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Variability patterns and genetic determination of the tolerance to metal-rich acid mine drainage by planktonic invertebrates

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Aos meus pais

pelo seu exemplo e seu amor incondicional

“ If you can’t fly, run.

If you can’t run, walk.

If you can’t walk, crawl.

But by all means, keep moving! ”

Martin Luther King, Jr. (1957)

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Abstract

Genetic erosion in natural populations due to pollution is an emergent problem, with repercussion in the future of these populations, since lower genetic variability difficults its ability to adapt to new environmental changes. Organism's response to a particular stressor may occur at the behavioral level, by avoidance from contaminated sites, or at the metabolic level, by hormonal or enzymatic adjustments. More sensitive genotypes might be eliminated if the stressor works as a selective pressure. Studies with populations inhabiting at contaminated sites exhibited loss of genetic diversity. However, when genetic analysis are included in the evaluation of genetic variability, some cases revealed a lack of agreement between the two approaches *i.e.,* evaluating a population through selectable markers, genetic erosion was detected, while, through neutral markers, did not significant decreased. To clarify this lack of agreement, it is necessary to take into account other variables. A population genetic variability is not weakened only by natural selection, but also by genetic drift, inbreeding and emigrations. Furthermore, mutations and immigrations may increase the genetic variability. This work aimed at evaluating the influence of factors that justify the occurrence of genetic erosion without significant loss of genetic variability in zooplankton populations exposed to an acidic effluent enriched in metals. Selectable markers and neutral markers were used for the evaluation of genetic variability in a copepod natural population (*Copidodiaptomus numidicus*), from a reference site. As target zones, for neutral markers, were selected ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) were selected, both consisting of alternating of conserved and variable sequences, conducive to polymorphisms' occurrence and, therefore, more likely to find "contaminant-indicative bands" by PCR-RFLP. A lethal test was conducted with a

single concentration of copper ($0.5mgL^{-1}$) and, using a"time-to-dead assay", different categories of tolerance were found. The LT_{50}value was 28.5 hours, with a confidence interval from 23.8 to 34.2 hours. The results of PCR-RFLP did not allow the distinction between sensitive organisms (those who died until 2 hours of exposure) and tolerant organisms (those who survived until 64 hours of exposure), because the bands' patterns of DNA fragments were the same for both groups.

Second, the mutagenic potential of acid mine drainage effluent (from de São Domingos mine, Portugal) was evaluated, to test if contaminant-induced mutations could occur at pertinent concentrations, overestimating the genetic diversity evaluated through neutral markers. It was used the *Allium cepa* test of cytotoxicity and genotoxicity. Mutagenicity was determined by the observation of micronuclei and chromosomal breaks in meristematic cells and was registered at the lowest AMD concentrations used (0.1 and 1% AMD). The mutagenic effect of the AMD persisted after the cessation of exposure (24 and 48 hours recovery tests). Those results indicated that the mutagenic potential of AMD may have contributed to the increased genetic variability of an impacted population of *D. longispina* reported by Martins et al. (2009) and Silva et al. (2010).

Third, the quantitative PCR (q-PCR) technique was used to profile *D. magna* gene expression, over 168 hours, corresponding to the growth from neonate up to the first reproductive stage (eggs in the brood pouch). A first approach to determin the baseline variability of target genes (Mt, Fer, COI, ND2, LDH, IMP, Vtg, and EcR) and of the reference genes (Act, GAPDH and UbC) was made, by comparing the obtained profiles with physiological processes during growth.

Most of the genes had an increased expression at 72 and 144 hours, which corresponds to eggs provisioning at 1st and 2nd brood, meaning that ovaries

maturation is a complex prossess involving various genes and metabolic pathways. The sharp decrease that occurs at 168 hours revealed the influence of the embryonic development process, and consequently, for a later analysis, eggs or embryos inside the brood pouch should be removed prior to RNA extraction. The genes of glicolysis and electron transport pathway (COI, ND2 and LDH) showed a higer basal variability and over extended 95% confidence intervals. No relation was found with the EcR gene expression pattern and ecdysis periods during *D. magna* growth, revealing that this EcR isoforme should have a preponderant role in embryonic development.

