead and, in a lesser degree, with copper, zinc, cadmium and iron. Concentration of iron in sediments was significantly positively correlated with both copper and zinc in sediments. These significant correlations (ρ-values ≥ 0.80) can be related with the presence of iron-oxides which are controlling sorption of metals, in this case copper and zinc (Burton, 1991; Yu *et al*., 2001). Although concentrations in sediments were less variable between seasons when compared with metal levels in surface waters, some degree of variation was found. The metal binding dynamics in sediments is dependent on several factors, such as pH, redox potential, sorption/desorption potential, presence of manganese and iron oxides and others, along with the dynamics of the system (e.g. flow-induced resuspension) (Burton, 1991). In fact, those iron-oxides and other iron-phases in sediments are unstable precipitates, which can be either transported as colloids downstream during high flow conditions or, on the other hand, can be transformed to more stable mineral forms (España *et al*., 2005).

In the São Domingos aquatic surrounding system, both spatial and temporal heterogeneity of metal pollution occurred. This heterogeneity was expected mainly due to the dynamics of the system, highly dependent on climatic conditions. Other hydrological and chemical assessments conducted in the area have also revealed that the referred ecosystem is affected by episodic disturbance events (flash floods) of dramatic extent additionally to both metal and pH stress (Gerhardt *et al*., 2004; Janssens de Bisthoven *et al*., 2005).

The use of environmental quality guidelines facilitates site-specific evaluation as well as the comparison with other metal-contaminated areas (Bird *et al.*, 2003). By the parameters analysed station F confirmed to be an acceptable reference station as concentrations of nutrients and dissolved metals were within the accepted levels of quality for surface waters (MA, 1998). The only exceptions were related with arsenic and lead concentrations in sediments, which were slightly above the SQGVs defined by CCME for the protection of aquatic life (CCME, 2001). Station DM, in turn, showed some degree of concern relative to the concentrations of sulphate ions (290.7 mg l^{-1}) and dissolved manganese (1.0 mg l^{-1}) in September, which were above the Portuguese maximum admissible value of 250 mg l^{-1} for sulphate ions and the Portuguese maximum recommended value (0.05 mg l^{-1}) for manganese, respectively. Data concerning metals in sediments for DM station were more critical, with concentrations of copper, cadmium, zinc, lead and arsenic fairly above the guideline values (USEPA, 2000).

2.4.2. Toxicity of waters

In general, significant acute toxicity to *D. magna* was found for all tested waters (with the exception of stations DM and F). This high toxicity was associated with high levels of dissolved metals and low pH values. Indeed, copper, cadmium, lead, iron and zinc, were found in concentrations far above the water quality guideline values established for protection of aquatic life and human health (USEPA, 2006). Barata and colleagues (1999) reviewing existing information on acute responses of *D. magna* to metals reported 48 h-LC_{50} values ranking from 0.002 to 0.05 mg l^{-1} for cadmium and from 0.01 to almost 0.1 mg l^{-1} for copper. In this study, the lowest concentration of dissolved copper found (considering stations A, C, D, DJ and E) was 2.10 mg l^{-1} (station E, September), which is much higher than any of the 48-h LC_{50} values found in the review of Barata *et al.* (1999). Regarding dissolved cadmium, the highest value found (0.18 mg l^{-1}) was above the highest 48 h-LC_{50} value reported for cadmium (Barata *et al.*, 1999). Dissolved zinc was found in the studied sites in concentrations between 4.18 and 60 mg l^{-1}, which were above the 48 h-LC_{50}s values for zinc found in the literature, usually between 1 and 3 mg l^{-1} (Barata *et al.*, 1998; Diamantino *et al.*, 2001; Erten-Unal

et al., 1998). For sites A, C and E the LC_{50}s in March were higher than the ones found in September, which can be related with a decrease in dissolved metal concentrations. Although a similar decrease in the concentrations of dissolved metals have been observed in sites D and DJ (between sampling periods), LC_{50}s determined for both September and March were similar. This can be attributable to several factors. First, toxicity of metals to freshwater organisms has been shown to be dependent on the free metal ion species, which vary dramatically with hardness and dissolved organic carbon concentration, which control metal bioavailability (De Schamphelaere and Janssen, 2002; Di Toro *et al.*, 2001). Second, along with the combined effects of each metal there's a need to take into account the complexity of acid mine drainage itself. The chemical and biochemical complexities involved when organisms are exposed to mixtures of metals along with synergistic and other effects derived from chemical interactions makes the interpretation of toxicity effects a hard task. In addition to high dissolved metal concentrations, iron precipitation could have also contributed to the high toxicity of waters observed. Iron precipitation took place due to the rise in pH in sediment samples with different proportions of test waters and ASTM water (mainly 25%, 50% and 75% treatments). In fact, it was observed in the bottom of test beakers from these sediment samples a visible orange precipitate indicating precipitation of iron hydroxides. The precipitation of these iron hydroxides can have detrimental effects on organisms, such as disruption of intestine membranes, clogging of the digestive tract, and coating of gill surfaces (Gerhardt, 1993). Soucek and co-workers (2000) studying the toxicity of iron precipitates in the laboratory to 5-day old *D. magna* found that with pure ferric hydroxide precipitate as bottom substrate, all of the test organisms died within 48-hours. Contamination of surface waters was documented in early investigations conducted in the area (Lopes *et al.*, 1999; Pereira *et al.*, 1999; Pereira *et al.*, 2000) and in all the toxic potential of acid waters to *D. magna* and *C. dubia* was confirmed.

2.4.3. Toxicity of sediments

In general, all tested sediments (with the exception of the ones from stations DM and F) elicited detrimental effects to both *D. magna* and *C. riparius*. In September, all

individuals exposed to sediments collected at sites A, C, D, DJ and E in the whole-sediment test with *Daphnia magna* were dead at the second day. This could be mainly due to pH values in the overlying water, since a significantly positive correlation was found between pH and survival of *D. magna* for September tests. Regarding March sediments, a significantly negative correlation was found between copper levels in sediments and survival of *D. magna*. Both dissolved and sediment-bound fractions could have affected daphnids. In fact, daphnids can be affected directly by metals in sediments since those organisms behave as nonselective epifaunal zooplankton, being frequently observed on the sediment surface and, in this way, are easily exposed through ingestion to particulate-bound contaminants (ASTM, 2000). Fertility was more sensitive to sediment contamination than growth (body length). In fact, significant differences in fertility were found in March for daphnids exposed to sediments DM and E, whereas body length was not significantly different between sediment samples. Differences in fertility were found for DM and E between stations in both sampling events, which could be attributable to differences in metal composition of both water-column and sediment and differences in metal bioavailability in both compartments. Regarding the value of using zooplankton species to evaluate sediment toxicity potential, Stemmer and coworkers (1990) argued that their routes of exposure do not reproduce those of benthic invertebrates, which are exposed to greater sediment surface area and interstitial water. However, comparison studies of species sensitivity in whole sediment tests have shown daphnids sensitivity to be similar (Sasson-Brickson and Burton, 1991) or to be even higher than some benthic species (Giesy *et al*., 1990).

The toxicity of metal contaminated sediment to *C. riparius* larvae was reflected by the significantly reduced survival, larval growth and biomass that occurred in the impacted stations, compared to the reference ones. Significant correlations occurred between chironomid survival and metal concentrations in sediments, which is indicative that survival was related with metals' contamination in sediments. In whole-sediment tests with *C. riparius*, all individuals from sediments from sites A, C and D were dead at the end of the test in September. This was due to water pH values, and lead and arsenic concentrations in sediments, since significant correlations between pH and chironomid survival (positive correlation) and between lead/arsenic concentrations and chironomid survival (negative correlations) were found for September tests. In fact, lead and arsenic

were the metals found in higher concentrations in September sediments, with sediment levels of lead and arsenic being far above the consensus-based PECs according to CSST and the SQGVs according to CCME. Survival in March, in turn, was significantly correlated with pH (positive correlation) and with zinc and iron levels in sediments (negative correlations) and only in sediment A with total mortality occurred. These results are indicative that *C. riparius* survival was highly affected by metal concentrations in sediments. The high sensitivity of *C. riparius* larvae to metal contamination in the sediment was expected since chironomids are benthic (bottom-dwelling) organisms and functionally are collector-gatherers, feeding on detritus from sediments (Vos, 2001). Significant reductions in larvae growth and biomass were found for most impacted stations, compared to the reference site (F). Both *C. riparius* growth and biomass were significantly positively correlated with pH in the overlying water. The metal binding dynamics in sediments is dependent on several factors, such as pH, redox potential, sorption/desorption potential, presence of manganese and iron oxides and others, along with the dynamics of the system (e.g. flow-induced resuspension) (Burton, 1991). The increase of pH in the water column could have contributed for the precipitation of metals (exchanged from and onto the sediment after the addition of ASTM medium to the contaminated sediments) in the surface of sediments (e.g. in the form of metal-carbonate complexes), reducing their bioavailability. Differences in toxicity could have also been related with differences in the organic matter content of sediments, but in general organic matter content was low and similar among sites. Overall results from toxicity tests allowed the discrimination between stations in terms of toxicity to both *D. magna* and *C. riparius*.

2.4.4. Station E as a heterogeneous environment

Station E was the chosen station for collecting *D. longispina* individuals for tolerance development studies to metals copper and zinc (following chapters) and this seasonal study have illustrated the temporal heterogeneity of this site in terms of physical-chemical characteristics. From September to March, differences in water and sediment chemistry were clear. An increase in pH, along with decreases in conductivity, nitrites, sulphates, phosphates and dissolved metals (e.g. cadmium, iron, zinc) occurred

from September to March. In the *D. magna* acute tests with water, an increase in the LC$_{50}$ from September to March revealed a decrease in the toxic conditions in March. Regarding whole-sediment tests (*D. magna* and *C. riparius* exposures) total mortality was observed in September whereas in March the percent survival was not significantly different from reference, although the fertility and growth have been affected.

2.5. Conclusion

Acid mine drainage from São Domingos mine have been causing a highly chemical impact in its surrounding aquatic system. Differences in water and sediment chemistry for the impacted sites were found between the dry (September) and the wet (March) periods. This trend was also observed on the laboratory toxicity tests done with both water and sediment collected in the two different periods. *D. magna* survival was highly affected by the toxicity of the waters from all the sites excepting reference and the station in the Mosteirão tributary, upstream the pH-mixing zone; survival, fertility and growth of *D. magna* along with survival and growth of *C. riparius* were negatively influenced by metal contamination in the stations located nearby the São Domingos mine. The obtained results illustrate the temporal heterogeneity of this aquatic system in terms of contamination and help to characterize the station selected for cladoceran sampling (Chapter 3 and Chapter 4) as a metal polluted heterogeneous environment.

Appendix.1. Average concentrations of dissolved trace elements and respective standard deviations (in brackets) for the sampling sites (mg l^{-1}, n=3 determinations).

Metal		A	C	D	DJ	E	DM	F
Cu	Sep	11.0 (0.308)	8.40 (0.0880)	3.20 (0.0315)	5.00 (0.0697)	2.10 (0.0160)	3.90×10^{-3} (8.0×10^{-5})	2.90×10^{-3} (1.5×10^{-4})
	Mar	8.07 (0.0796)	3.14 (0.0326)	2.55 (0.0212)	2.37 (0.0209)	2.11 (0.0196)	2.52×10^{-3} (4.0×10^{-5})	1.30×10^{-3} (8.0×10^{-5})
Zn	Sep	7.70 (0.151)	22.0 (0.162)	60.0 (0.548)	32.0 (0.319)	12.0 (0.161)	0.039 (0.36×10^{-3})	0.024 (0.62×10^{-3})
	Mar	4.74 (0.0540)	6.57 (0.0471)	5.15 (0.0529)	4.65 (0.0461)	4.18 (0.0427)	b.l.	b.l.
As	Sep	b.l.	0.160 (0.00106)	4.60 (0.0420)	0.290 (0.0018)	0.010 (2.3×10^{-4})	b.l.	b.l.
	Mar	0.0107 (0.0010)	0.0020 ($5.0 10^{-5}$)	0.150 (2.0×10^{-5})	0.0449 (0.0022)	0.0076 (1.5×10^{-4})	0.00107 (1.0×10^{-5})	0.0045 (9.0×10^{-3})
Cd	Sep	0.024 (8.0×10^{-5})	0.097 (2.8×10^{-5})	0.180 (4.3×10^{-4})	0.170 (2.9×10^{-4})	0.058 (5.0×10^{-4})	b.l.	b.l.
	Mar	0.020 (0.0023)	0.038 (7.8×10^{-4})	0.034 (0.0011)	0.030 (0.0022)	0.027 (7.9×10^{-4})	b.l.	b.l.
Pb	Sep	3.0×10^{-3} (5.0×10^{-5})	0.420 (0.0025)	0.044 (1.8×10^{-4})	0.011 (9.0×10^{-5})	0.049 (3.9×10^{-4})	6.1×10^{-3} (0.00)	b.l.
	Mar	2.2×10^{-3} (2.0×10^{-5})	0.154 (0.0058)	0.180 (0.0048)	0.160 (0.0018)	0.139 (8.9×10^{-4})	2.0×10^{-4} (0.00)	b.l.
Fe	Sep	56.0 (1.276)	88.0 (0.739)	78.0 (0.704)	48.0 (0.567)	16.0 (0.211)	b.l.	b.l.
	Mar	40.9 (0.898)	20.7 (0.165)	25.9 (0.154)	21.6 (0.123)	11.3 (0.089)	0.0362 (0.0015)	b.l.
Al	Sep	140 (2.818)	98.0 (1.115)	270 (1.039)	150 (1.850)	60.0 (0.868)	0.047 (0.0010)	0.051 (0.0038)
	Mar	82.8 (1.198)	30.1 (0.257)	23.6 (0.189)	22.0 (0.231)	20.0 (0.155)	0.0113 (9.0×10^{-5})	2.15 (2.0×10^{-5})
Mn	Sep	8.10 (0.180)	7.70 (0.080)	22.0 (0.225)	19.0 (0.248)	9.90 (0.118)	1.0 (0.0072)	6.0×10^{-3} (2.6×10^{-4})
	Mar	4.70 (0.0457)	2.14 (0.0328)	1.69 (0.0341)	1.57 (0.0278)	1.48 (0.0237)	0.0103 (8.0×10^{-5})	b.l.
Si	Sep	49.0 (1.424)	21.0 (0.191)	0.062 (0.601)	0.019 (0.132)	0.042 (0.525)	0.0061 (0.0250)	3.70×10^{-4} (7.2×10^{-3})
	Mar	33.8 (0.560)	8.05 (0.0866)	8.12 (0.0740)	7.71 (0.0326)	7.50 (0.0652)	0.892 (0.0129)	1.42 (0.02178)
Cr	Sep	0.037 (4.8×10^{-4})	0.033 (2.0×10^{-4})	0.079 (5.0×10^{-4})	0.030 (2.0×10^{-4})	0.0078 (5.0×10^{-5})	0.0039 (4.0×10^{-5})	0.0036 (3.0×10^{-5})
	Mar	0.033 (3.4×10^{-4})	0.015 (1.4×10^{-4})	0.012 (1.3×10^{-4})	0.012 (9.0×10^{-5})	0.0083 (5.0×10^{-5})	6.2×10^{-4} (5.0×10^{-5})	6.7×10^{-4} (0.00)

Appendix.1. *(cont.)*

Metal		A	C	D	DJ	E	DM	F
Ni	Sep	0.210 (3.4×10⁻³)	0.150 (1.2×10⁻³)	0.570 (2.7×10⁻³)	0.290 (2.1×10⁻³)	0.140 (1.0×10⁻³)	0.0030 (3.0×10⁻⁵)	0.0022 (7.0×10⁻⁵)
	Mar	0.183 (2.0×10⁻³)	0.057 (1.7×10⁻³)	0.049 (7.7×10⁻⁴)	0.046 (8.7×10⁻⁴)	0.041 (1.1×10⁻⁴)	8.4×10⁻⁴ (7.0×10⁻⁵)	5.5×10⁻⁴ (1.0×10⁻⁵)
Se	Sep	0.010 (1.2×10⁻⁴)	0.0035 (3.9×10⁻⁴)	b.l.	0.0015 (6.5×10⁻⁴)	0.0014 (3.4×10⁻⁴)	0.0016 (1.5×10⁻⁴)	b.l.
	Mar	0.012 (2.3×10⁻⁴)	0.0020 (1.6×10⁻⁴)	0.0019 (6.0×10⁻⁵)	0.0017 (1.2×10⁻⁴)	0.0016 (2.9×10⁻⁴)	3.8×10⁻⁴ (6.0×10⁻⁵)	4.0×10⁻⁴ (7.0×10⁻⁵)
Sn	Sep	0.008 (4.0×10⁻⁵)	0.0038 (9.0×10⁻⁵)	b.l.	0.0014 (2.0×10⁻⁵)	0.013 (7.0×10⁻⁵)	0.0060 (8.0×10⁻⁵)	0.0036 (9.0×10⁻⁵)
	Mar	b.l.	b.l.	0.0083 (1.7×10⁻⁴)	0.0044 (1.0×10⁻⁴)	b.l.	0.0010 (7.0×10⁻⁵)	b.l.
Ag	Sep	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.
	Mar	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.

Abbreviations: b.l. - below detection limit

Appendix.2. Average trace elements concentrations in the particulate fraction for the sampling sites (µg l⁻¹, n=6 determinations). Si, Ni, Se, Sn and Ag were below detection limits.

Metal		A	C	D	DJ	E	DM	F
Cu	Sep	10.5 (0.49)	12.3 (0.34)	4.40 (0.21)	5.3 (0.067)	2.8 (0.30)	3.2 (1.29)	b.l.
	Mar	6.56 (0.34)	10.50 (0.56)	3.79 (0.11)	3.89 (0.06)	b.l.	1.5 (0.08)	b.l.
Zn	Sep	b.l.	24.2 (1.91)	62.5 (0.28)	19.7	b.l.	30.2 (17.9)	b.l.
	Mar	b.l.	14.2 (0.90)	42.1 (0.98)	11.8	b.l.	b.l.	b.l.
As	Sep	b.l.	7.60 (2.37)	2818.2 (238.87)	16.1 (0.62)	b.l.	b.l.	b.l.
	Mar	b.l.	6.23 (1.30)	1918.5 (138.22)	b.l.	b.l.	b.l.	b.l.
Cd	Sep	b.l.	0.1 (0.004)	0.20 (0.01)	0.20 (0.009)	b.l.	b.l.	b.l.
	Mar	b.l.	0.2 (0.008)	0.2 (0.02)	0.15 (0.008)	b.l.	b.l.	b.l.
Pb	Sep	1.8 (0.28)	24.8 (0.75)	3.7 (0.30)	2.9 (0.05)	b.l.	18.8 (1.73)	0.90 (0.16)
	Mar	0.9 (0.09)	14.7 (0.55)	1.7 (0.60)	1.5 (0.03)	b.l	9.87 (0.73)	0.67 (0.06)
Fe	Sep	138 (16.87)	381 (21.65)	4673.6 (491.69)	1056.6 (48.70)	147.6 (8.78)	116 (0.46)	b.l.
	Mar	b.l.	189 (11.85)	2623.5 (311.80)	857.1 (48.79)	179.4 (4.90)	b.l.	b.l.
Al	Sep	183.0 (16.05)	231.6 (34.18)	305.6	240.4 (38.22)	51.9 (11.73)	140.7 (7.05)	673.5 (92.0)
	Mar	145.0 (9.05)	98.6 (14.12)	217.9	200.6 (18.72)	41.0 (5.73)	110.1 (2.05)	573.5 (32.0)
Mn	Sep	5.90 (0.22)	7.80 (0.35)	18.7 (1.90)	14.9 (0.61)	8.5 (1.27)	215.9 (19.1)	195.4 (16.92)
	Mar	4.90 (0.09)	6.70 (0.56)	12.5 (1.10)	9.87 (0.31)	5.8 (0.27)	115.6 (9.80)	115.6 (11.90)
Cr	Sep	0.70 (0.27)	0.60 (0.067)	0.90 (0.07)	0.50 (0.03)	0.40 (0.02)	0.50 (0.02)	1.0 (0.02)
	Mar	0.50 (0.17)	0.46 (0.050)	0.70 (0.05)	0.30 (0.01)	0.30 (0.01)	0.20 (0.01)	0.89 (0.02)

Abbreviations: b.l. - below detection limit

Appendix.3. Average sediment trace elements concentrations for the sampling sites ([a] mg Kg^{-1}; [b] %, n=3 determinations).