The normalization process may be responsible for diferences at gene expression profiles. The metal transporters group (Mt and Fer) showed more significant results when normalized with geNorm than with BeastKeeper methodologies. The IMP was the gene most affected by copper exposure, being up-regulated at 6, 24, 48, 72, and 168 hours, with a down-regulation at 96 hours of exposure. Despide the inositol relation with growth processes, it also may act as a defense against copper oxidative damage.

Keywords

Genetic erosion; natural populations; mechanisms of tolerance; acid mine drainage (AMD); contaminant-indicative bands (CIB); mutagenicity; gene expression

Resumo

A erosão genética em populações naturais devido à contaminação ambiental é um problema crescente, com implicações no futuro dessas mesmas populações, pois quanto menor for a sua variabilidade genética, mais comprometida fica a sua capacidade de adaptação a novas alterações do meio. A resposta dada pelos organismos a um determinado factor de *stress* pode ocorrer a nível comportamental, por evasão dos locais contaminados, ou a nível metabólico, por alterações hormonais ou enzimáticas. Pode ainda ocorrer eliminação de genótipos mais sensíveis se o factor de *stress* actuar como pressão selectiva. Em estudos de ecotoxicologia evolutiva têm sido reportados casos de erosão genética, porém a inclusão de metodologias de genética molecular na avaliação da variabilidade genética revelou alguma falta de concordância entre as duas abordagens, isto é avaliando uma população através de marcadores seleccionáveis verificou-se erosão genética, mas através de marcadores neutros a variabilidade genética dessa população não apresenta decréscimos significativos. Para esclarecer esta falta de concordância é necessário ter em conta outras variáveis, pois a variabilidade genética de uma população, principalmente se ela for uma população de dimensões reduzidas, não é diminuída apenas pela selecção natural mas também por deriva genética, cruzamentos preferenciais (*inbreeding*) e fluxo génico (emigrações). Por outro lado factores como as mutações e imigrações podem aumentar a variabilidade genética.

O objectivo geral deste trabalho foi compreender os padrões de variabilidade e investigar os mecanismos de determinação genética da tolerância a drenagem ácida de minas em invertebrados planctónicos. Para esse efeito o plano de trabalho foi dividido em três partes experimentais (capítulos II, III e IV). No capítulo II foram

usados marcadores seleccionáveis e marcadores neutros para a avaliação da variabilidade genética, de uma população natural de invertebrados dulçaquícolas habitando um local de referência sem fontes de contaminação antropogénicas. Como zonas alvo para os marcadores neutros foram seleccionadas o DNA ribossómico (rDNA) e o DNA mitocondrial (mtDNA) ambos compostos por regiões conservadas e regiões variáveis, propensas à ocorrência de polimorfismos e, por conseguinte, com maior probabilidade de encontrar "bandas indicadoras de contaminação" por PCR-RFLP. O organismo escolhido foi o copépode *Copidodiaptomus numidicus* um invertebrado zooplanctónico com reprodução sexuada. Foi realizado um ensaio letal com uma única concentração de cobre (0,5mgL^{-1}), tendo sido determinadas categorias de tolerância através do tempo decorrido até à morte. O valor de TL_{50} foi de 28,5 horas, com um intervalo de confiança de 23,8 a 34,2 horas. A abordagem através de PCR-RFLP não permitiu a distinção entre organismos sensíveis (que morreram até às 2 horas de exposição) e tolerantes (vivos após 64 horas de exposição), tendo sido obtidos padrões de bandas de DNA iguais entre os grupos.

No capítulo III, foi avaliado o potencial mutagénico do efluente de drenagem ácida de minas (Mina de São Domingos, Portugal) e a possibilidade da ocorrência de mutações induzidas pelo contaminante e passíveis de sobrestimar a diversidade de perfis genéticos, obtidos através de marcadores neutros. Foi usado o teste de citotoxicidade e genotoxicidade de *Allium cepa* e foi registada a ocorrência de mutações, por observação de micronúcleos e quebras cromossómicas em células meristemáticas. Os resultados obtidos nas baixas concentrações de efluente (0,1 e 1% de efluente), tal como nos testes de recuperação de 24 e 48 horas, permitem afirmar que o potencial mutagénico do AMD pode ter aumentado a variabilidade

genética da população de *D. longispina* referida por Martins et al. (2009) e Silva et al. (2010).

No capítulo IV, foi, usada a técnica de PCR quantitativo (q-PCR) para traçar o perfil de expressão de genes responsivos à exposição a metais, ao longo de 168 horas, correspondendo ao crescimento de *D. magna* desde neonato até ao primeiro estádio reprodutivo, com ovos no marsúpio. Foi feita uma primeira abordagem para a determinação da variabilidade basal dos genes alvo (Mt, Fer, COI, ND2, LHD, IMP, Vtg e EcR) e dos genes de referência (Act, GAPDH e UbC), comparando os perfis obtidos com processos fisiológicos. A maioria dos genes analisados teve um aumento da expressão às 72 e 144 horas, o que corresponde ao provisionamento de ovos correspondentes à 1ª e 2ª ninhada, significando que ocorre um pico de transcrição no início da maturação dos ovários. A diminuição nítida, que ocorre às 168 horas revela a influência do processo desenvolvimento embrionário, pelo que para uma análise posterior, os ovos ou embriões dentro do marsúpio devem ser removidos antes da extracção de RNA. Os genes relacionados com a obtenção de energia (COI, ND2 e LDH) apresentaram uma variabilidade basal com intervalos de 95% de confiança mais alargados. O padrão de expressão do gene EcR não permitiu detectar os momentos de muda de exosqueleto nem do crescimento de *D. magna*, revelando que o isoforme de ECR analisado deve ter um papel mais preponderante no desenvolvimento embrionário do que no crescimento.

O processo de normalização pode influenciar os resultados finais de expressão génica numa avaliação dos efeitos de contaminantes. No grupo dos transportadores de metais foram detectadas mais variações significativas quando os dados foram normalizados pelo método geNorm do que pelo método BeastKeeper.

No ensaio com cobre os resultados mostraram que o gene IMP foi o mais afectado sendo a sua expressão induzida às 6, 24, 48, 72 e 168 horas e inibida às 96 horas de exposição, podendo estar relacionado com um efeito protectivo contra danos oxidativos do cobre pelo inositol.

Palavras-chave

Erosão genética; populações naturais; mecanismos de tolerância; drenagem ácida de minas (AMD); bandas indicadoras de contaminação (CIB); mutagenicidade, expressão génica.

Chapter 1

General Introduction

Natural populations are subjected to pressures induced by environmental changes, either being caused by cyclical alternation of the seasons or atypical, such as habitat destruction. Chemical contamination is one of the main causes of populations' imbalance and Ecotoxicology continues to evolve in order to better predict how populations respond to contamination challenges (Truhaut 1977). In recent decades, alternative methods have been designed and improved to achieve higher levels of ecological relevance and knowledge integration. Furthermore, cause/effect relationships between environmental stressors and their action on aquatic ecosystems can now be better established as well as a better knowledge of the organisms' mechanisms of tolerance to contaminants was achieved. Although contaminants affect individual organisms, their consequences can be reflected at the population level and at higher biological levels of organization (e.g. populations can be influenced by indirect stress exposure such as predator-prey interactions (Adams 2003)).

Contamination intensity and time of exposure may involve different stress responses that might vary from evasion and increased tolerance, by restoring homoeostasis (behavioral, physiological and morphological adjustments at the individual level), to organisms' death and, consequently, the loss of the most sensitive phenotypes (Badyaev 2005). The conceptual workframe in figure 1.1 summarizes the interpretation of Hoffmann and Parsons (1991) on the stress response at the individual level and possible implications at the population level with increasing exposure duration. The first response to occur will be a behavioral one t allowing organisms to evade. During an early detection of stress (e.g. trough nervous receptors), organisms can escape to uncontaminated sites or befall into

dormancy, possibly reducing metabolic and energetic costs to fight stress. Dormancy works as a temporal barrier to stress effects. This stress-induced strategy, because it benefits evaders, can be incorporated genetically into a population, as a type of selection — selection for stress special or temporal evasion (Hoffmann and Parsons 1991).