Metal		A	C	D	DJ	E	DM	F
Cu [a]	Sep	423.4 (2.90)	926.7 (8.07)	192.0 (2.61)	233.3 (2.90)	160.0 (0.5)	177.2 (1.31)	16.37 (0.22)
	Mar	332 (1.34)	396 (1.89)	217 (1.01)	706 (2.98)	206 (1.25)	93.6 (0.98)	11.2 (0.34)
Zn [a]	Sep	189.6 (1.22)	282.4 (1.80)	141.3 (1.61)	154.2 (0.67)	109.0 (0.33)	216.1 (1.87)	72.60 (0.26)
	Mar	204 (1.13)	169 (1.34)	106 (1.20)	166 (1.10)	122 (1.34)	128 (1.14)	34.1 (0.26)
As [a]	Sep	1651 (11.91	3414 (25.49	3999 (43.68)	5714 (31.57)	1050 (8.50)	185.5 (0.76)	46.8 (1.08)
	Mar	1500 (15.46)	1400 (13.90)	3650 (23.40)	598 (10.40)	1190 (12.89)	76.3 (1.29)	32.1 (0.46)
Cd [a]	Sep	0.25 (0.01)	0.86 (0.05)	0.75 (0.01)	0.63 (0.03)	0.25 (0.01)	0.58 (0.01)	b.l.
	Mar	0.25 (1.01)	1.17 (0.08)	0.56 (0.02)	0.39 (0.01)	0.31 (0.01)	0.33 (0.04)	0.06 (0.00)
Pb [a]	Sep	5565 (61.43)	7322 (74.68)	3309 (52.67)	3415 (46.90)	685 (21.04)	1440 (32.23)	187.2 (16.1)
	Mar	3980 (26.89	5280 (43.86)	2340 (25.55)	579 (10.41)	532 (8.90)	268 (12.65)	124 (15.34)
Fe [b]	Sep	8.93 (0.15)	22.4 (0.34)	7.13 (0.15)	6.90 (0.11)	6.65 (0.08)	4.43 (0.10)	1.53 (0.02)
	Mar	9.59 (0.24)	7.95 (0.09)	7.67 (0.12)	8.05 (0.15)	7.74 (0.23)	3.72 (0.06)	0.94 (0.08)
Al [b]	Sep	4.59 (0.09)	3.57 (0.06)	5.40 (0.13)	5.53 (0.11)	7.61 (0.12)	6.44 (0.13)	4.65 (0.05)
	Mar	7.43 (0.08)	6.77 (0.14)	6.03 (0.07)	6.74 (0.17)	8.42 (0.18)	7.70 (0.11)	4.86 (0.10)
Mn [a]	Sep	42.48 (0.33)	51.17 (0.60)	73.45 (0.98)	98.07 (0.77)	167.0 (0.66)	1847 (39.32)	241.6 (1.29)
	Mar	58.7 (0.43)	107 (0.27)	84.2 (0.32)	203 (0.56)	205 (0.76)	1030 (15.67)	340 (1.43)
Si [b]	Sep	20.55 (0.27)	9.06 (0.09)	25.93 (0.43)	19.81 (0.24)	26.40 (0.26)	28.75 (0.53)	40.43 (0.41)
	Mar	23.1 (0.11)	15.5 (0.14)	24.6 (0.23)	8.27 (0.11)	20.2 (0.27)	30.6 (0.43)	35.6 (0.22)

Appendix.3. *(cont.)*

Metal		A	C	D	DJ	E	DM	F
Cr [a]	Sep	59.75 (0.71)	43.04 (0.48)	61.18 (0.95)	61.40 (0.31)	74.10 (0.34)	73.81 (0.83)	34.49 (0.22)
	Mar	74.2 (1.01)	63.7 (0.56)	52.8 (0.33)	62.6 (0.35)	76.4 (0.44)	68.1 (0.56)	20.7 (0.12)
Ni [a]	Sep	8.37 (0.10)	8.18 (0.06)	10.57 (0.16)	11.24 (0.10)	21.80 (0.27)	31.96 (0.33)	13.27 (0.04)
	Mar	11.8 (0.23)	17.8 (0.12)	8.63 (0.09)	17.8 (0.13)	27.1 (0.17)	31.8 (0.11)	7.83 (0.09
Se [a]	Sep	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.
	Mar	21.1 (0.21)	9.98 (0.09)	7.48 (0.08)	b.l.	b.l.	b.l.	b.l.
Sn [a]	Sep	79.66 (0.56)	380.2 (2.83)	142.4 (1.43)	98.43 (1.43)	93.57 (0.21)	27.10 (0.69)	26.87 (0.16)
	Mar	343 (43.90)	1330 (23.0)	478 (11.9)	b.l.	b.l.	b.l.	b.l.
Ag [a]	Sep	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.	b.l.
	Mar	5.95 (0.05)	16.7 (1.20)	1.93 (0.05)	b.l.	b.l.	16.1 (1.10)	b.l.

Abbreviations: b.l. - below detection limit

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CHAPTER 3

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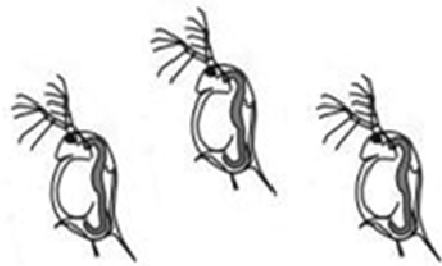
Genetic costs of tolerance to metals in *Daphnia longispina* populations historically exposed to a copper mine drainage
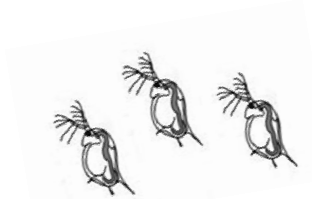
Chapter

3

Genetic costs of tolerance to metals in *Daphnia longispina* populations historically exposed to a mine drainage

Abstract

The present study was conducted to assess three microevolutionary aspects of adaptation to pollution in a *Daphnia longispina* population historically exposed to an acid mine drainage from an abandoned pyrite mine: pollution mediated effects in acute tolerance to copper (Cu) and zinc (Zn); pollution-mediated effects on genetic variability of tolerant and physiological traits related to fitness (feeding rates); and fitness costs of tolerance measured as genetic trade-offs between tolerance and feeding rates under none and low levels of contamination. These objectives were addressed by comparing broad sense heritabilities and genetic correlations using up to 20 distinct clonal lineages randomly obtained from two populations: one located in a water reservoir contaminated by the acid mine drainage, and the other located in a nearby clean water reservoir. Results showed that only sensitive and resistant lineages to Cu were present in the reference and contaminated site, respectively. For Zn, however, both populations had a similar distribution pattern of sensitivities. Heritability values for tolerant and feeding traits across metal exposure levels was similar in both populations being in most cases greater than 50%. Fitness costs of tolerance were illustrated by lower feeding rates of the tolerant population compared to the reference one and negative genetic correlations between mean clonal feeding rates and median clonal survival time in control conditions (no added Cu or Zn). The results obtained thus support the view that tolerance to pollution is ecologically costly.

3.1. Introduction

Long-term ecological consequences of genetic changes due to pollution, microevolutionary, or genetic erosion effects *sensu* Medina *et. al.* (2007) and Van Straalen and Timmermans (2002) respectively, is rarely considered into the ecological risk assessment process despite the fact that genetic variation is one of the pillars of biodiversity and evolution (Bagley *et al*., 2002; Hoffmann and Parsons, 1997; Medina *et. al.,* 2007; Posthuma and Van Straalen, 1993; Van Straalen, 1999; Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002). Nevertheless, several studies have demonstrated that pollutant-induced selection can alter the genetic integrity of a population by replacing original genotypes with others that are different, not only in terms of tolerance to the pollutant at play, but also to other environmental variables (Hoffmann and Parsons, 1997; Medina *et al.,* 2007; Posthuma and Van Straalen, 1993; Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002). Pollutant-induced selection of resistant genotypes can have long-term ecological impacts, such as the reduction in the overall genetic variability, which in turn can lead to the loss of heterozygosity and increased sensitivity to novel environmental stresses (Hoffmann and Parsons, 1997; Medina *et al.,* 2007; Posthuma and Van Straalen, 1993; Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002). Nevertheless, there is little empirical evidence supporting the general view that selection processes lead to negative evolutionary side effects (Barata *et al*., 2002; Medina *et al.,* 2007; Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002). By predicting that the development of resistance to toxic stress involves a cost theories on adaptive change imply that resistant genotypes should be at a disadvantage in the absence of stress (Harper *et al*., 1997; Sibly and Calow, 1989). Several studies, however, have shown that fitness costs of resistant genotypes are often due to negative pleiotropy or genetic linkage (Shirley and Sibly, 1999; Van Straalen and Hoffmann, 2000). Thus, the classical idea of trade-offs based on energy allocation is not well supported (Sibly and

Calow, 1989; Shirley and Sibly, 1999). According to Van Straalen and Hoffman (2000), the best evidence for fitness disadvantages of tolerance comes from pesticide resistance that involves structural mutations with pleiotropic effects. In heavy metal studies on invertebrates, similar effects were reported in midges, mites, and insects, and it was hypothesized that fitness costs might be due to interactions between selected metal tolerance and metabolism of essential elements. In fact, the essential metal deficiency hypothesis postulates that the cost will be manifested because individuals, by means of their tolerance mechanism, are less efficient at metal uptake or utilization, and the essential metal deficiency will occur as a result of the low metal levels found in nontoxic environments (Harper *et al*., 1997; Van Straalen and Hoffmann, 2000). Nevertheless, there are many cases where such costs have not been reported, suggesting that the relationship between resistance and fitness costs is difficult to determine. Another problem in determining costs of tolerance is to distinguish costs from other causes that may also alter characteristics of populations in metal-polluted habitats (Posthuma and Van Straalen, 1993). *Daphnia longispina* O.F. Muller field populations located in the aquatic system surrounding the abandoned cupric pyrite São Domingos Mine (South Portugal) offer an excellent system to study ecological costs and other microevolutionary consequences of adaptation to pollution. This aquatic system include sites unaffected and impacted by heavy metals (Lopes *et al*., 2004, 2005). Furthermore, reported information also indicates that *D. longispina* populations affected by São Domingos acid drainage are equally diverse genetically and include more Cu-tolerant genotypes to Cu than those from upstream reference sites (Martins *et al*., 2007, 2009). Nevertheless, other microevolutionary consequences of adaptation to pollution such as alterations of genetic variability of tolerance and fitness related traits and the existence of trade-offs between these traits across environments have not been fully studied. The present study addresses three microevolutionary aspects of adaptation to pollution: first, whether pollution affects tolerance to Cu and Zn; second, whether pollution affects genetic variability of tolerance and physiological fitness traits; and third, whether genetic trade-offs occur between tolerance and physiological fitness traits under low levels of contamination. The physiological fitness examined in this study was the feeding response as *Daphnia* feeding rates are directly linked to resource acquisition for somatic growth and reproduction (Barata and Baird, 2000). The first objective was

addressed comparing the nature and genetic range of tolerance to Cu and Zn of up to 20 clonal lineages randomly obtained from two populations: one located in a water reservoir contaminated by the acid mine drainage and the other located in a nearby clean water reservoir. The second objective was assessed by determining broad sense heritabilities of the above-mentioned tolerance traits and of the feeding responses. Finally, objective three was achieved by studying how feeding rates of sensitive and tolerant clones vary across increasing levels of Cu and Zn.

3.2. Material and Methods

3.2.1. Site description

In March and June 2004, *D. longispina* individuals were sampled from two different and hydrologically disconnected reservoirs belonging to the aquatic system surrounding the São Domingos mine in the southeast of Portugal, Alentejo: one population was sampled in the Chança River reservoir, impacted with acid mine drainage (referred as impacted population, I); and the other population was sampled in the Tapada Grande reservoir, a reference site located 5 km away from the contaminated one (referred as reference population, R). This reservoir has no history of contamination by heavy metals being monitored by the government with the objective to serve the local water needs (Figure 3.1). For a detailed description of sampling sites see Chapters 1 and 2. *Daphnia longispina* individuals were sampled in the field with the aid of a 250-μm mesh size plankton net and then transferred to 5-l capacity glass containers containing local water. At each sampling site, pH, conductivity, dissolved oxygen, and temperature were measured using a WTW Multi 340i handheld meter (Weilheim, Germany). Suspended solids were determined in the lab from 1-l water samples following American Society for Testing and Materials (ASTM) standard methods (ASTM, 1998). Concentrations of total recoverable metals (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Co, and Zn) were determined from 100-ml acidified water samples from both sampling sites by a Perkin-Elmer model Elan 6000 inductively coupled plasma-mass

spectrometer. Quality assurance was performed using calibration standards, certified reference material IAEA/W-4 (Simulated Fresh Water) and reagent blanks. Additionally, Rhenium was used as an internal standard to correct for any nonspectral interferences. Detection limits were calculated from blank measurements (n=5) these values being 0.1 mg.l^{-1} for Cd, Co, Pb; 0.2 mg.l^{-1} for Cu, Ni; 1 mg.l^{-1} for Al, As, Fe, Mn; and 5 mg.l^{-1} for Zn.
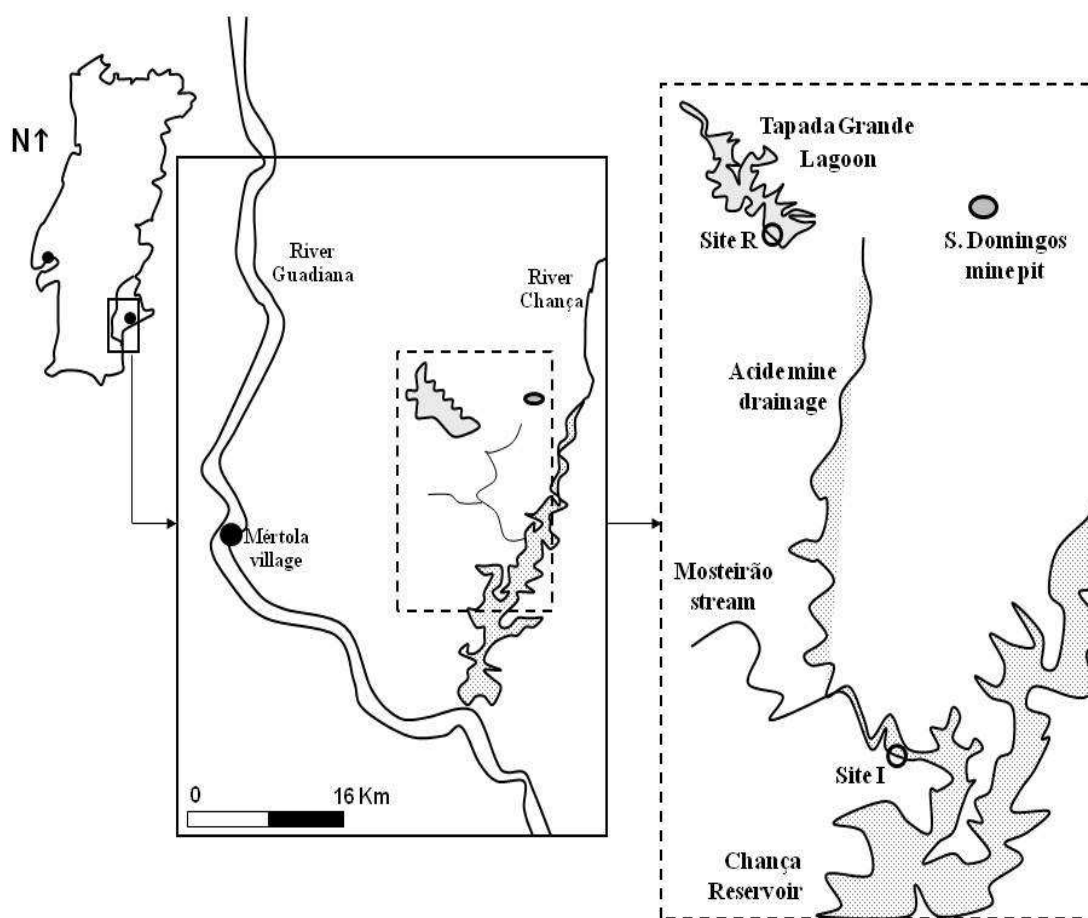
Figure 3.1. Studied area showing the location of reference (R) and impacted (I) field sampling sites in Portugal.

3.2.2. Culture conditions

Daphnia populations were taken to the laboratory in less than 24 h in local water from their capture site. In the laboratory, adult females bearing eggs were isolated in 150-ml glass vials containing filtered local water, acclimatized to laboratory conditions and raised as individual clonal lineages. From each of these populations (R and I) 20 to 27 clonal lines where established in the laboratory and maintained (by means of asexual reproduction) under controlled conditions of temperature (20±1°C) and photoperiod (16:8 h light:dark) for, at least 15 generations prior to assays. Single *D. longispina* females were maintained in ASTM hard water (ASTM, 1998) enriched with a standard organic extract (Barata and Baird, 2000). Animals were fed every other day with *Chlorella vulgaris* Beijerinck (3×10^5 cells ml^{-1}). The culture medium was changed three times a week and neonates were removed within 24 h of being born.

3.2.3. Test chemicals

Stock solutions of Cu and Zn were prepared by adding analytical reagent-grade salts of copper chloride and zinc sulphate ($CuCl_2.2H_2O$, $ZnSO_4.5H_2O$), respectively, to nanopure water (Milli-Q; 18 MV cm^{-1} resistivity). Copper chloride and zinc sulphate reagents were supplied by Sigma-Aldrich. Nominal test concentrations were subsequently prepared by adding aliquots of each metal stock solution to the ASTM hard water medium. For each experimental trial inductively coupled plasma-mass spectrometer measured concentrations of Cu and Zn in water at the beginning and end of tests were within 10% of nominal levels.

3.2.4. Tolerance to lethal levels of copper and zinc

Tolerance to Cu and Zn was assessed for each of the two populations in at least 20 single clones randomly selected from each population. Toxicant responses were determined from time to death tests (96-h duration) of *D. longispina* individuals exposed to a single concentration of Zn (1.5 mg.l^{-1}) and to two concentrations of Cu (25 and 75 μg.l^{-1}) plus a control treatment. Two concentrations for Cu were used due to the observed large differences in responses of the two populations (Figure 3.2). A total of 10 individuals (24-h old) per clone were exposed to each metal concentration. The

assays were performed by introducing two individuals in 50-ml glass containers containing 20 ml of test solution, in a total of five replicates per treatment and the mortality was checked every 8 h during 4 d. The test medium was renewed once in the middle of the test to minimize changes of initial exposure levels. Tests were run at controlled conditions of temperature (20±1°C) and photoperiod (16:8 h light:dark).

3.2.5. Feeding tests

To test if tolerance was ecologically costly (environmental trade-offs) the feeding rate responses of at least 15 clones from each population were assayed. Feeding tests were conducted using none and two concentrations of copper (0.5 and 5 μg l^{-1}) and zinc (0.1 and 1 mg l^{-1}). Due to the large number of treatments and obvious difficulties in maintaining all clonal lineages reproducing at the same time, each metal was assayed separately in different trials with different clones. The assays were performed by exposing a total of twenty five 4-day-old juveniles (in five replicates with 5 neonates each) per concentration (for each metal and clone) in 50-ml glass vials containing 20 ml of metal solution plus *C. vulgaris* at 3×10^5 cells ml^{-1}d^{-1}. Three blanks (vials with algae but without organisms) were also included per treatment to assure that algal concentration did not changed during the assay. At the start of the experiment, juveniles were distributed randomly among the five replicates of each feeding treatment and allowed to feed for a period of 24 h. Juveniles were transferred immediately after their third moult to avoid moulting during the experiment. Assays were carried out in the darkness (to minimize algal growth) at controlled conditions of temperature (20±1°C) (Barata and Baird, 2000). At the end of the test, individuals were removed and algae concentration was determined by spectrophotometry (in a Jenway 6505 UV/Vis. Spectrophotometer). Individual feeding rates (cells animal^{-1} h^{-1}) were determined as the change in cell density during 24 h and converted to proportional feeding rates relative to control treatments following Barata and Baird procedures (Barata and Baird, 2000). Cell density was estimated from absorbance measurements at $\lambda = 440$ nm using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

3.2.6. Data analysis

3.2.6.1. *Assessing differences in tolerance and feeding rates among populations*

Among populations interclonal differences were not the main focus of interest, therefore replicated values of the clones were averaged and entered as the lowest level of replication. Due to the presence of censored data (not all animals died during the assays) in lethal tests performed with copper and zinc, survival responses of the studied populations were compared by the Gehan-Wilcoxon test (Zar, 1996). Feeding rate responses of populations across Cu and Zn were compared by two way ANOVA analyses. Prior to analyses data was $\log_e$ transformed to meet ANOVA assumptions of normality and variance homocedasticity (Zar, 1996).

3.2.6.2. *Assessing differences in tolerance and feeding rates among clones within populations*

Genetic variation of a trait among treatments (environments) was compared using broad sense heritabilities: $H = VG/VT$, where VG and VT are the genetic and total variance of a trait. For each environment, the genetic (VG) and the environmental components of the variance (VE) were estimated using the method of the moments with appropriate accounting for unequal sample sizes among clones (Lynch and Walsh, 1998). Construction of confidence intervals and hypothesis testing was performed using nonparametric random bootstrap resampling (1000 samples) with replacement of clones for tolerance traits (Lynch and Walsh, 1998). H was considered significantly different from zero if the 5th percentile was >0. For feeding rates H values, and their associated confidence intervals were determined using parametric one way ANOVA analyses performed in log e transformed data (Lynch and Walsh, 1998). The population mean of H for a given treatment was considered significantly different from other if its 5% and 95% confidence intervals not overlap (i.e. equivalent to a two-sided test at the 0.05 significance level).

3.2.6.3. *Assessing fitness costs*

To assess properly trade-offs between tolerant traits and feeding responses across increasing metal levels two different analyses were performed. Firstly, it was necessary to show the existence of substantial genetic variation of feeding responses across the studied metal exposure levels by performing within each population a two-way ANOVA considering clone and metal exposure levels as random and fixed factors, respectively. Secondly, genetic relationships between tolerance to copper and zinc were determined from product-moment correlations among clonal means considering: median survival times of juveniles at 25, 75 μg l^{-1} of copper and 1.5 mg l^{-1} of zinc and mean feeding rates at 0, 0.5, 5 μg l^{-1} for Cu, 0, 0.1, 1 mg l^{-1} for Zn . Prior to analyses data were loge transformed. All analyses were performed with the aid of SPSS statistical package (SYSTAT, Chicago, IL, USA).

3.3. Results

3.3.1. Water physical-chemical characterization

Water physical-chemical parameters varied largely between sites and sampling months. On March, during the main rain season, water collected from the impacted site, presented lower pH, higher conductivity and higher metal levels than those at the reference site (Table 3.1). Within the analysed metals, levels of Cu and Zn showed the greatest differences, being from one to two orders of magnitude greater at the impacted location. During the drain season on June, both the impacted and reference site presented similar values for most physicochemical parameters.

3.3.2. Population differences

Cumulative clonal survival curves are shown in Figure 3.2. Gehan-Wilcoxon survival tests denoted significant differences ($p<0.05$, $\chi^2_3= 66.3$) of populations across Cu exposures (Figure 3.2A). Most individuals of clones from the population I did not die at 25 μg l^{-1} of Cu, whereas those of reference population R died within 96 h at this

concentration. On the other hand, at 75 μg l^{-1} of Cu the median survival time of the clones from the contaminated site was 52 hours, while all individuals from the reference population died during the first eight hours of the test (< 8 hours). For Zn, despite that survival tests also indicated greater survival of clones from the I population ($p<0.05$, $\chi^2_1= 4.6$), differences in tolerance between both populations (median survival time of 32 and 42.4 hours for R and I, respectively) were less pronounced (Figure 3.2B).

Table 3.1. Water physical and chemical parameters measured in the field, at the reference (R) and impacted sites (I), during collection of clones in March (Mar) and June (Jun) (* below detection limit)

Parameters	Sites			
	R		I	
	Mar	Jun	Mar	Jun
pH	7.2	7.5	6.0	6.9
T	19.0	23.6	18.8	23.5
Cond	250	241	450	339
Oxygen	9.0	9.2	8.9	9.2
SS	2.0	6.4	1.4	1.2
Al	620	295	248	559
As	<1*	1.4	<1*	5.3
Cd	<0.1*	<0.1*	2.9	<0.1*
Co	0.3	0.1	11.7	0.2
Cr	0.3	10.7	0.2	6.7
Cu	2.8	4.2	188.0	9.6
Fe	292	147	488	870
Mn	59.9	12.9	177.1	15.5
Ni	3.0	1.1	5.7	1.1
Pb	0.6	63.2	7.4	48.6
Zn	5.5	529.1	<5*	<5*

Abbreviations: Cond – conductivity (μS cm^{-1}); oxygen – dissolved oxygen (mg l^{-1}); T – Temperature (°C); SS – suspended solids (mg l^{-1}); Metals – concentrations in μ l^{-1}; Al = aluminium; As = arsenic; Cd = cadmium; Co = cobalt; Cr = chromium; Cu = copper; Fe = iron; Mn = manganese; Ni = nickel; Pb = lead; Zn = zinc.

Feeding rates of individuals from the impacted population were significantly ($p<0.05$) and marginally ($0.05<p<0.1$) smaller than those of the reference one during the first (Cu) and second (Zn) experimental trial, with neither Cu nor Zn showing any effect on the overall population performance (Figure 3.3 A, C, population, Cu or Zn, Interaction effects in Table 3.2). Nevertheless, within each of the studied populations there was

substantial (p<0.05) genetic variability in feeding responses of clones within and across Cu and Zn exposure levels (Figure 3.3 B, D, Table 3.2).
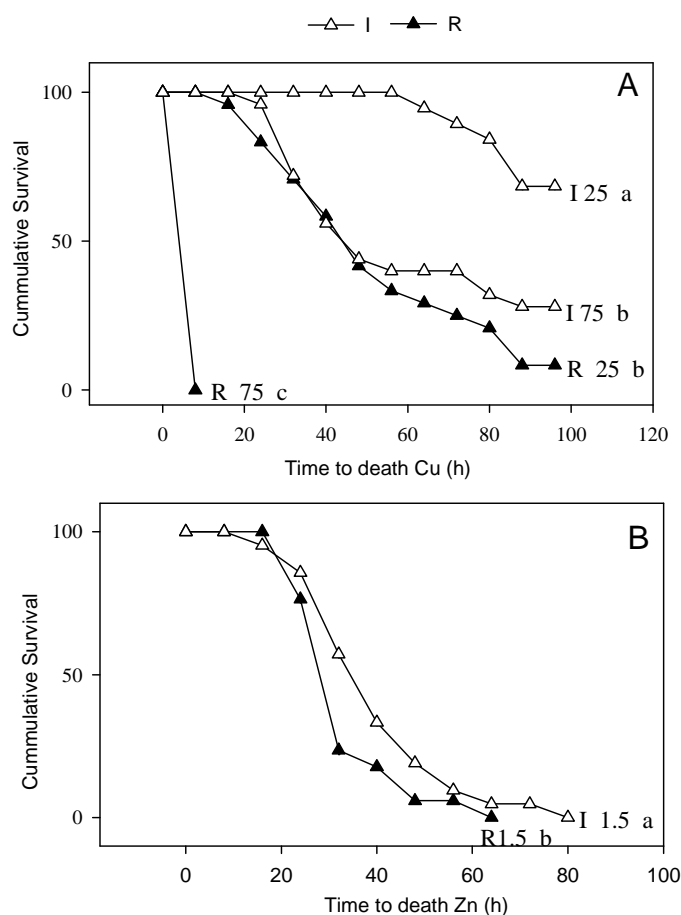
Figure 3.2. Lethal responses of reference (R) and impacted (I) *D. longispina* populations across 96-h acute exposures to metals. These included: survivorship curves (expressed as probability values) at 25 and 75 μg.l^{-1} of copper (A), and at 1.5 mg.l^{-1} of zinc (B). Toxicant concentrations are depicted after population abbreviations. Different lowercase letters denote significant (p<0.05) differences following Gehan-Wilcoxon tests.

Table 3.2. Analysis of variance results testing for *Daphnia longispina* population differences in feeding responses across Cu and Zn exposure levels (*p< 0.05) [a]

Source	df	F	Source	df	F
Testing for population differences					
Cu	*2, 84*	0.2	Zn	2, 69	2.4
Population	1, 84	5.6*	Population	1, 69	3.1
Interaction	2, 84	0.6	Interaction	2, 69	0.1

[a] *When comparing populations, clonal means were used as the lowest level of replication and population and metal levels as fixed factors. Only F ratios and degrees of freedom (df) are depicted. Differences in df are due to missing values)*
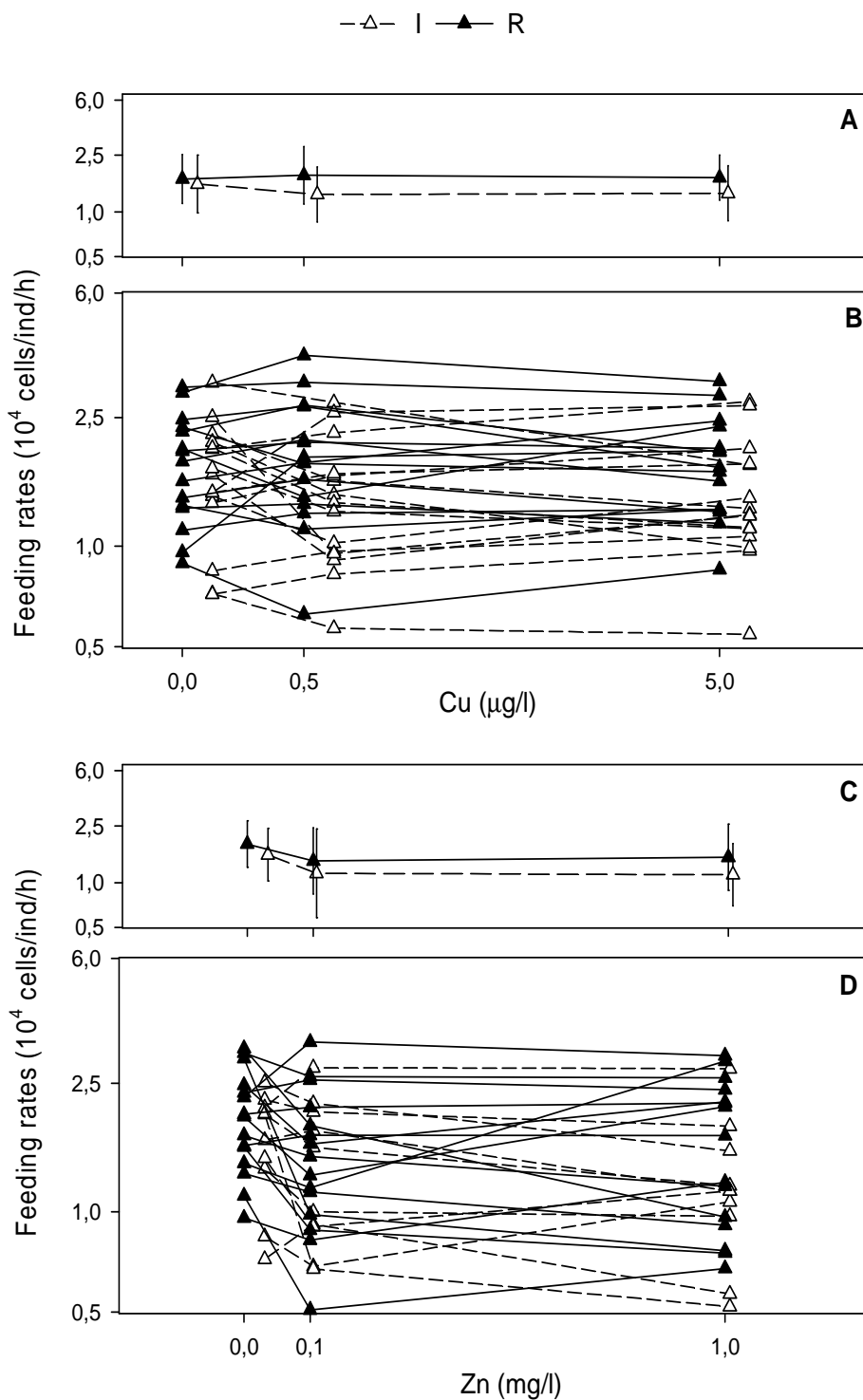
Figure 3.3. Population (A,C) and interclonal (B,D) variation in feeding responses across Cu and Zn exposure levels. Errors bars are standard errors. In graphs B,D each symbol corresponds to a single clonal mean. For clarity, values from the impacted population (I) have been switched slightly to the right. Axes are depicted in log e scale. I = impacted; R = reference.

3.3.3. Genetic variability

A further characterization of genetic variability within environments denoted significant levels ($p<0.05$) of interclonal variation in feeding and survival responses in all the studied treatments (Table 3.3). Interestingly both populations had similar H values for feeding and tolerant traits and behave similarly, showing higher heritability levels in feeding rates under Cu and Zn exposures relative to controls (95% CI of H did not overlap).

Table 3.3. Analysis of variance results testing for interclonal differences in feeding responses across Cu and Zn exposure levels (*$p < 0.05$) [a]

Source	df	F	Source	df	F
Population – R					
Cu	2, 28	1.2	Zn	2, 28	2.2
Clone	*14, 28*	9.7*	Clone	14, 28	4.6*
Interaction	28, 235	2.7*	Interaction	28, 233	4.8*
Population – I					
Cu	2, 28	0.24	Zn	2, 28	1.4
Clone	14, 28	5.1*	Clone	9, 18	4.2*
Interaction	28, 210	3.2*	Interaction	18, 63	6.6*

[a] *Clones and metal levels were included as random and fixed factors, respectively. Only F ratios and degrees of freedom (df) are depicted. Differences in df are due to missing values.*

3.3.4. Genetic correlations

Genetic relationships (product–moment correlations) among *ln* transformed tolerance traits (median survival time) and mean feeding rates in absence (control) and in the presence of low to moderate levels of Cu and Zn varied between populations (Figure 3.4).

First, tolerance to Cu was significantly related with that of Zn only in the impacted population (Figure 3.4A). Second, in both populations tolerances to Cu and Zn were inversely related with feeding rates in control treatments (in absence of metals, Figures 3.4B and E) and tended to decrease (being less negative) with increasing levels of metals (Figures 3.4C, D, F, and G). It is important to note, however, that negative genetic correlations in control treatments were consistent across experimental trials only in the impacted (I) population (Figures 3.4B and E). Moreover, inverse correlations with increasing metal levels were much more obvious in the impacted population.

Table 3.4. Broad sense heritability values (H) and their 95% confidence intervals (CI) of tolerance (time to death) and feeding responses of clones within the studied treatments [a]

Exposure levels	H	5% CI	95% CI		H	5% CI	95% CI
Experiments with Cu ($\mu g\ l^{-1}$)							
R	Feeding			**I**	Feeding		
Ctr	35	30	40.1	**Ctr**	43	31.3	54.6
0.5	74.2	69.6	78.8	**0.5**	63.9	59.6	68.1
5	69.4	66.7	72.2	**5**	73.6	69.3	77.8
	Tolerance				Tolerance		
25	54.8	37.1	63.6	**75**	62.8	49.8	70.3
Experiments with Zn ($mg\ l^{-1}$)							
R	Feeding			**I**	Feeding		
Ctr	34.5	29.5	39.4	**Ctr**	48.6	36.5	60.6
0.1	77.6	74.1	81.2	**0.5**	87.5	73.2	101.7
1	83.8	77.5	90.1	**5**	71.7	64.7	78.7
	Tolerance				Tolerance		
1.5	63.1	35.4	73.3	**1.5**	58.2	36.9	68.5

[a] R and I correspond to the reference and impacted population; Ctr, control treatment. All H values obtained for feeding and survival responses were significant ($p<0.05$, based on one-way analysis of variance and inspection of 95% CI, respectively).