Other behavioral responses, prompted by the presence of contaminants, have been used in contamination assessment (Hebel et al. 1997). For example, alterations in feeding rate (Castro et al. 2004, Domene et al. 2007, Agra et al. 2010), reproductive behavior (Kiørboe 2007, Seuront 2011), and mobility (Untersteiner et al. 2003, Kwok and Leung 2005, Breckels and Neff 2010) have been used as sublethal endpoints. These complex alterations are caused by hormonal induction of enzyme activities, repercuting on reproduction rates, affecting population dynamics, genetic variability or, even, its long term maintenance (Knops et al. 2001, Kiørboe 2007, Amiard-Triquet 2009, Seuront 2011). Hoffmann and Parsons (1991) considered them as a biochemical escape that may be responsible for another type of selection — selection for tolerance via hormonal modulation (Fig. 1.1). e Organisms are able to acclimate to environmental changes, within a given range. Acclimation may occur as a short-time response involving the preexisting mechanisms of metabolic pathways through changes in substrate concentration and effectors. For example, multienzyme systems have regulatory enzymes that conduct adjustments in metabolic rates by controlling the substrate availability in such a way that it triggers the activity of enzymes in a given sequence along the metabolic pathway. Alosteric enzymes change their metabolic rates in response to effectors (molecules that bind to the alosteric site by changing enzyme conformation, enhancing or inhibiting its activity). Acclimation may also occur as a later response

by altering patterns of gene expression and synthesis of specific proteins, (e.g. stress-proteins, isozymes) or by alterating the degradation rate of some enzymes. When organisms under stress can restore homeostasis, thus resulting in an increased stress tolerance, selection for tolerance via acclimation can occur. However, if organisms' metabolic efficiency diverges within the population and only the most tolerant genetic polymorphisms cope with the stress exposure, then natural selection or selection for tolerance via metabolic changes can take place (Fig. 1.1), and the population may significantly lose genetic diversity.