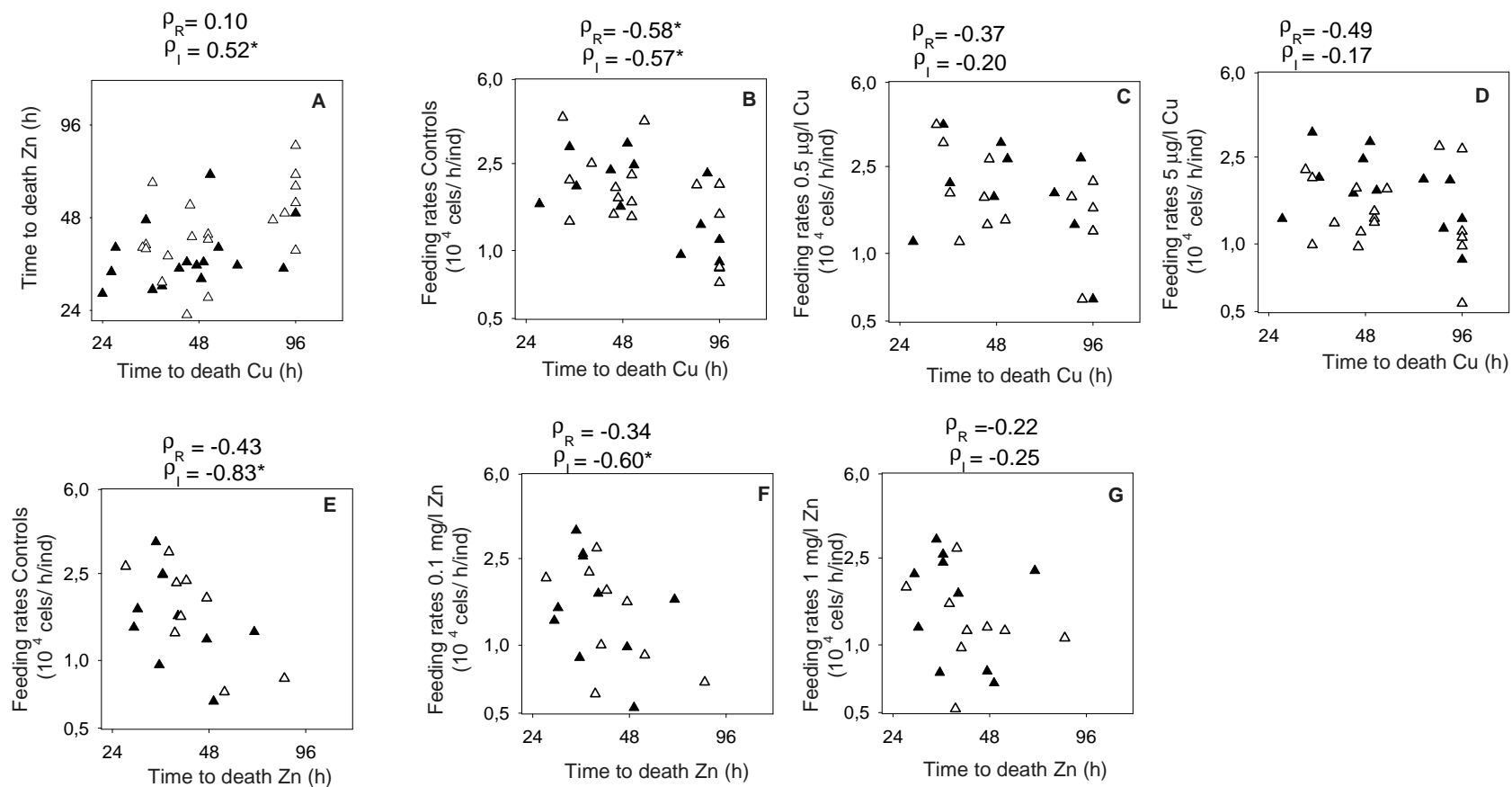**Figure 3.4.** Genetic correlations between tolerance traits (time to death; A) and between tolerance and feeding rates measured at increasing sublethal levels of Cu and Zn (B–G). Each symbol corresponds to a single clone. Results and correlation values within reference (R; filled symbols) and impacted (I; open symbols) populations are depicted separately. Axes are depicted in log e scale. (p <0.05).

3.4. Discussion

Contamination of waterbodies by metals originating from the mining and smelting of metal ores is a problem affecting many areas worldwide and it has been responsible for the elimination of several species, whereas others have shown their adaptive strength by surviving and reproducing in those metal polluted environments (Groenendijk *et at*., 2002).

São Domingos mine is an example of an abandoned mining system that has been an ideal model for ecotoxicological studies (De Bisthoven *et al*., 2004; Gerhardt *et al*., 2004; Pereira *et al*., 1999) and for studies about adaptation occurring in natural populations exposed to historical chemical stress (Lopes *et al*., 2004, 2005; Martins *et al*., 2007, 2009). In the present study measured contaminant levels in water evidenced a strong metallic pollution gradient from reference to the impacted site in March where most *D. longispina* individuals were collected. In June however, metallic contaminant levels were only slightly higher at the impacted Chança River reservoir. These and previous studies evidenced that the studied metallic contaminants are heterogeneously distributed both spatial and temporally within the Chança River reservoir (Pereira *et al*., 1995). Metallic levels are the highest nearby mine effluent discharges or after rainfalls, decreasing dramatically towards distant reaches within the reservoir or during dry periods (i.e. June) when mine discharges are absent. Unfortunately, there is no reported ecological data on *D. longispina* population dynamics in Chança reservoir but assuming that they occur along all reservoir reaches, at least from March to June, it is reasonable to assume the they may be exposed to different levels of metallic pollution and hence developed different degrees of tolerance to metallic pollution.

The first purpose of the present study was to assess and compare the genetic range of tolerances to acute copper and zinc exposures in two *D. longispina* populations differing in their habitats with respect to the heavy-metal contamination history. The higher tolerance of the population from the impacted site (I) to the tested metals (compared to the reference R population) illustrated the occurrence of a change in the

frequencies of clonal lineages in the metal exposed population. Specifically, only sensitive lineages to Cu were present in the reference population, whereas in the contaminated site, only copper tolerant individuals were present (Figure 3.2A). For Zn, however, both populations had a similar distribution pattern of sensitivities (Figure 3.2B), but only in the impacted one tolerance to Zn was genetically correlated with that of Cu (Figure 3.4.A). These results were in concern with those obtained years ago by Lopes *et al.* (Lopes *et al.*, 2004, 2005) who studying the response to copper and zinc of other clones collected from the same sites also reporter greater tolerances to copper of those from I population and a significant correlation between tolerance to Cu and Zn. Therefore differences in acute tolerance to Cu but not to Zn between the two studied populations are likely to be related to selective pressures experienced in its local habitat leading to local adaptation (Fox, 1995; Medina *et al.*, 2007). Recently Martins *et al.* (Martins *et al.*, 2007, 2009) using allozyme and amplified fragment length polymorphism (AFLP) analyses showed that differences in resistance to copper of up to 20 clones or 360 females of *D. longispina* obtained from the same populations studied here, were unrelated with genetic differentiation. According to Cousyn *et al.* (Cousyn *et al.*, 2001) the previous results indicate that directional selection for tolerance rather than changes in genetic drift or gene flow may explain the observed differences in tolerance. Development of tolerance to metals through genetic adaptation has been documented for a diversity of animal *taxa*. In terrestrial organisms genetic adaptation has been found in isopods (Donker *et al.*, 1993) and springtails (Posthuma *et al.*, 1993). For aquatic organisms, evidence of a genetic basis for tolerance to metals have been reported in chironomids (Groenendijk *et al.*, 2002; Postma *et al.*, 1995), oligochaetes (Vidal and Horne, 2003) and daphnids (Lopes *et al.*, 2004, 2005; Ward and Robinson, 2005). In contrast to many other environmental factors, metal contamination represents a strong and stable directional selection pressure for metal-exposed populations making the metal adaptation often a very quick process expected to be determined, to a large extent, by single major genes in contrast to adaptation to natural stressors (Posthuma and Van Straalen, 1993; Posthuma *et al.*, 1993).

Another purpose of this work was to assess microevolutionary detrimental effects of adaptation such as decreased levels of genetic variability in fitness related traits and ecological costs of tolerance (Medina *et al.*, 2007). Heritability results

evidenced similar high levels of genetic variation in tolerance to Cu and Zn in both populations (H>50%) These findings imply that both the reference and impacted populations may still show response to selection upon metal-pollution. When considering physiological traits related with fitness such as feeding rates, both populations showed also substantial levels of genetic variation in plasticity across increasing Cu and Zn exposure levels, high heritability levels and a significant increase of genetic variation under Cu and Zn exposure. These results agree with previous work performed with *D. magna* field populations exposed to cadmium and with the response of other organisms to stress, thus supporting the view that in many cases stress increase rather than decrease the expression of genetic variability (Hoffmann and Parsons, 1997; Barata *et al.*, 2002). Nevertheless, according to Hoffmann and Parsons (Hoffmann and Parsons, 1997) there is a narrow range between stress exposure levels that affect heritable variation and those that cause extinction. Results for Zn corroborate the previous argument since while 1 mg/l of Zn was able to enhance the genetic variability of feeding responses in the studied population, at just 1.5 mg/ Zn was lethal. Finally, the results reported in Figures 3.3 and 3.4 provided two lines of evidence for fitness cost: lower feeding rates of the tolerant population compared to the reference population and negative genetic correlations between mean clonal feeding rates and median clonal survival time in control conditions (no added Cu or Zn). Interestingly negative genetic correlations were also observed in the reference population between tolerance to copper and feeding rates, thus indicating the existence of genetic trade-offs for adaptation to Cu and Zn. These findings may prevent fixation of tolerant genes and also explained the observed high degree of genetic variability in tolerance to the studied metals in both populations (Hoffmann and Parsons, 1997; Posthuma *et al.*, 1993). Contrary to us Lopes *et al*. (Lopes *et al*., 2004) studying the same populations reported that under control conditions both populations grew and reproduced similarly. Nevertheless, it is important to consider that the previous authors compared individuals within populations rather than clonal means, which may underestimate the contribution of clones maximizing interindividual variability (Barata *et al*., 2002). According to Van Straalen and Hoffman (2000) and Harper *et al*. (1997), there are two hypotheses to explain how a cost of metal tolerance could be manifested. One is the trade-off hypothesis, which postulates that the tolerance mechanism may be expensive in terms of energy and other

resources; in this way, increased investment in this tolerance mechanism may initiate a trade-off between the benefits of the adaptation and the costs arising from a decreased expenditure in other processes, thereby reducing individual fitness in a non-adaptive environment. Another explanation is related with the metal deficiency hypothesis, which associates the tolerance mechanism with a lower efficiency of metal uptake or utilization, which in turn will reduce individual fitness in a clean environment by means of an essential micronutrient deficiency. The variation of the correlation values between lethal tolerance and the feeding rates across metal levels showed that costs can be associated with altered physiological processes that enable tolerant organisms to cope with the presence of excess levels of Cu, which corroborates the metal essentiality hypothesis. In the studied *D. longispina* populations, tolerances to Cu and Zn were inversely related with feeding rates in absence of metals and tend to decrease (being less negative), specially in the impacted population, with increasing levels of metals. Both copper and zinc are vital metals for animals' normal development and reproduction (Da Silva and Williams, 1991). Zn is incorporated in metallothioneins and in other metabolic compounds and it has the function of stabilizing biological molecules and structures (Canli, 2005; Da Silva and Williams, 1991; Muyssen and Janssen, 2002). Cu is incorporated in at least thirty different enzymes, including those responsible for oxygen binding and oxygen transport in crustaceans (Canli, 2005; Muyssen and Janssen, 2002). The tested concentrations probably were lower than the natural bioavailable concentration range of such elements in their natural habitat, which in turn may be closer to the tested ones. Another study suggesting the existence of costs associated with a micronutrient defficiency is the one of Postma and his co-workers (Postma *et al.,* 1995). They showed that the reduced fitness in cadmium-adapted *Chironomus riparius* populations reared in a clean environment was due to an increased dependency on high metal concentrations of the essential metal zinc. They provided evidence that costs of tolerance resulted from interactions between zinc and cadmium metabolism. In summary the results reported here and those from other studies (Lopes *et al*., 2004, 2005; Nelson *et al*., 2007, 2009) evidenced the following microevolutionary effects of long term metallic pollution in *D. longispina* populations: Adaptation to Cu and probably to other environmental factors associated to acid mine drainage did not affect genetic differentiation, neither the expression of genetic variation

in tolerant and fitness related traits but there was evidence of enhanced fitness costs of tolerance in the impacted population. Findings such as the lack of fixation of tolerant genotypes in the impacted population and the observed high degree of genetic variability within and across metal exposure levels for the studied fitness related trait (feeding rates) are in concern with previous results and life history and evolutionary theories under stress (Hoffman and Parsons, 1997; Medina *et al*., 2007; Van Straalen and Timmermans, 2002). Finally the observed changes in genetic environmental trade-offs across environments suggested pre-adaptation of tolerant genotypes to live under moderate levels of metallic pollution. To end, it is important to pointed out that our genetic variability estimates included both heritable and non heritable (i.e. maternal effects) variation and hence do not provide information about the extent to which observed responses are inherited from parents to offspring (Lynch and Walsh, 1998). Nevertheless, during the main growing season, *Daphnia* species reproduce mainly parthenogenetically producing genetically identical offspring. Under this circumstances selection will mainly act on interclonal variation (Lynch, 1983).

3.5. Conclusion

In this study it was possible to show the microevolutionary effects of long term metallic pollution in *D. longispina* populations. One of the study's outcomes was that adaptation to copper and possibly to other environmental factors did not affect genetic differentiation, neither the expression of genetic variation in tolerant and fitness related traits. Besides that, there was evidence of enhanced fitness costs of tolerance in the impacted population. These costs were illustrated by lower feeding rates of the tolerant population compared to the reference one and negative genetic correlations between mean clonal feeding rates and median clonal survival time in control conditions Nevertheless, the observed changes in genetic environmental trade-offs across environments suggested pre-adaptation of tolerant genotypes to live under moderate levels of metallic pollution.

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CHAPTER 4

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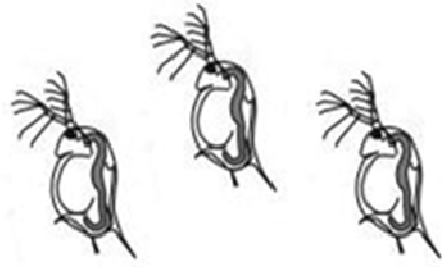
Life-history responses to sublethal levels of copper using *Daphnia longispina* clones
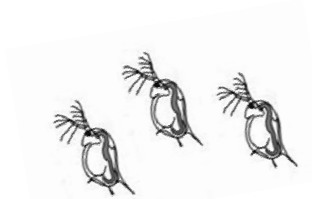
Chapter

Life-history responses to sublethal levels of copper using *Daphnia longispina* clones

4

Abstract

The present work was conducted to assess life-history responses to sublethal copper contamination in four *Daphnia longispina* clones. Two of the clones came from a reference site (R clones) and the other two from a historically copper-exposed site (I clones). Feeding, oxygen consumption, growth and reproductive responses were compared. Results from feeding and respiration experiments showed that for most clones feeding rates were significantly inhibited at increasing copper concentrations whereas respiration rates remained relatively constant being inhibited only at exposure copper levels close to lethality. The tolerant clone from the impacted population instead increased its feeding rates and oxygen consumption rates at the highest copper concentrations tested. Regarding reproduction, the tolerant clone from impacted population increased or maintained its fitness performance with increasing copper concentrations at expenses of maturing earlier, increasing its daily reproduction rates and being smaller (having lower somatic growth). The other clones, irrespectively of their overall sensitivity to copper and origin, showed a similar response: copper decreased their fitness delaying maturation and decreasing reproduction and somatic growth. Overall, data showed that tolerance manifested by *D. longispina* clones at lethal copper levels was also evident at sublethal concentrations, with the tolerant clone from impacted population showing higher tolerance to copper for all the parameters compared to the rest of clones.

4.1. Introduction

Research into biological stress responses and adaptation to stress has becoming an even more important and progressing field owing to the growing impact of anthropogenic activities on natural environments (Bijlsma and Loeschcke, 2005; Forbes and Depledge, 1996; Hoffmann and Parsons 1991; Scott, 1995). Stressful environmental conditions are those that lead to a sharp reduction in fitness of individuals which potentially limit survival or reproduction significantly (Bijlsma and Loeschcke, 2005; Evendsen and Depledge, 1997; Hoffmann and Hercus, 2000). Organisms when faced with a change in environmental conditions may migrate, become extinct or adapt (Bijlsma and Loeschcke, 2005; Hoffmann and Hercus, 2000). Adaptation is the process of change in an organism to conform better to the new environmental conditions. This implies that the organism (or group of organisms) acquires characteristics (involving changes in morphology, physiology or behavior) that improve their survival and reproductive success in the particular environment (Bijlsma and Loeschcke, 2005). Changes can occur phenotypically (phenotypic adaptation) – changes of phenotype within a certain genotype according to prevailing environmental conditions or genetically (genotypic adaptation), being, in this case, the result of heritable changes in genes due to the selection pressure exerted by the environment (Bijlsma and Loeschcke, 2005; Evendsen and Depledge, 1997; Hoffmann and Parsons 1991). The persistence of a population in a stressful environment is associated with natural selection acting at the individual level, with stress being a selective force responsible for the evolutionary changes occurring in that population. Nevertheless, the ability of natural populations to evolve is dependent on the existence of sufficient genetic variation in order to adaptive changes to occur (Ashley *et al*., 2003; Hoffmann and Parsons 1991). Metal contamination is an example of a stress arising from human activities and often reduces the fitness of organisms to such an extent that species diversity is strongly reduced and the species which do survive have an increased tolerance to the metals involved (Donker *et al*., 1993; Hoffmann and Parsons 1991; Postma *et al*., 1995). The adaptations allowing the survival of organisms to a metal or other toxicant can be

classified as either mechanisms of decreased response to the metal (mechanisms of target insensitivity to the toxicant) and mechanisms of decreased exposure to the metal namely mechanisms of avoidance, reduced uptake, increased detoxification, increased elimination and increased sequestration (Belfiore and Anderson, 1998; Klerks and Weis 1987; Posthuma and Van Straalen, 1993; Taylor and Feyereisen, 1996). One of the general features of metal stressed systems is the increase in energy expenditure, since energy balance can shift from maintenance and production to repair and recovery (Hoffmann and Parsons 1991; Parsons, 2005). Although metal stress may decrease energetic efficiency (fitness), organisms tend to become increasingly adapted to the resource status of their habitats by an increasingly energetic efficiency (Parsons, 2005). The evolutionary response – tolerance – is the direct cause of the improvements, and the fitness is indirectly affected (Posthuma and Van Straalen, 1993). There are three possible reasons for altered performance of fitness characteristics and only one has positive implications for populations, which is the selection for life-history characteristics associated with an improved performance. In this way, reduced adult survival and increased reproductive allocation is expected to induce earlier maturation and increased reproduction. For metal-exposed populations, life-history theory thus predicts that the evolutionary response to chronic exposure to metals will consist of earlier maturation and an increased reproductive effort. The other two possible outcomes for altered performance of fitness include negative pleiotropic effects of genes and genetic linkage disequilibrium, which usually involve fitness cost of tolerance in uncontaminated environments (Posthuma and Van Straalen, 1993; Van Straalen and Hoffmann, 2000; Van Straalen and Timmermans, 2002).

Copper is an essential metal to living organisms, in part through its fundamental role in electron transport, respiration, growth and development (Atienzar *et al*., 2001; Grosell *et al*., 2002; Long *et al*., 2004). Although its essentiality, copper it's a toxicant in aquatic systems when present in elevated concentrations in the water so the emission of this metal into the environment is currently under regulation (Grosell *et al*., 2002). In aquatic systems copper bioavailability and speciation are dependent on the type and concentration of organic and inorganic ligands present, the pH, temperature, alkalinity and hardness, which in turn influence the copper toxicity to aquatic organisms (Long *et al*., 2004; Meador, 1991; Suedel and Rodgers, 1996). Additionally, the sensitivity of

each species, the age structure of its population as well as its genetic variation contributes to the specific influences on copper toxicity to aquatic organisms. Some works have shown the occurrence of adaptation to copper-contaminated environments through the acquisition of a genetically-determined tolerance (Barata *et al.*, 2000; Klerks and Weis 1987; Lopes *et al.*, 2004).