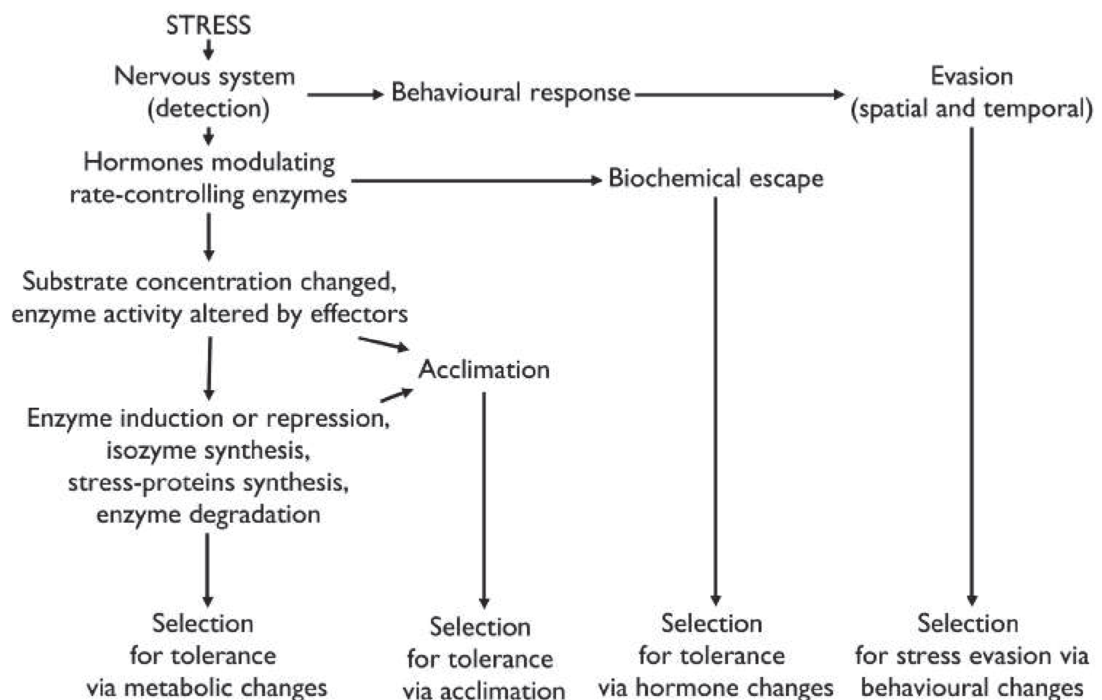

Figure 1.1. Conceptual framework for environmental stress responses related with increasing time and the type of selection that can occur at each response type. Evasion can be a spatial occurrence when organisms are able to avoid stress by leaving the stressed site or can be a temporal occurrence when organisms enter into dormancy. Acclimation is considered as a type of phenotypic plasticity allowing organisms to recover homoeostasis during stress exposure, with increased stress tolerance acquisition (Adapted from Hoffmann and Parsons 1991).

The loss of genetic diversity in a population by the elimination of the most stress sensitive phenotypes (increased mortality and/or reduced reproductive rate) leads to alterations on its genetic structure by genetic erosion (Belfiore and Anderson 2001, Futuyma 2005, Bach and Dahllöf 2012). Genetic erosion has been used as a synonym for the loss of genetic diversity, also as a result of directional selection for tolerant traits, due to contamination or, more generally, due to human pressure on ecosystems (Ouborg et al. 1991, van Straalen and Timmermans 2002, Nowak et al. 2009, Bijlsma and Loeschcke 2012, Ribeiro et al. 2012, Ribeiro and Lopes 2013). This process has been studied in molluscs (Gajardo et al. 2002), insects (Schmitt and Seitz 2002), collembolans (van Straalen and Timmermans 2002), cladocerans (Lopes et al. 2004a, Ribeiro and Lopes 2013), and fish (Knaepkens et al. 2004).

Van Straalen and Timmermans (2002) made a critical review of studies assessing contaminant effects on natural populations that supported the "genetic erosion hypothesis", arguing that those studies did not present sufficient data about habitat fragmentation, population size, migrations, preferential mating, and their potential pressure on genetic variability of the population (Fig. 1.2). Contaminants may exhibit genotoxic effects by acting on organisms' DNA, (either germinative or somatic cells). Although only mutations in germinative cells can be transmitted to successive generations, somatic cell mutations are also important in populations under stress, as they can induce a decrease in vital the capacity of the organism, for example through diseases or tumors (Lynch 2008, 2010), increasing mortality and decreasing the reproductive rate (Fig. 1.2). Physiological effects observed at the individual level, or ecological effects as food limitation, predator-prey or parasitism

interactions (Adams 2003), can also cause alterations in populations' genetic structure through changes in mortality and/or reproductive rates (Fig. 1.2).