The specific objectives of this study are: to select the most sensitive and the most tolerant clones from two *D. longispina* populations proved to differ in lethal tolerance to the metal copper; and to determine if tolerance manifested by those clones at lethal copper levels was also evident at sublethal copper levels. The above mentioned objectives were addressed by studying the sublethal tolerance to copper in two clonal lineages from each population, chosen by their high and low tolerance to lethal levels of copper. The comparison of feeding, oxygen consumption, reproduction and growth allowed the assessment of sublethal tolerance to copper and the possible existence of differences in the sublethal tolerance assessed.

4.2. Material and Methods

4.2.1. Study populations and culture conditions

Twenty clonal lineages of *D. longispina* were selected for this study. These new lineages were isolated again from two field populations of *D. longispina* inhabiting a reference site (site R: pH 7.4, conductivity 210 μS cm^{-1}, dissolved oxygen 9.5 mg l^{-1}) and an acid mine drainage historically impacted site (site I: pH 6.3, conductivity 550 μS cm^{-1}, dissolved oxygen 8.50 mg l^{-1}) located at the aquatic system surrounding S. Domingos mine in the southeast of Portugal, Alentejo. For a detailed description of the study site and sampling methodology see Chapter 2. The field campaign occurred in March 2005. The twenty lineages were maintained in laboratory cultures under controlled conditions of temperature (20±1 ℃) and photoperiod (16 light: 8 dark) for more than 15 generations prior to bioassays. Cultures were maintained in 1 liter glass vials with 800 ml of ASTM hard water (ASTM, 2002) enriched with a standard organic extract Marinure 25 (Glenside, Stirling, UK) (Baird *et al.*, 1989). Daphnids (30

organisms per culture vial) were fed every other day with the green algae *Chlorella vulgaris* Beijerinck (3×10^5 cells ml^{-1}) and its culture medium was changed three times a week. The born neonates in cultures were removed within 24 h of release. Neonates from the fifth or sixth broods were used to replace the old cultures.

4.2.2. Test chemical

A stock solution of copper was prepared by adding analytical reagent-grade salt of copper chloride dihydrate ($CuCl_2\cdot 2H_2O$) (supplied by Sigma-Aldrich, Germany) to deionized water (Milli-Q, Bedford, MA, USA). The stock solution was stored at 4 ºC protected from light during the experiments. Nominal test concentrations were subsequently prepared by adding aliquots of each metal stock solution to the ASTM medium. Duplicate water samples were taken from acute tests and from reproduction tests for copper analysis by inductively coupled plasma-mass spectrometry (ICP-MS). A total of 44 exposures were analysed.

4.2.3. Preliminary assessment of acute toxicity

Acute toxicity was estimated to gauge the sensitivity of *D. longispina* clones to the metal copper. Based on the 48-h LC_{50} values, two *D. longispina* clones with extreme genotypes (on the basis of their relatively high or low acute tolerance to copper) will be selected within each population to be used in the feeding, respiration and reproduction experiments. Selected clones from the reference population will be designated hereinafter as R-sen and R-tol, the sensitive and the tolerant one respectively; the clones from the population I (from the site impacted with metals) will be designated hereinafter as I-sen and I-tol, the sensitive and the tolerant respectively. Acute toxicity bioassays were performed with neonates from two *D. longispina* populations under controlled conditions of temperature (20±1ºC) and photoperiod (16 light: 8 dark). Third to fifth brood neonates (<24 h old) were used in the 48 hour static immobilization tests, which were performed in accordance with the OECD 202 guideline (OECD, 2004). Six chemical concentrations plus a negative control (ASTM only) were used for the experimental setup. The complete concentration series for copper treatments was 0,

12.5, 25, 50, 100, 200 and 400 µg l^{-1}. A total of twenty-five individuals with less than 24 hour old were exposed per treatment and assays were performed by introducing five individuals in 150 ml glass vials containing 100 ml of test solution, in a total of five replicates per treatment. The measured toxicological endpoint was mortality as identified by immobility and was checked at 24 and 48 hour of exposure. Duplicate water samples (100 ml) were also included to measure pH, oxygen levels and metal concentrations at the beginning and at the end of the tests. Dissolved oxygen concentration was measured with a WTW 330 oxygen meter (WTW, Weilheim, Germany) and the pH with a WTW 330i pH meter (WTW, Weilheim, Germany).

4.2.4. Chronic toxicity experiments

4.2.4.1. *Feeding tests*

The assays were performed by exposing a total of twenty five four-day old juveniles (in five replicates with 5 neonates each) per concentration (for each metal and clone) and eight copper concentrations were used. At the start of the experiment, juveniles were distributed randomly among the five replicates consisting in 150 ml glass vials containing 100 ml of metal solution plus *Chlorella vulgaris* at 3×10^5 cells ml^{-1}d^{-1}. Three blanks (vials with algae but without organisms) were also included per treatment to assure that algal concentration did not changed during the assay. Juveniles were transferred immediately after their third moult to avoid moulting during the experiment and were allowed to feed for a period of 24 hours. Assays were carried out in the darkness (to minimize algal growth) at controlled conditions of temperature (20±1ºC). Duplicate water samples were also included to measure pH and oxygen concentration at the beginning and at the end of the tests as described above. At the end of the test individuals were removed and algae concentration was determined by spectrophotometry (in a Jenway 6505 UV/Vis. spectrophotometer). Individual feeding rates (cells animal^{-1} h^{-1}) were determined as the change in cell density during 24 h according to the method given by Allen *et al.* (1995) and converted to proportional feeding rates relative to control treatments following Barata *et al.* (2000). Cell density

was estimated from absorbance measurements at $\lambda= 440$ nm using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

4.2.4.2. Respirometry tests

Five concentrations of copper (5, 10, 20, 40 and 80 $\mu g\ l^{-1}$) plus a control were tested. The oxygen consumption experiments were performed using standard respirometry methods with 50-ml gastight syringes (Hamilton, USA). Three syringes were used per treatment and were filled with 30 ml of the appropriate test solutions and with five four day-old juveniles of *D. longispina*, the remaining air was expelled from each syringe, which were left in the dark in a water bath (20 ºC) for 24 hrs. After the first 2 hours of exposure, initial O_2 concentrations were measured with Oxygen meter (Model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow) and after 24 hrs from the start of the test, the final O_2 concentrations were measured in the same way. The oxygen consumption was given by the differences in the oxygen content of water before ($T_0= 2h$) and after ($T_{final}= 24h$) the exposure period and the respiration rate is expressed as and $\mu g\ O_2\ ml^{-1}$ consumed per organism per hour. In a separated experiment three blank controls (syringes with no organisms) were tested for every treatment to correct for the O_2 ambient depletions due to factors other than organism's respiration. The depletion in the oxygen content on these blank controls was used as a correction factor for the appropriate treatments. For the respiratory experiments daphnids (<12 hour-old) were isolated and maintained in ASTM, with *C. vulgaris* and organic extract until they reached the four days.

4.2.4.3. Reproduction tests

Twenty replicates of one juvenile (<24 h) were exposed to at least seven copper concentrations, ranging from no copper addition (control) to 240 $\mu g\ l^{-1}$. Each test vessel contained 50 ml of test medium. Test solutions were renewed every other day and *C. vulgaris* was supplied every day in a concentration of 3.0×10^5 cells ml^{-1}. Age and clutch size at first reproduction, reproduction rates (daily offspring production per female) and

the intrinsic rate of increase (r, equation 1) were recorded. The intrinsic rate of increase was computed iteratively from the Lotka equation

$$\sum_{x=0}^{\infty} e^{-rx} l_x \qquad m_x = 1 \text{ (equation 1)}$$

where l_x is the proportion of the females surviving to age x (days) and mx is the number of female juveniles produced per surviving female between the ages x and $x+1$. The age at birth was set to 0. The 95% confidence intervals were estimated by the Jackknife method according to Meyer *et al.* (1986). Size of experimental organisms was also measured in the start (30 randomly selected neonates) and at the end of the experimental period (21 days) to assess effects on somatic growth. Body length measurements were performed using a stereomicroscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer. Duplicate water samples were also included to measure pH, oxygen levels and metal concentrations at the beginning and at the end of the tests.

4.2.5. Data analysis

The number of immobilized organisms from each acute test was plotted against the copper concentrations, and a 48-h LC_{50} with a 95% confidence interval (CI) was calculated by the standard probit procedure (Finney, 1971). Survival responses during the 21-day exposures were compared by the Gehan-Wilcoxon χ^2 test (Zar, 1996). Somatic growth rate was calculated according to Burns (Burns, 1995) as (ln BL_t- ln BL_0)/Δt, where BL_0 and BL_t are body length of organisms at day 0 and day 21 respectively, and time Δt = 21 day. Quantile plots and Shapiro-Wilk tests did not denoted significant ($p<0.05$) deviations from normality of measured data. Two way ANOVA using clone and Cu as fixed factors followed by post hoc Tukey's multiple comparison tests testing for differences between different clones and different copper treatments relatively to control were performed. In some variables variance heteroscedasticity was corrected by square or log transformation (Zar, 1996). Overall, a difference in sublethal endpoints was reported as statistically significant at $p < 0.05$.

4.3. Results

4.3.1. Chemical analysis

Results showed that measured concentrations varied in general less than 5% from the nominal concentrations. So, all calculations were based on nominal concentrations.

4.3.2. Acute toxicity

In the acute immobilization tests performed all pH and dissolved oxygen values were within the protocol requirements. The calculated LC_{50} values for the 48-h exposure are showed in Table 4.1. Eight in the ten clones tested from population R showed Cu-LC_{50} values below 30 μg l^{-1} whereas all clones from I population exhibited Cu-LC_{50} values above 35 μg l^{-1}. The highest LC_{50} found in I population (358.4 μg l^{-1}) was almost 4 times higher than the highest LC_{50} value found for the R population. Clonal differences for acute responses in population I were high compared to those reported for population R. For example, the 48-h LC_{50} of extreme genotypes were 38 to 358.4 μg l^{-1} for population I whereas the highest clonal differences observed in the LCs obtained for population R were 18 to 95.1 μg l^{-1}. Clones were categorized according to the observed degree of lethal tolerance to copper. Using the copper concentrations tested, a category system with six categories was created: 48h-LC_{50}s between 0 to 12.49 μg l^{-1} – "extremely sensitive"; 12.5 to 24.9 μg l^{-1} – "very sensitive"; 25 to 49.9 μg l^{-1} – "sensitive"; 50 to 99.9 μg l^{-1} – "tolerant"; 100 to 199.9 μg l^{-1} – "very tolerant"; 200 to 400 μg l^{-1} – "extremely tolerant". Two I-clones were characterized as extremely tolerant, five as tolerant and three as sensitive. Five R-clones were characterized as very sensitive, four as sensitive and one as tolerant. According to the obtained copper LC_{50}s, clones I3 (LC_{50} = 38.0 μg l^{-1}) and I10 (with LC_{50} =358.4 μg l^{-1}) were selected as I-sen and I-tol, the sensitive and the tolerant clones from population I, respectively. Clones R5 (LC_{50} = 18.0 μg l^{-1}) and R10 (with LC_{50} =95.1 μg l^{-1}) were selected as R-sen and R-tol, the sensitive and the tolerant clones from population R, respectively.

Table 4.1. Concentrations of copper (μg l⁻¹) impairing survival 50% on R and I *D. longispina* clones (LC$_{50}$s). LC$_{50}$s are represented with the 95 % confidence interval (CI) values inside brackets. (? = Values could not be computed).

I clones	Cu LC$_{50,\,48h}$ (μg l⁻¹)	R clones	Cu LC$_{50,\,48h}$ (μg l⁻¹)
I1	318.6 (281.0–358.4)	R1	23.2 (14.9–25.1)
I2	59.0 (50.3–70.5)	R2	25.2 (15.7–42.9)
I3	38.0 (33.2–43.4)	R3	21.9 (19.2–24.6)
I4	58.4 (50.8–68.4)	R4	23.7 (?–?)
I5	80.7 (70.0–93.0)	R5	18.0 (15.8–20.5)
I6	75.1 (62.4–93.6)	R6	40.4 (23.6–74.5)
I7	38.3 (32.7–45.6)	R7	26.1 (17.5–37.7)
I8	48.5 (?–?)	R8	25.8 (18.0–38.7)
I9	58.1 (50.6–70.9)	R9	20.04 (13.9–22.6)
I10	358.4 (318.1–403.9)	R10	95.1 (64.3–151.6)

4.3.3. Feeding tests

In the feeding experiments, observed mortality was always below 10% with the exception of the feeding experiment treatment of 80 μg l⁻¹ in which mortality was higher than 50% for both R clones (R-sen and R-tol). High mortality at 80 μg l⁻¹ prevented feeding determination at this concentration. Two-way ANOVA denoted significant ($p<0.05$) clonal differences within (clone) and across Cu (interaction) exposure levels (Table 4.2). For the "I-tol" clone, post hoc multiple comparison tests showed no differences in feeding rates at higher exposure levels of copper whereas for the rest of the clones significantly differences occurred, with a common trend of significantly lower feeding rates at higher exposure levels (Figure 4.1). Comparing feeding rates in "I-clones", "I-tol" clone showed always significantly higher feeding rates than "I-sen" clone, except for control conditions ($p < 0.05$). The comparison of feeding rates between R clones showed no significant differences for all the Cu concentrations tested, except the one of 5 μg l⁻¹, where the sensitive clone (R-sen) showed significantly higher feeding rate compared to the tolerant one (R-tol). The comparison of all clones showed a particular pattern for "I-tol". For instance, at control conditions "I-tol" clone showed a significantly lower feeding rate compared to the rest of clones, whereas the opposite occurred at 40 μg l⁻¹ of Cu.

Figure 4.1. Effects of copper and clone on feeding rates (mean + SE) of *D. longispina* clones from I population (A) and from R population (B). Statistically differences between clones (within each Cu concentration) are assigned in the graphs, using different letters (a, b, c, d). Asterisks denote Cu treatments statistically different relatively to the control (within each clone) (Post-hoc tests: $p < 0.05$).

Table 4.2. Analysis of variance results testing for effects of clone and copper and their interaction in *Daphnia longispina* feeding responses (ns – no significant; * - significant at $p < 0.05$; ** - significant at $p < 0.001$).

Source	df	F	p	Significance
Feeding				
Cu	*7, 280*	5.2	0.000	**
Clone	*3, 280*	12.1	0.000	**
Interaction	*13, 280*	3.5	0.000	**

4.3.4. Respirometry tests

In the respirometry experiments, observed mortality was always below 10% except for sensitive I and R clones, which was > 40%. Accordingly, two way ANOVA tests were restricted to 0-40 μg l^{-1} of copper. Exposure to increasing concentrations of copper caused different response patterns in terms of oxygen consumption (Figure 4.2, Table 4.3). *D. longispina* "I-tol" clone showed an increase in oxygen consumption when exposed to sublethal concentrations of copper. In contrast, the other three studied clones showed decreased oxygen consumption rates at increasing Cu concentrations (Figure 4.2, Table 4.3).
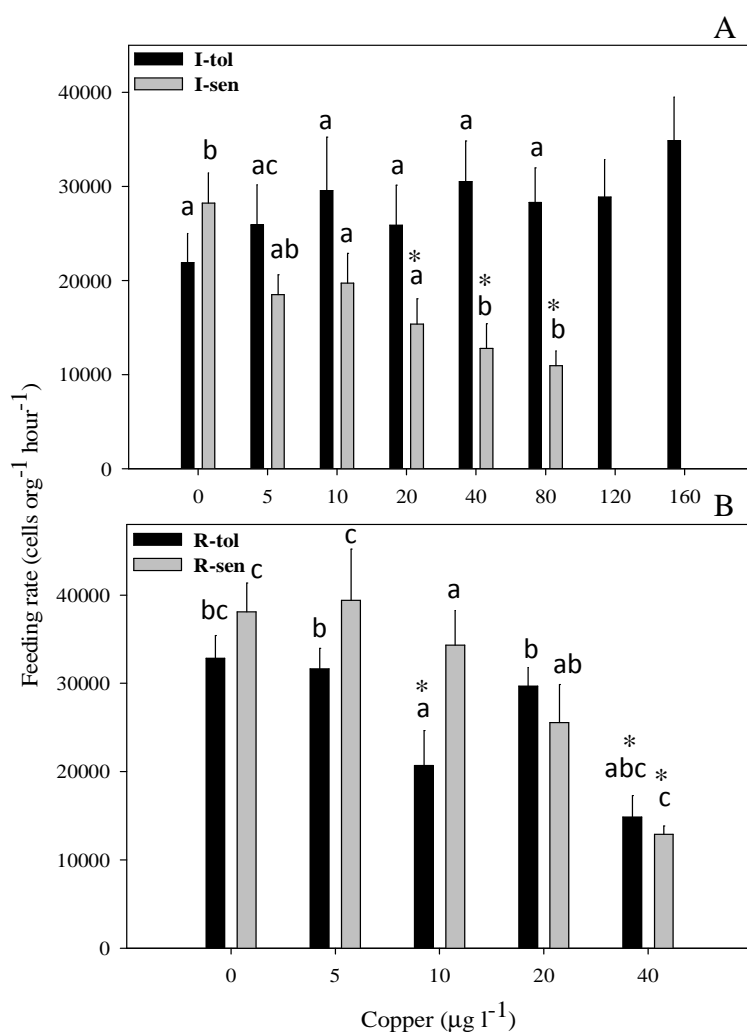

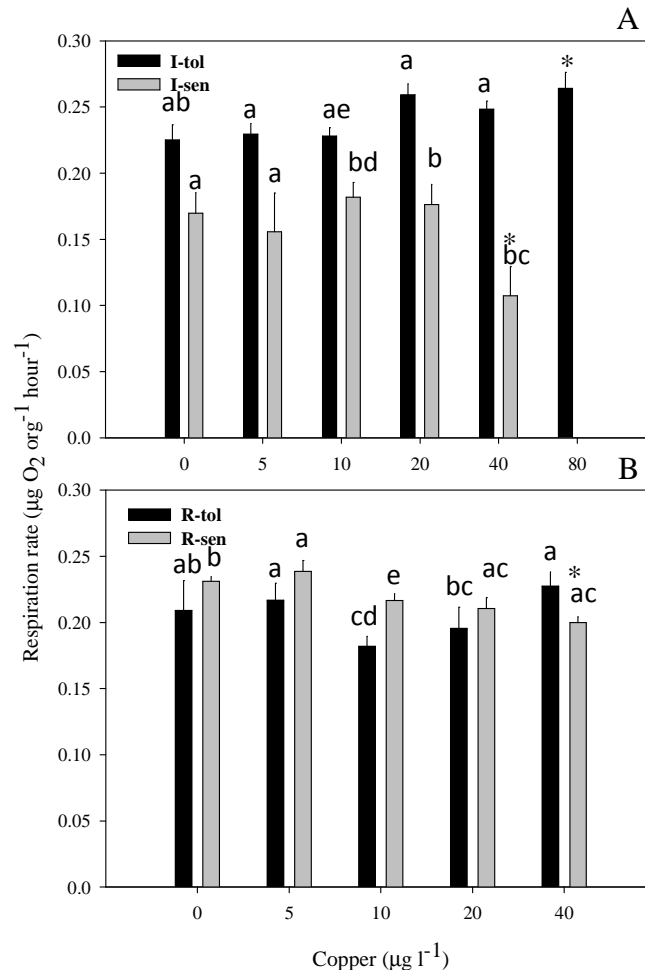**Figure 4.2.** Effects of copper and clone on respiration rates (mean + SE) of *D. longispina* clones from I population (A) and from R population (B). Statistically differences between clones (within each Cu concentration) are assigned in the graphs, using different letters (a, b, c, d). Asterisks denote Cu treatments statistically different relatively to the control (within each clone) (Post-hoc tests: *p* < 0.05).