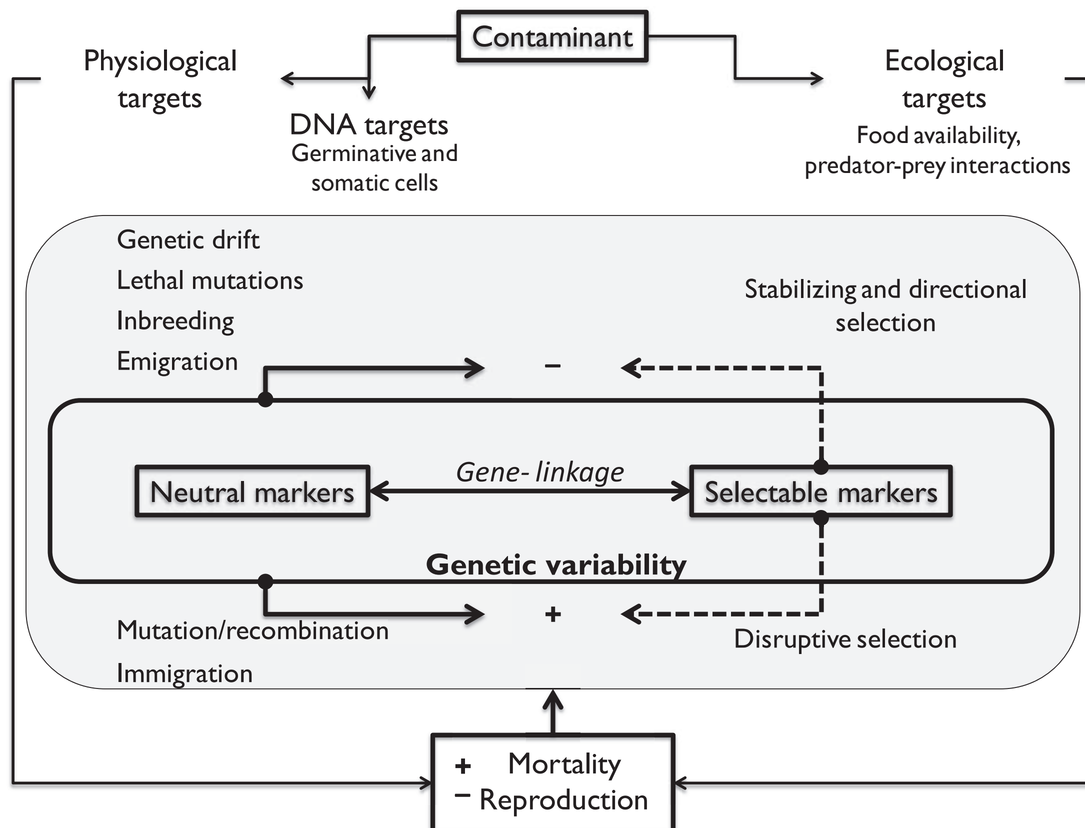

Figure 1.2. Contaminant targets and their effects on natural populations' genetic structure. Genotoxic, physiological and ecological effects on population mortality/reproductive rates may do genetic structure adjustments (+ increase; - decrease). Markers are traits that may respond to selective pressure (selectable markers), or may be unresponsive to the selective pressure (neutral markers) except when linked with selectable markers (gene-linkage). Mutations, genetic drift, migrations, and preferential mating (inbreeding) could take action on both types of markers (Adapted from van Straalen and Timmermans 2002, Adams 2003, and De Wolf et al. 2004).

A selectable marker is defined as a partially or fully genetically determined trait giving some advantage or disadvantage to organisms living at an environment under a stress factor, which means that a selective pressure have a direct action on that trait (van Straalen and Timmermans 2002, Ribeiro et al. 2012, Ribeiro and Lopes 2013). Neutral markers are traits that are indifferent to selective pressures, unless they are linked to selectable markers (gene linkage). Natural selection may increase or decrease genetic variability populations' depending on selective type.

Stabilizing selection is typical of relatively stable environments, pressing extreme traits and leading to a decrease of genetic diversity while disruptive pressure confers an advantage to extreme traits, leading to an increase in genetic diversity. Directional selection takes place when the selective pressure shifts the population's distribution trait towards one extreme, as illustrated by an impacted population of *Daphnia longispina* where the most sensitive categories were eliminated and the population acquired an higher tolerance to copper and acid mine drainage (AMD) (Lopes et al. 2004a, Ribeiro et al. 2012). Other factors that influence populations' genetic variability, affecting both selectable and neutral markers, are genetic drift (including bottleneck and founder effects), non random mating (mainly inbreeding), gene flow, and mutations (van Straalen and Timmermans 2002, Hoffmman and Willi 2008, Ribeiro and Lopes 2013) (Fig. 1.2). The occurrence of genetic drift (also bottlenecks) may decrease genetic variability but its effect is more relevant on small populations due to the random fluctuation of allele frequencies which leads to an increase in homozygosity and eventually to allele fixation (Kirk and Freeland 2011, Bijlsma and Loeschcke 2012).