Comparing clones from each population, "I-tol" clone showed also significantly higher respiration rates compared to the "I-sen" clone, at 10, 20 and 40 µg l^{-1} of copper. Comparing R-clones, significant differences only occurred at 10 µg l^{-1} of copper, with R-tol showing a significantly lower respiration rate compared to the R-sen clone.

Table 4.3. Analysis of variance results testing for effects of clone and copper and their interaction in *D. longispina* respiration rate responses (ns: no significant; *: significant at $p < 0.05$; **: significant at $p < 0.001$)

Source	df	F	p	Significance
Respiration				
Cu	*4, 80*	0.85	0.498	ns
Clone	3, 80	32.9	0.000	**
Interaction	12, 80	2.7	0.004	*

4.3.5. Reproduction tests

In the 21-day reproduction tests survival was significantly affected at 240 and 80 µg l^{-1} of copper in clones "I-tol" and "I-sen", "R-tol" and "R-sen", respectively ($p < 0.05$; $\chi^2(3) > 25.0$). Two-way ANOVAS showed effects of Cu and clonal differences in life-history and fitness traits among clones within and across Cu concentrations (Table 4.4, Figure 4.3). Copper and clone significantly affected age and clutch size at first reproduction, reproduction and growth rates and *r* responses (Table 4.4). Copper affected differently the studied clones. Reproductive parameters (somatic growth, reproductive rate, *r*) of "I-tol" clone were only impared at 160 and/or 240 µg l^{-1} of Cu, whereas for the "I-sen" one, maturation was significantly delayed and somatic growth was significantly reduced at 80 µg l^{-1} compared to control conditions. Regarding R population, Cu significantly affected reproduction rate and *r* in the sensitive clone (R-sen) at the concentration of 5 µg l^{-1} compared to control, whereas somatic growth was significantly reduced at 20 µg l^{-1} of Cu. In the "R-tol" clone, instead, Cu only significantly affected somatic growth and reproduction rate at 80 µg l^{-1} of Cu, whereas *r* was significantly affected at 10 µg l^{-1} of Cu. Regarding clone effects, clones from population I ("I-tol" and "I-sen") at control conditions (0 µg l^{-1} of Cu) were characterized by reproducing significantly earlier than R ones producing smaller clutches at first reproduction (significantly smaller for I-sen at $p < 0.05$) and

consequently having lower reproduction rates. Comparing tolerant clone versus sensitive one (inside each population), tolerant clones mature earlier and increase reproduction at higher copper concentrations, whereas in the sensitive clones Cu increased maturation ages and decreased reproduction. R clones showed higher somatic growth rates than I ones in the tested Cu concentrations with the exception of 80 µg l^{-1} of Cu, where I-tol showed a significantly higher somatic growth rate compared to clones from reference site (R-tol and R-sen). The above mentioned life-history traits resolved in differing fitness responses (r) of clones across Cu concentrations. Despite of showing similar r values in control conditions (non significant differences at Cu = 0), they showed different responses to Cu. Females of the "I-tol" clone keep their r similar with increasing Cu concentration, whereas the remaining clones decreased their r values.

Table 4.4. Analysis of variance results testing for effects of clone and copper and their interaction on the different parameters analyzed (ns – no significant; * - significant at p <0.05; ** - significant at p <0.001)

Source	df	F	p	Significance
Age at first reproduction				
Cu	5, 411	17.2	<0.001	**
Clone	3, 411	23.0	<0.001	**
Interaction	15, 411	3.84	<0.001	**
Clutch size at first reproduction				
Cu	5, 411	1.82	0.107	ns
Clone	3, 411	9.23	<0.001	**
Interaction	15, 411	2.08	0.010	*
Reproduction rate				
Cu	5, 411	8.57	<0.001	**
Clone	3, 411	6.25	<0.001	**
Interaction	15, 411	9.09	<0.001	**
Intrinsic rate of increase (r)				
Cu	5, 440	52.0	<0.001	**
Clone	3, 440	40.8	<0.001	**
Interaction	15, 440	9.81	<0.001	**
Somatic Growth rate				
Cu	5, 379	7.47	0.001	*
Clone	3, 379	8.00	0.002	*
Interaction	15, 379	15.0	0.000	**
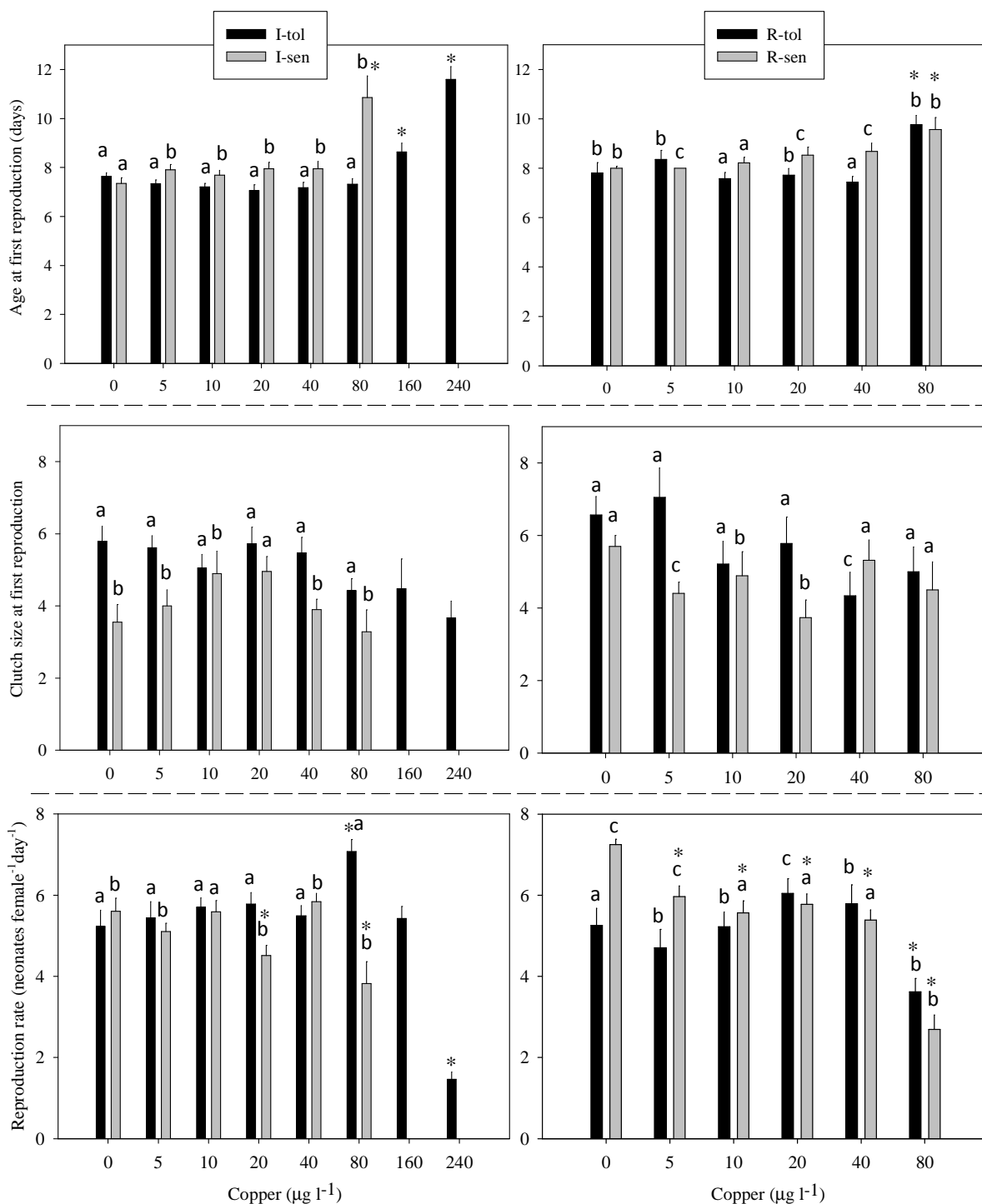
Figure 4.3. Effects of copper and clone on age and clutch size at first reproduction (mean+SE) and on reproduction rate (mean+SE) of *D. longispina* clones from I and from R populations. Statistically differences between clones (within each Cu concentration) are assigned in the graphs, using different letters (a, b, c, d). Asterisks denote Cu treatments statistically different relatively to the control (within each clone) (*p* < 0.05).

Figure 4.4. Effects of copper and clone on somatic growth rate (mean + SE) and on intrinsic rate of increase (mean + SE) of *D. longispina* clones from I population and from R population. Statistically differences between clones (within each Cu concentration) are assigned in the graphs, using different letters (a, b, c, d, e) Asterisks denote Cu treatments statistically different relatively to the control (within each clone) ($p < 0.05$).

4.4. Discussion

The first attempt of this study was to select sensitive and tolerant clones from two *D. longispina* populations. Results obtained in the 48-hour acute tests illustrated that I clones (from the field population historically exposed to metal contamination) exhibited a higher tolerance to lethal levels of copper relative to R clones. Five in a total of ten clones tested from population R showed Cu-LC_{50} values below 25 µg l^{-1} whereas

seven out of ten I clones exhibited Cu-LC$_{50}$ values above 50 μg l^{-1}. As for chapter 3, differences in lethal tolerance were evident in organisms acclimatized for more than 15 generations to laboratory conditions, which denotes a genetic basis as differences persisted after controlling for environmental and maternal influences through acclimatization (Barata and Baird 1998; Bossuyt and Janssen, 2004; Klerks and Weis, 1987; Lopes *et al.*, 2004). Based on the system defined to categorize clones according to the observed degree of lethal tolerance to copper, extremely tolerant clones were only found in population I and very sensitive clones were absent. These results illustrate the directional selection imposed by metal exposure through a shift in population genotype frequencies (Klerks and Weis, 1987; Spurgeon and Hopkin 2000). Thus evolutionary adaptation is most likely to be responsible for the clonal variaton in response to copper in the present study. Lopes and coworkers (2004) also observed the eradication of *D. longispina* sensitive lineages in a population from a metal impacted site, but contrary to the present study they found extremely tolerant and very tolerant clones in the reference *D. longispina* population. Survival of a fraction of a population, followed repeated or extreme exposure to a chemical stress (e.g. metal contamination), may lead to the genetic selection of segments of the population capable of stress avoidance, stress detoxification or repair or compensation for injury, whereas the least fit organisms are eliminated (Fox, 1995; Hoffman and Parsons 1994; Hoffman and Hercus, 2000; Spurgeon and Hopkin 2000). In cyclical parthenogenetic *Daphnia* species microevolution of ecologically relevant traits is expected to take place quickly (Declerck *et al.*, 2001; Haap and Köhler, 2009; Weber and Declerck, 1997) indicating a strong potential to adapt to environmental pollutants (Barata *et al.*, 2002; Hairston *et al.*, 1999).

The second purpose of this study was to determine if tolerance manifested by clones at lethal copper levels was also evident at the sublethal level and to assess different life strategies in the clones. This assertion was undertaken through the comparison of feeding and respiration rates, reproduction and growth and *r* values between those tolerant and sensitive clones. Results reported showed a clear distinct pattern of responses between the most tolerant clone ("I-tol") and the rest of tested clones and between I and R populations. Results from feeding and respiration experiments showed that for most clones but I-tol one, feeding rates were significantly

inhibited at increasing copper concentrations whereas respiration rates remained relatively constant being inhibited only at exposure copper levels close to lethality. Clone I-tol instead increased its feeding rates and oxygen consumption rates at the highest copper concentrations tested. Probably this different performance of I-tol clone is associated to enhancement of metabolic demands due to detoxification processes (i.e. metallothionein synthesis or other pathways). In fact, basal oxygen consumption rates in clone I-tol were greater than in the rest of clones. This latter finding also agrees with the physiological cost theory of Sibly and Calow (1989) about the existence of individual costs associated with altered physiological processes which enable resistant organisms to cope with the toxic stress. Accordingly to these authors detoxification mechanisms may divert energy from other fitness traits, such as growth or reproduction (Sibly and Calow, 1989). Indeed, the survival of an organism under stress conditions crucially depends on its ability to balance energy demand and energy supply (Calow and Forbes, 1998; Sibly and Calow 1989). The minimum requirement for short-term survival is provision of sufficient energy to cover basal maintenance needs such as maintenance of cellular homeostasis and systemic functions, including ventilation and circulation. On a long-term basis (especially on the time scale required for population survival), additional energy is needed to cover other costs, such as reproduction, growth or locomotion (to escape predators, to find food or a mate). Environmental stressors such as toxic metals can shift this equilibrium, resulting in energy deficits due to elevated maintenance costs (e.g. for detoxification or repair of the cellular damage) or direct interference with energy conservation (e.g. mitochondrial function and/or cytosolic ATP-producing pathways) (Calow and Forbes 1998; Sibly and Calow 1989). In either of the cases, such shifts in the energy balance may lead to fitness costs and trade-offs at the whole-organism and population levels. Other studies have theoretically and experimentally analyzed the association of tolerance and fitness cost due to an increased energy demand in detoxification processes. In the study of Lukasik and Laskowski (2007), a multi-generation exposure of the flour beetle (*Tribolium confusum*) to copper showed that animals bred for ca. 10–13 generations in copper-contaminated medium had higher maintenance costs than their counterparts originating from the uncontaminated medium. The result from this study illustrates that significant change in energy budgets may occur even after relatively short selection in small laboratory

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cultures. In another study Cherkasov *et al.* (2006) showed that both protein synthesis rate and oxygen consumption required to cover the costs of protein synthesis increased in eastern oysters (*Crassostrea virginica*) exposed to cadmium.

Regarding reproduction, tolerant clones matured earlier at expenses of producing smaller broods and having lower somatic growth rates than sensitive clones. Interestingly under none or low copper exposures both populations showed equivalent fitness (*r* values). The most significantly finding, however, is the observed different responses to Cu of the most tolerant clone relative to the other ones. In particular, "I-tol" clone increased or maintained its fitness performance with increasing copper concentrations at expenses of maturing earlier, increasing its daily reproduction rates and being smaller (having lower somatic growth). The other clones, irrespectively of their overall sensitivity to copper and origin, showed a similar response: copper decreased their fitness delaying maturation and decreasing reproduction and somatic growth. These results agree with the observed life-history performance of artificially selected lines of the fruit fly (*Drosophila melanogaster*) exposed to cadmium (Shirley and Sibly, 1999). These laboratory studies on the fruit fly have demonstrated that flies reared on a medium polluted by cadmium evolved resistance to the metal and showed higher survival and fecundity when exposed to this polluted environment (Shirley and Sibly, 1999). However, in the work of Shirley and Sibly resistant lines paid a fitness cost in unpolluted environments, with reduced fecundity and weight relative to non-resistant flies, which would likely translate to a rapid loss of the resistant genotype in unpolluted environments. A similar resistance-related fitness costs have been observed in annelid worms, with cadmium-resistant genotypes from polluted sediments displaying very slow somatic growth relative to worms from cleaner sites (Levinton *et al.*, 2003). Whether or not tolerance to copper was costly in fitness terms was not evident in the present study. Clonal intrinsic rate of population growth rate responses, considered to be a good estimate of fitness in the laboratory, did not differ in non exposed treatments. Although in control treatments mortality did not occur, in real field situations under fish or invertebrate predation differences in size at first and subsequent clutches may have strong effects on survival. For example, under fish predation clones reproducing earlier at smaller size should be favored, whereas the opposite trend will be selected under invertebrate predation (Barata *et al.*, 2002; Declerck and Weber, 2003;

Taylor and Gabriel, 1992). In fact, according to the model of Taylor and Gabriel (2002), the optimal *Daphnia* life strategy (with maximal fitness) in the presence of a predator invoking mortality of larger/older individuals is characterized by a small adult body size and a high allocation of energy to reproduction. Conversely, in the presence of a predator selecting for small/young prey, the model predict the highest fitness if allocation to growth is high, resulting in a large adult body size. In this study clones from population I and R will show greater fitness under fish and invertebrate predation under no copper, respectively. Unfortunately the dominant predatory pressure under the studied system is unknown, although fish (mainly cyprinids) and probably invertebrates are present in both locations (Godinho *et al*., 1998; Ribeiro *et al*., 2007).

Regarding behavioral and physiological traits, reported results evidenced a close link between feeding and oxygen consumption rates and life-history responses. Clones showing impaired or increasing feeding under copper exposure also showed their fitness impaired or increased. Results of respiration rates are less conclusive since for most clones but the tolerant one, respiration rates were quite constant being inhibited only at exposure Cu levels close to lethality.

4.5. Conclusion

In this study it was possible to show that the adaptation to the stressful environment involved the acquisition of tolerance not only to lethal levels of copper but also to sublethal levels. In fact, historical exposure to metal contamination conferred a higher genetically determined tolerance to sublethal levels of copper in the tolerant clone. Reported results evidenced that the referred clone increased its feeding rate in the presence of copper and matured earlier at expenses of producing smaller broods and having lower somatic growth rates. Changes in life-history traits in the tolerant clone were not accompanied by the existence of fitness costs associated under optimal conditions (absence of copper). The present study provided further evidence confirming the importance of genotype by environment interactions in the clonal *D. longispina* response to sublethal levels of copper.

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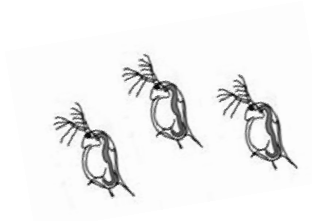ZAR J. H. (1996). Biostatistical Analysis 3rd Ed., Prentice Hall International, Upper Saddle River, NJ, USA.

CHAPTER 5

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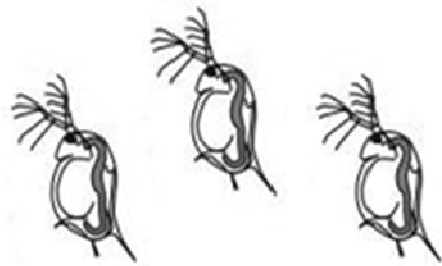
Influence of multigenerational acclimation to sublethal concentrations of copper on tolerance in *Daphnia longispina*

Chapter

5

Influence of multigeneration acclimation to sublethal concentrations of copper on tolerance in *Daphnia longispina*

Abstract

The present study was conducted to assess the effects of a multigenerational acclimation to copper in a *Dapnhia longispina* clone. In the previous chapter it was reported that those *D. longispina* clones historically exposed to copper that showed a higher tolerance matured earlier, had greater reproductive rates and hence higher fitness performance when exposed to copper. The aim here was to test if physiological adaptation to copper across several generations may lead to the same outcome. To test this hypothesis a single cone of *Daphnia longispina* originated from the reference population was chronically exposed to copper over three consecutive generations and its life-history performance evaluated. Results illustrate that physiological adaptation to copper across several generations only increased marginally acute tolerance to copper. Besides that, a high variation in life-history traits was observed not only between copper treatments, but also among generations. For instance, generation had a significantly influence on the observed pattern of age at first reproduction and interact with copper in the observed variation of time and clutch size at first reproduction. These findings document the limited significance of single generation surveys in standard ecotoxicological studies. Besides that, as survival remained constant in this study and the reproductive rates increased, it can be hypothesized that adaptation to copper in the tested *Daphnia longispina* clone involve a trade-off between maturing earlier at expenses of growth, although this parameters has not been tested.

5.1. Introduction

Exposure of organisms to sublethal concentrations of metals in the environment can lead to changes at all levels of biological organization, from biochemistry and physiology of individual organisms to whole communities' structure and function (McGeer *et al*., 2000; Posthuma and Van Straalen, 1993; Weis *et al*., 2001). Numerous studies have documented negative effects of metals and other environmental pollutants on life-history traits of ecotoxicological model species in the laboratory (Walker *et al*., 2001). Although laboratory bioassays provide a powerful tool for toxicity assessment, they mostly consider proximate single-generation effects on life-history responses towards chemical exposure (Vogt *et al*., 2007). In contrast, natural populations are chronically exposed to metals across multiple generations since metals persistent pollutants (Paumen *et al*., 2008). There are numerous factors that can influence long-term response to metal exposure in the field. Populations may adapt to metal-polluted environments but at expenses of physiological and genetic costs that may lead to extinction (Bickham *et al*., 2000; Gillis *et al*., 2002; Shirley and Sibly, 1999; Van Straalen and Timmermans, 2002; Vogt *et al*., 2007). Multigenerational studies, thus, can provide valuable insight into the long-term response of organisms to chemically induced stressors (Janssen *et al*. 2000; Lagisz and Laskowski, 2008; Paumen *et al*., 2008; Van Brummelen *et al*., 1996). In fact, multigeneration experiments allow to exploring long-term effects such as the detrimental effects of delayed reproduction, of maternal pollutants transmitted to the offspring or increasing levels of DNA damage (Janssen *et al*. 2000; Lagisz and Laskowski, 2008; Paumen *et al*., 2008; Van Brummelen *et al*., 1996). For these reasons, it is essential to investigate multigeneration metal exposures responses. Tolerance of organisms to metals can be mediated by either physiological mechanisms of decreased response (target insensitivity to the toxicant) or mechanisms of decreased exposure (avoidance, reduced uptake, increased detoxification, increased

elimination and increased sequestration) (Belfiore and Anderson, 1998; Klerks and Weis 1987; Posthuma and Van Straalen, 1993; Taylor and Feyereisen, 1996). Metal tolerance is well documented in many plant and animal *taxa* and is thought to be among the best observed examples of organismal evolution to natural and anthropogenic stress (Posthuma and Van Straalen, 1993). In animal species, metal tolerance has been studied mostly in aquatic and terrestrial invertebrates (Donker *et al.*, 1993; Groenendijk *et al.*, 2002; Leblanc, 1982; Lopes *et al.*, 2004). Multigeneration metal exposure can mimic field situations thus providing more systematic results than single generational studies (Muyssen and Janssen, 2004). The choice of test animals is an important factor to successfully perform multigenerational studies. *Daphnia* is small in size and has a relatively short life cycle. The time required for 24-h old daphnids to have their first offspring is approximately 9 to 12 d. Hence, it is an ideal candidate for those kind of tests. There are several studies evaluating the toxicity of metal compounds across several generations of *Daphnia magna*, including copper (Bossuyt and Janssen, 2003, 2004, 2005), cadmium (Bodar *et al.*, 1990; Guan and Wang, 2006; Muyssen and Janssen, 2004; Ward and Robinson, 2005), zinc (Muyssen and Janssen, 2004), nickel (Münzinger, 1990; Pane *et al.*, 2004), chromium (Münzinger and Monicelli, 1992) and mercury (Tsui and Wang, 2005). A recent review from Tsui and Wang (Tsui and Wang, 2007) addressing the development of tolerance to toxic metals in *D. magna* emphasized that multigeneration exposure of metals in other species of *Daphnia* should also be evaluated. In fact, the use of *D. magna* has been criticized by some authors who argue that this cladoceran has a limited geographical range and low sensitivity to toxic chemicals when compared to other cladoceran species. Moreover, it was proved in the work of Koivisto and coworkers (Koivisto *et al.*, 1992) that sensitivity to copper of small cladoceran species was higher than that of *D. magna*. In another study, Shaw and colleagues showed that *D. magna* exhibited higher tolerance to both cadmium and zinc toxicity than other *Daphnia* species (Shaw *et al.*, 2006). Regarding the metal copper, Bossuyt and Janssen (2004) reported over 12 fold differences in tolerance across twenty cladoceran species.

In the previous chapter it was reported that those *D. longispina* clones historically exposed to copper that showed a higher tolerance matured earlier, had greater reproductive rates and hence higher fitness performance when exposed to copper. The

aim of the present study is to test if physiological adaptation to copper across several generations may lead to the same outcome. To test this hypothesis a single clone of *Daphnia longispina* originated from the reference population was chronically exposed to copper over three consecutive generations and its life-history performance evaluated.

5.2. Material and Methods

5.2.1. Culturing of *D. longispina*

The *Daphnia longispina* R-sen clone was maintained in laboratory under controlled conditions of temperature (20±1 ºC) and photoperiod (16 light: 8 dark) for more than 15 generations prior to bioassays. The culture was maintained in 1 liter glass vials with 800 ml of ASTM hard water (ASTM, 2002) enriched with a standard organic extract Marinure 25 (Glenside, Stirling, UK) (Baird *et al.*, 1989). Daphnids (30 organisms per culture vial) were fed every other day with the green algae *Chlorella vulgaris* Beijerinck (3×10^5 cells ml^{-1}) and its culture medium was changed three times a week. Newborn neonates in cultures were removed within 24 h of release. Neonates from the fifth or sixth broods were used to replace the old cultures.

5.2.2. Test chemical

A stock solution of copper was prepared by adding analytical reagent-grade salt of copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) (supplied by Sigma-Aldrich, Germany) to deionized water (Milli-Q, Bedford, MA, USA). Stock solution was stored at 4 ºC protected from light during the experiments. Nominal test concentrations were subsequently prepared by adding aliquots of each metal stock solution to the ASTM medium. Copper test solutions were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS).

5.2.3. Experimental design

In the parental generation (F_0) reproduction test, 20 neonates (< 24 h old) per copper concentration were individually transferred from the bulk culture to 60-ml glass beakers containing 50 ml of the test medium. Five copper concentrations (5, 10, 20, 40 and 80 μg l^{-1}) plus a control treatment (ASTM only) were tested. To start another experimental generation (F_1), 20 neonates (< 24 h old) from the third brood of the parental generation (F_0) were collected from each exposure copper concentration and individually transferred to 60-mL beakers containing 50 ml of the same medium, plus the control. Subsequently, these newborn daphnids (F_1) were exposed to the same copper concentrations as generation F_0. Two more experimental generations were conducted with third brood newborns generation F_1 (F_2 experimental generation) and F_2 (F_3 experimental generation). Test solutions were renewed every other day and *C. vulgaris* was supplied every day in a concentration of 3.0×10^5 cells ml^{-1}. Tests were checked daily for eventual mortality. Age and clutch size at first reproduction and the reproductive rate responses (offspring/female/day) were recorded and used to determine the per capita rate of population increase (r) using the Euler- Lotka equation:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1$$

where r stands for the per capita rate of population increase (day^{-1}), x for the age class (days), l_x for the probability of surviving to age x (0…n), and mx for the fecundity at age x. Uncertainties were estimated according to the Jackknife technique (Meyer *et al*. 1986).

The experimental design is provided in Figure 5.1. To assess tolerance to copper across generations, acute experiments were conducted with neonates from the fourth brood of the first generation and third generation. Forty-eight hour (48-h) static immobilization tests were performed in accordance with the OECD 202 guideline (OECD, 2004). Six copper concentrations (12.5, 25, 50, 100, 200 and 400 μg l^{-1}) plus an ASTM negative control were used. Acute tests were performed by quadruplicated using 5 newborn <24 h neonates exposed to 100 ml of medium. The measured toxicological endpoint was mortality as identified by immobility and was checked at 24 and 48 hour of exposure.

Dissolved oxygen and pH were monitored along the acute and chronic tests for validation purposes (OECD 1998, 2004).
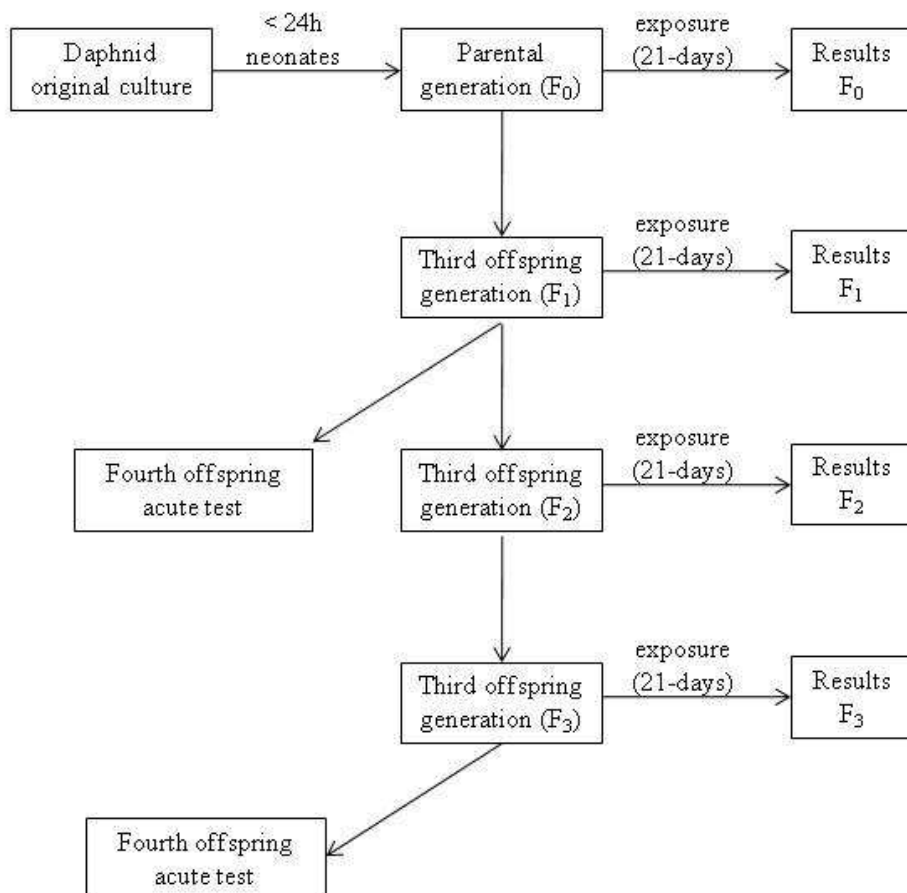
Figure 5.1. Experimental design of the multigenerational toxicity test.

5.2.4. Data analysis

Probit analysis (Finney, 1971) allowed the calculation of the 48-hr LC_{50} immobilisation values with the respective 95% confidence limits, for both F_1 and F_3 generation daphnids. For each *Daphnia* population, a two-way analysis of variance (two-way ANOVA) was used to assess the significance of the effects of generation and copper concentration, as well as their interaction, on each of the life-history endpoints and on the population growth estimate (Zar, 1996). Copper and generation were set as fixed and random factors. Prior to analyses data were *ln* transformed to meet ANOVA

assumptions of normality and homocedasticity. One-way ANOVAs, followed by post-hoc multi-comparisons test, were carried out to achieve differences in copper treatments relatively to the control within each generation and to assess differences in generations within each copper treatment (Zar 1996). A significance level (α) of 0.05 was used in all the performed ANOVAs (Zar 1996). All analyses were performed using SPSS statistical package (SYSTAT).

5.3. Results

Either the acute or the chronic tests fulfilled the validity criteria recommended in the standardised protocols (OECD 1998, 2004). Dissolved oxygen and pH did not change significantly during the assays or with the increase of copper concentrations (min-max: 7.45-8.37 pH; 7.3-8.1 mg l^{-1} O_2).

Effects of copper on survival during the 21-day exposures for parental (F_0) and the three subsequent generations of *D. longispina* (F_1, F_2 and F_3) are showed in Figure 5.2. Survival was always equal or higher than 80% at all copper exposures in all the generations tested, being the 20 μg l^{-1} Cu exposure of the F_2 generation the only exception, with a 70% survival at the end of the 21 days.

Effects of copper and generation on age and clutch size at first reproduction, reproduction rates and *r* are showed in Figure 5.3. Two-way ANOVAs showed always significant copper effects with either significant generation or interaction effects on all life history traits studied (Table 5.1). Age at first reproduction was the trait most affected by exposure to consecutive generations with F3 organisms maturing earlier. For instance, 1-way ANOVA showed significant differences between generations, with generation F3 showing a significantly lower age at first reproduction comparing to F0, F1 and F2 for the highest copper exposure (80 μg l^{-1}) (Pos-hoc tests; $p < 0.05$). Clutch size and reproduction rates, however, showed a more complex pattern without a clear trend. According to two-way ANOVA results, generation didn't have a significant effect in the clutch size and reproduction rate (at $p > 0.05$), but showed interaction effects modeling the way how copper influenced the referred parameters.

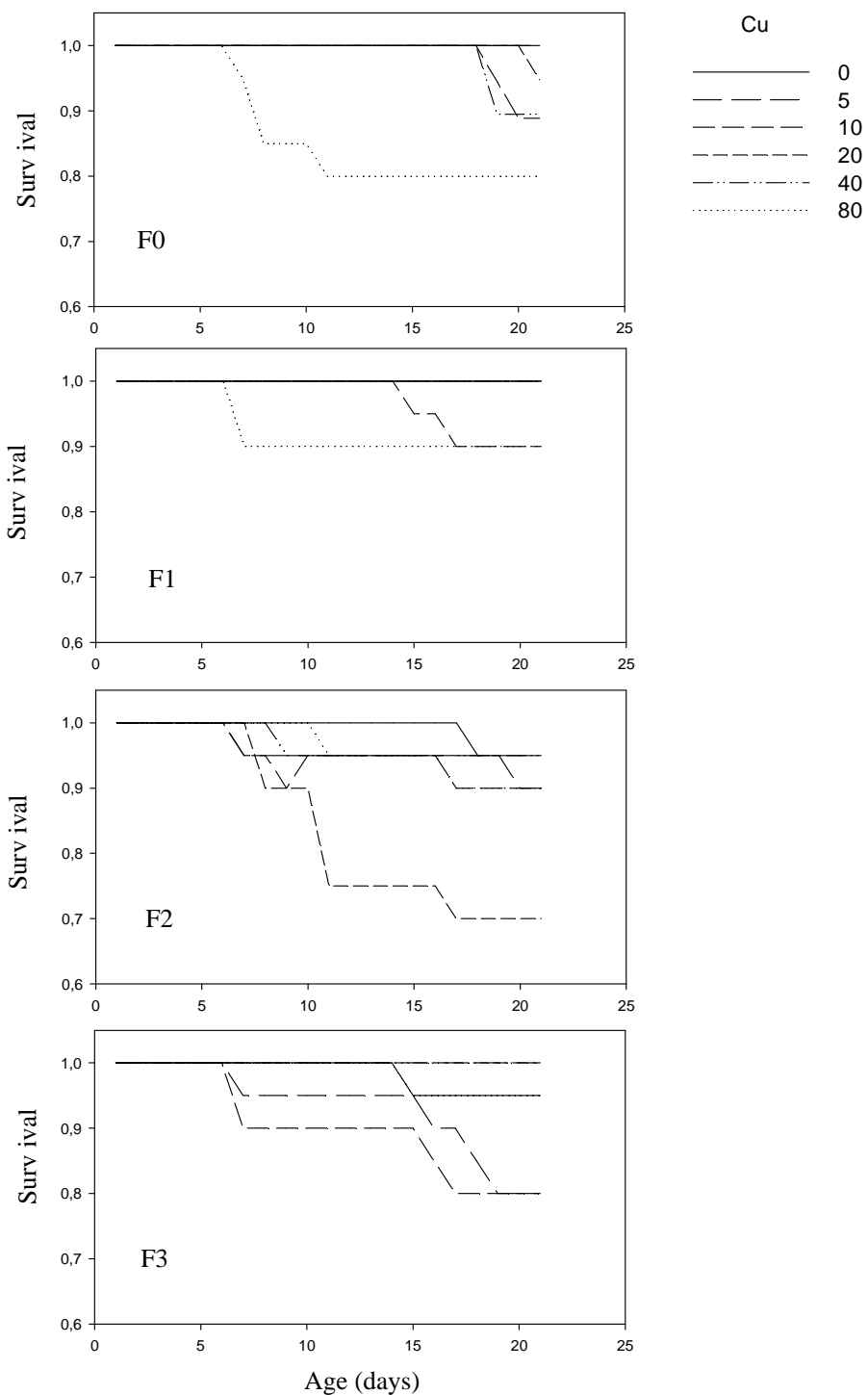**Figure 5.2.** Effects of copper on survival during the 21-day tests (for the generations F0, F1, F2 and F3).
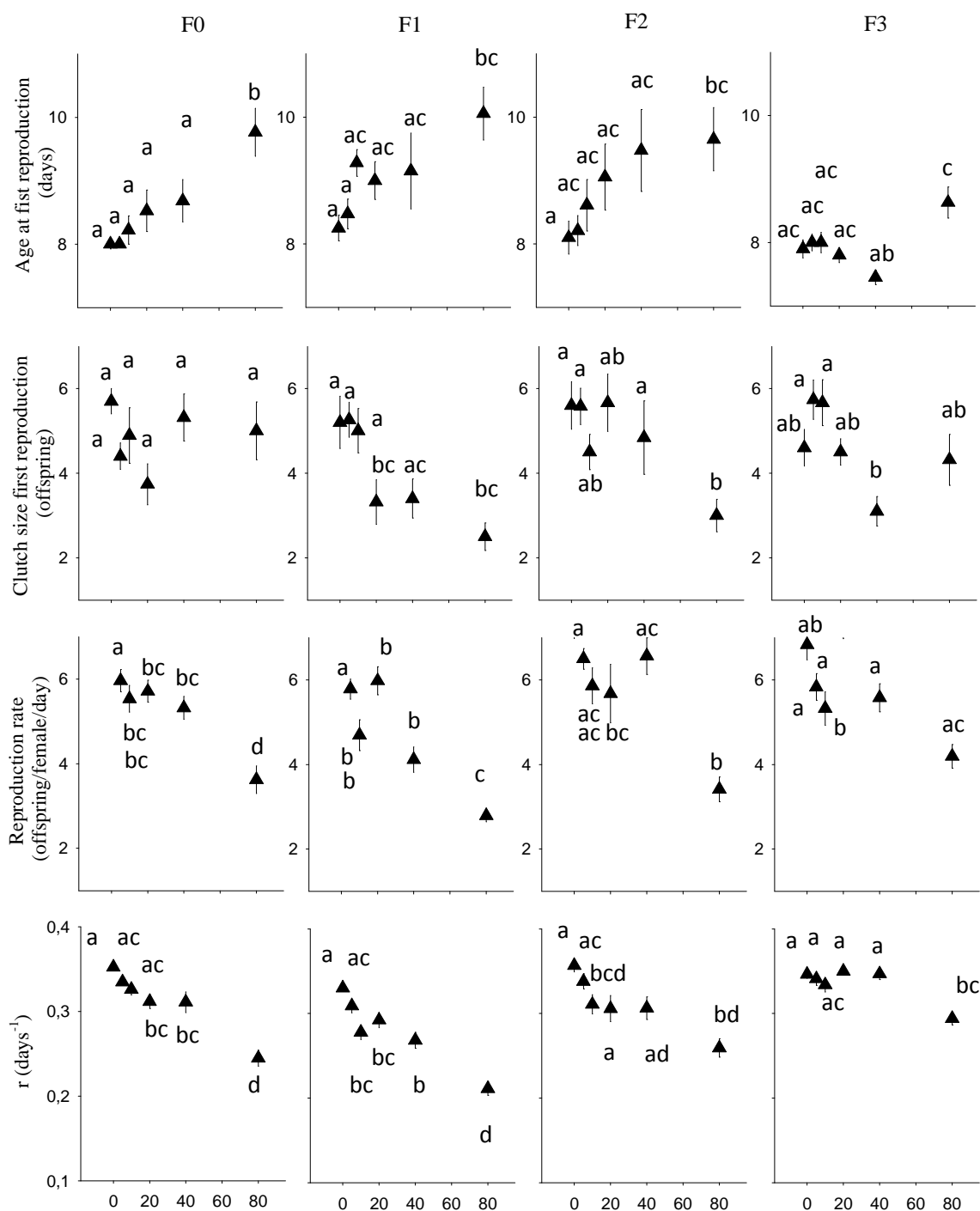
Figure 5.3. Effects of copper on life history parameters (mean + SE) for the consecutive generations F0, F1, F2 and F3. Within each generation different letters indicate significant *(p<0.05)* differences across exposures (In reproductive rate, values for Cu treatments 5 and 10 are overlapped)

Table 5.1. Effects of copper and generation on life history parameters age and clutch size at first reproduction, reproduction rate and r

Source	df	F
Age at first reproduction		
Generation	3,15	12.66*
Copper	5,15	10.13*
Interaction	15,433	1.102
Clutch size at first reproduction		
Generation	3,15	2.804
Copper	5,15	6.511*
Interaction	15,433	2.801*
Reproduction rate		
Generation	3,15	6.848
Copper	5,15	55.11*
Interaction	15,433	3.070*
r		
Generation	3,15	39.17*
Copper	5,15	53.74*
Interaction	15,451	2.212*

The effect of copper on population growth rates decreased with increasing generations. In fact, for generation F0, copper at 20 µg l^{-1} have significantly reduced the *r* value compared to control (1-way ANOVA; Pos-hoc test; $p < 0.05$), whereas in generation F3, a significantly reduction in the *r* value compared to control occurred only at the highest copper exposure (80 µg l^{-1}). Generation have also a significant effect in the observed populations growth rates. In fact, generation F3 showed significantly higher population growth rates compared to the other generations (F0, F1, F2) at the highest copper concentration tested (80 µg l^{-1}) (1-way ANOVA; Pos-hoc test; $p < 0.05$) whereas at control conditions (Cu = 0 µg l^{-1}), population growth rate of F3 was not significantly different from F0 or F1 population growth rates.

Acute tolerance of neonates from F3 were only marginally greater than those of F1 as shown by overlapping 95% CI of LC$_{50}$s (Table 5.2).

Table 5.2. Acute (48-h LC50) toxicity with confidence interval of F1 and F3 generations of D. longispina as a function of the copper concentration

Generation	copper concentration (µg l^{-1})					
	0	5	10	20	40	80
1	22.51	22.51	22.97	26.09	22.51	32.27
	(9.70-24.62)	(9.70-24.62)	(6.66-25.16)	(17.84-38.86)	(9.71-24.62)	(21.88-47.58)
3	22.07	26.09	28.63	30.26	35.34	47.42
	(10.44-24.18)	(17.84-38.86)	(23.67-34.45)	(25.25-36.20)	(29.40-42.98)	(28.96-78.72)

5.4. Discussion

This work tested the hypothesis that physiological acclimatization to copper across generations may lead to greater tolerance and higher fitness to the studied metal. The copper concentrations used in this mutigenerational life-table experiment (from 5 to 80 µg l^{-1}) encompassed a wide range of concentrations typically found in natural freshwater systems, including polluted and unpolluted waters (Bossuyt and Janssen, 2003; Bossuyt and Janssen 2004). Regarding the effects of the chosen copper range on successive *D. longispina* generations, no significant effects were observed on survival, which has remained relatively constant during the long-term copper exposure. In contrast there were significant effects on reproduction parameters, such as time and clutch size at first reproduction and reproductive rate. High variation in life-history traits was observed not only between copper treatments, but also among generations throughout the multigenerational study. For instance, generation had a significantly influence on the observed pattern of age at first reproduction and interact with copper in the observed variation of time and clutch size at first reproduction. These findings document the limited significance of single generation surveys in standard ecotoxicological studies. Although conditions were kept constant throughout the study and equal concentrations of copper were applied in all generations, a comparison of

different generations (e.g. F_2 and F_3) leads to completely different conclusions concerning the effects of the chosen copper concentration on *D. longispina* reproductive parameters. There are two reasons why organisms may develop tolerance to metals. One is that they may acclimate to the metal at some early stage in their life cycle (Klerks and Weis, 1987; Posthuma and Van Straalen, 1993). Upon exposure to sublethal concentrations of the metal, physiological responses may arise that promote increased tolerance compared to individuals that have not been exposed. As this response is induced as a result of metal exposure, acclimation-based tolerance is not passed on to an organism's offspring. Other alternative is the development of a genetically-based tolerance, which means that adaptation to the presence of the metal occurs through the action of natural selection on genetically based individual variation in tolerance. This selection for genetically tolerant organisms increases the proportion of resistance alleles in the population since the transfer of its tolerance to the offspring occurs (Bodar *et al*., 1990 Klerks and Weis, 1987; Posthuma *et al*., 1993). In this study, population growth rates at all copper treatments investigated tended to increase over time (more evident from F_2 to F_3). This increase can most likely be explained by maturing earlier in the respective groups. According to Shirley and Sibly (1999), adaptation to metals can involve tradeoffs between maturing earlier at expenses of survival, maturing earlier at expenses of growing less and maturing earlier at expenses of fecundity. As survival remained constant it can be hypothesized that adaptation to copper in *Daphnia longispina* involve a trade-off between maturing earlier at expenses of reproduction and growth. As fecundity increased, it can be hypothesized that adaptation to copper in *D. longispina* tested in the study involved a trade-off between maturing earlier at expenses of growth, although this parameter was not measured in the study. A similar trade-off was illustrated in the work of Shirley and Sibly (1999) where *Drosophila melanogaster* populations were maintained for 20 generations on a cadmium-polluted medium and a between-environment trade-off was identified allowing *D. melanogaster* increased fitness in polluted environments but only at the cost of reduced growth and reproduction in unpolluted environments. Other multigenerational life-table experiments for other metals and *Daphnia* species showed the occurrence of adaptation although the trade-off hypothesis was not revealed. Münzinger (1990) studied the effect of nickel on seven generations of *D. magna* and found an adaptation toward nickel as the intrinsic rate of

population growth (*r*) increased in the exposed generations. Furthermore, Bodar *et al*. (1990) found that *D. magna* exposed to sublethal cadmium concentrations become more resistant to the toxic effects of cadmium and the degree to which exposed daphnids developed resistance to cadmium remained similar for three sucessive generations.

Results of the current study did agree with previous studies. In the present work acclimatization to copper only increased marginally acute tolerance to copper.

Effects of metal toxicity on invertebrates are complex and vary markedly between taxa. Whereas some species are able to develop a degree of tolerance to metals, through physiological acclimation or genetic adaptation (Clements, 1999; Bossuyt and Janssen, 2003) other species are not. As an example, Clements (1999) found that the nymphs of two mayflies species *Baetis tricaudatus* and *Rhithrogena hageni*, which had already been exposed to chronic levels of cadmium, copper and zinc, survived better and were less inclined to drift than nymphs that had not been exposed previously to the pollutants. *Taxa* can differ in sensitivity to metals depending on their life history stage. As an example, early instars of a chironomid midge *Chironomus tentans* tolerated 12-27 times higher concentrations of copper than late instar larvae (Gauss *et al*., 1985). Avoidance behavior is another response to the presence of toxic concentrations of metals. An understanding of both the timing and the basis of contaminant resistance is important for the design and interpretation of toxicity studies. Knowledge of the temporal scale of resistance could affect the design of a toxicity test whereas information regarding the ability of a species to adapt (acclimate) could influence its suitability for a particular test design (Vidal and Horne, 2003).

5.5. Conclusion

In this study it was possible to show the life-history effects of long-term acclimatization to copper in a *D. longispina* clone. One of the study's outcomes was that physiological adaptation to copper across several generations only increased marginally acute tolerance to copper. Besides that, a high variation in life-history traits was observed not only between copper treatments, but also among generations. For instance, generation

had a significantly influence on the observed pattern of age at first reproduction and interact with copper in the observed variation of time and clutch size at first reproduction. These findings document the limited significance of single generation surveys in standard ecotoxicological studies. Results of the current study also agree with previous studies such as the one from Shirley and Sibly (1999), which postulate that adaptation to metals can involve tradeoffs between maturing earlier at expenses of survival, maturing earlier at expenses of growing less and maturing earlier at expenses of fecundity. As survival remained constant in this study and fecundity increased, it can be hypothesized that adaptation to copper in the tested *Daphnia longispina* clone involve a trade-off between maturing earlier at expenses of growth.

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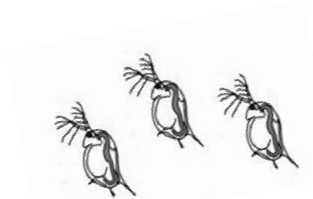
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CHAPTER 6

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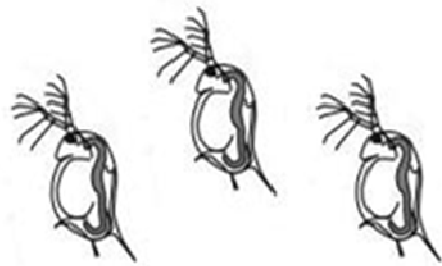
General Conclusions

Chapter
6

General Conclusions

Daphnia species play an important role in aquatic ecosystems and due to their life-cycle they offer unique possibilities for research on evolutionary and ecological questions (Hairston *et al*., 1999; Dudycha and Tessier, 1999; Innes and Singleton, 2000; Lynch, 1983). One of the studied issues during the last years has been *Daphnia* adaptation to chemical pollution. In fact, pollutants represent often a strong and stable directional selection pressure that can make adaptation a very quick process (Klerks and Levinton, 1989; Posthuma and Van Straalen, 1993; Posthuma *et al.,* 1993; Theodorakis and Shugart, 1997). This is especially true for metals since they are non degradable pollutants and tend to accumulate in water sediments and soil where they can persist for many years. Several works have highlighted the significance of microevolutionary processes in population responses to toxicants as well as of the evolutionary consequences of population exposure to toxicants (Barata *et al*. 2002; Depledge, 1994; Matson *et al*., 2006; Morgan *et al*., 2007; Shugart and Theodorakis, 1996). In resume, rapid genetic changes or microevolutionary processes associated to a genetically inherited increase in tolerance can be triggered by toxic substances like metals released to the environment (Klerks and Levinton, 1989; Medina *et al*., 2007; Theodorakis and Shugart, 1997). This PhD study enlarges the examples of naturally occurring populations that have developed tolerance to metals following long-term exposures (i.e. decades). In this PhD study, a natural population of a cladoceran species – *Daphnia longispina* – was chosen from a relatively well-known metal contaminated aquatic system, the freshwater system surrounding São Domingos mine. In Chapter 2 it has been shown that field populations of *Daphnia longispina* located in the water reservoir contaminated by the acid mine drainage are continuously exposed to seasonally varying concentrations of metals. In Chapter 3 a *Daphnia longispina* population

collected in this heterogeneous polluted reservoir along with another population from a nearby non-contaminated reservoir was maintained in the laboratory for several generations and genetic consequences of adaptation to both copper and zinc were assessed. A genetic analysis including changes in tolerance, genetic variation in tolerant and fitness related traits and genetic by environmental trade-offs (costs of tolerance) was done. Results showed that only resistant clonal lineages to copper were present in the contaminated site. This fact was also confirmed in Chapter 4 and can have implications at the population level if the loss of sensitive species is translated into a higher susceptibility to extinction (Evendsen and Depledge, 1997; Fox, 1995). Thus, the loss of sensitive species in resistant populations can cause deleterious effects at other levels of organization, such as communities and ecosystems, affecting its process rates and functional properties (Kinzig *et al*., 2002; Loreau *et al*., 2003; Medina *et al*., 2007). Also in Chapter 3 it was shown that heritability values for tolerant and feeding traits were similar in both populations being in most cases greater than 50% and fitness costs of tolerance were evidenced supporting the view that tolerance to pollution is ecologically costly. In fact the existence of costs associated with adaptation to pollution becomes important as these could affect the performance of the adapted population during the polluting event or could be observed as between-environment genetic trade-offs in unpolluted or recovered sites (i.e. cleaned). These costs will eventually change the physiology of organisms, thus the way how populations exploit their ecological niches and participate in the overall ecosystem functioning (Medina *et al*., 2007). In resume, the acquisition of genetically inherited tolerance can have ecological costs derived from genetic changes (i.e. loss of genetic variability), negative pleiotropy with fitness traits and/or from physiological alterations that enable resistant individuals to remain at the affected site. In Chapter 4 an exhaustive life-table response study was conducted for four *D. longispina* clones differing in their tolerance to lethal levels of copper to determine how adaptation to copper affected the life history strategies. Feeding, oxygen consumption, growth and reproductive responses were compared. Results from feeding and respiration experiments showed that for most clones feeding rates were significantly inhibited at increasing copper concentrations whereas respiration rates remained relatively constant being inhibited only at exposure copper levels close to lethality. The opposite occurred for the tolerant clone from impacted population which increased its feeding rates and oxygen consumption rates at the highest

copper concentrations tested. Regarding reproduction, tolerant clone from impacted population increased or maintained its fitness performance with increasing copper concentrations at expenses of maturing earlier, increasing its daily reproduction rates and being smaller (having lower somatic growth). The other clones, irrespectively of their overall sensitivity to copper and origin, showed a similar response: copper decreased their fitness delaying maturation and decreasing reproduction and somatic growth. Overall, data showed that tolerance manifested by *D. longispina* clones at lethal copper levels was also evident at sublethal concentrations, with tolerant clone from impacted population showing higher sublethal tolerance to copper for all the parameters compared to the rest of clones. Whether or not tolerance to copper was costly in fitness terms was not evident in the study in Chapter 4. Population growth rate responses, considered to be a good estimate of fitness in the laboratory, did not differ in non exposed treatments. Effects of copper across generations were then studied in Chapter 5 to assess possible variations in the tolerance to copper in a *D. longispina* clone continuously exposed to copper. One of the Chapter's outcomes was that physiological adaptation to copper across several generations only increased marginally acute tolerance to copper. Besides that, a high variation in life-history traits was observed not only between copper treatments, but also among generations. For instance, generation had a significantly influence on the observed pattern of age at first reproduction and interact with copper in the observed variation of time and clutch size at first reproduction. These findings document the limited significance of single generation surveys in standard ecotoxicological studies. Results of the current study also agree with previous studies such as the one from Shirley and Sibly (1999), which postulate that adaptation to metals can involve tradeoffs between maturing earlier at expenses of survival, maturing earlier at expenses of growing less and maturing earlier at expenses of fecundity. As survival remained constant in this study and fecundity and r increased, it can be hypothesized that adaptation to copper in *D. longispina* tested in the study involved a trade-off between maturing earlier at expenses of growth, although this parameter was not measured in the study.

Throughout this dissertation it was shown that cladoceran populations inhabiting metal polluted environments may persist as a consequence of the genetic adaptation to the metals involved. However, the acquisition of genetically inherited tolerance could have associated ecological costs. These results reinforce the need to integrate these issues when

assessing risks posed by chemicals to the environment. Several studies have shown that the safety margins currently applied in Environmental Risk Assessment (ERA) procedures may account for the genetic changes in tolerance generated by pollution-mediated microevolutionary processes (Barata *et al*., 2002; Jensen *et al*., 2001; Mazzeo *et al*., 1998). Barata *et al*. (2002) experimental studies with *Daphnia magna*, for instance, showed that the genetic range of tolerance traits to metals and pesticides of up to 160 naturally occurring *D. magna* genotypes did not exceed one order of magnitude. However, the consideration of the ecological costs associated to these genetic changes is one of the aspects that probably need to be incorporated into ERA procedures. In fact, the degree to which genetic changes generated by selection processes could lead to negative ecological costs is not well documented (Medina *et al*., 2007) so there is a need for more studies that can assess the existence of ecological costs associated with adaptation but also studies that can show changes in the ability of populations to cope with other environmental changes, the risk of extinction or the accumulation of spontaneous deleterious mutations (genetic load). Perspectives for future research are based in the need to detect and quantify interactions between toxicants and evolutionary processes and to incorporate these interaction relationships in the framework of Ecological Risk Assessment. These research goals can only be reached by an interdisciplinary approach where methodologies specific to the field of Ecotoxicology are combined with those committed to evolution, such as population genetic models (Roelofs *et al*., 2006; Steinberg *et al*., 2008; Weltje, 2003; Xie and Klerks 2002). Amplified fragment length polymorphism technique (AFLP), together with allozyme analysis, microsatellites, random amplification of polymorphic DNA (RAPD), mitochondrial DNA sequencing, and single nucleotide polymorphism (SNP) detection, are among the most recommended methodologies for population genetics analysis (Belfiore and Anderson, 2001; Bickham *et al*., 2000) and can be used to study selection and genetic adaptation of organisms to several types of contaminant stress (Theodorakis and Bickham, 2004). The recent advances in genomic and molecular tools such as DNA and RNA microarrays are now being used to study issues of environmental importance, with a strong focus on non-human organisms exposed to chemical stressors (Roelofs *et al*., 2009; Steinberg *et al*., 2008). In addition the study of evolutionary adaptations can be done in an integrative way in order to test whether regulatory mutations contribute to heritable changes in morphology, physiology and behavior. In a recent study

of Roelofs *et al.* (2009) performed with soil arthropod populations it was shown that selection for altered transcriptional regulation can be a powerful mechanism for microevolution to cadmium exposure. The application of transcriptomics showed a strong signature of stress-induced genome-wide perturbation of gene expression in reference population, whereas the tolerant animals maintained normal gene expression upon Cd exposure. In previous studies with the same populations tolerance to cadmium was correlated with heritable increase in metal excretion efficiency, less pronounced cadmium (Cd)-induced growth reduction and overexpression of the metallothionein gene. The integration of all these results confirmed the micro-evolutionary processes occurring in soil arthropod populations and suggests a major contribution of gene regulation to the evolution of a stress-adapted phenotype.

To conclude, while it's necessary to improve the understanding of ecological side effects of long-term adaptation to metals in natural populations, future studies that incorporate new genomic and molecular tools, new contamination scenarios involving chemical and natural stresses, preferably accounting for long-term effects, would be extremely valuable.

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