The set of studies on environmental effects of a metal-rich AMD, performed in natural populations of *Daphnia longispina*, for several years, has been filling the gaps concerning genetic erosion referred by van Straalen and Timmermans (2002) (Lopes et al. 2004a, b, 2005a, b, Martins et al. 2005a, Martins et al. 2005b, Lopes et al. 2006, Martins et al. 2007, Lopes et al. 2009, Martins et al. 2009, Silva et al. 2010, Ribeiro et al. 2012, Saro et al. 2012, Ribeiro and Lopes 2013). These studies were performed at Mina de São Domingos, a cupric pyrite mine located in south-eastern Portugal in the Guadiana River basin. The mining activity ended in 1967 but the drainage remains, preserving a high acidity (pH ≈ 2) due to the continuous oxidation

of residual ores in contaminated sediments, until the dilution at the Chança River reservoir (Fig. 1.3). This site has been considered as a model for studies of metal pollution on aquatic ecosystems because there are no other local sources of contamination (agricultural, industrial or urban); the contamination is restricted to the area circa the effluent path; and it is surrounded by uncontaminated landmarks with high biological diversity, including reference sites (Fig. 1.3).

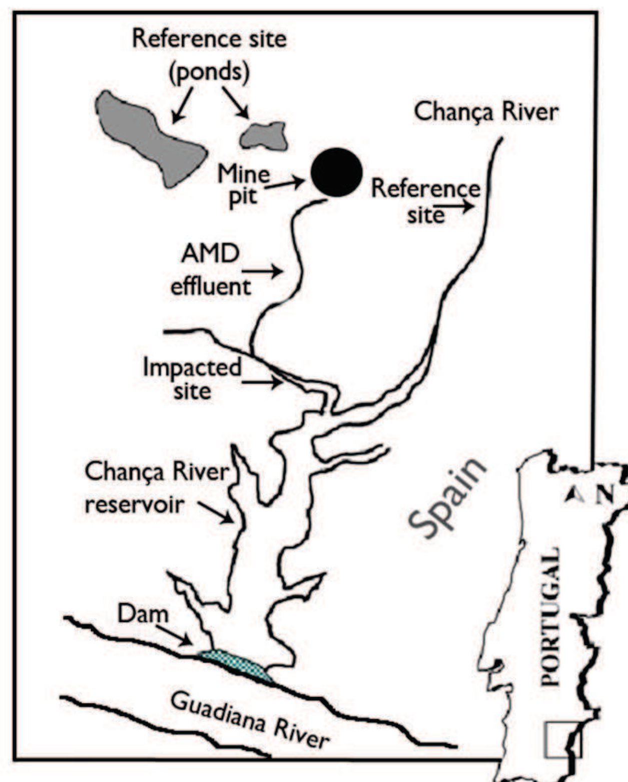

Figure 1.3. São Domingos mine location (Mértola, Alentejo, Portugal) and aquatic system scheme with the acid mine drainage effluent path from the mine pit to the Chança River reservoir and impacted and reference sites (Adapted from Lopes et al. 2004a).

Ribeiro et al. (2012) reviewed all the previous studies done at the São Domingos mine for data integration and critical analysis concerning contaminant-driven genetic erosion. Several conclusions were highlighted: i) genetic erosion was detected by selectable markers (lethal endpoints) and directional selection was confirmed as the main microevolution factor, by the lost of sensitive genotypes and an increased tolerance to copper and to AMD in impacted

population; ii) genetic drift was not a significant factor of genetic erosion; iii) genetic erosion was not confirmed using neutral markers because the comparison of DNA polymorphisms between reference and impacted population presented no significant differences; although tolerant categories more frequently presented a specific band wich could be considered a contamination indicative band (CIB), i.e. potentially associated with metal resistance polymorphisms. As further developments were needed to further support achievements and to clarify discrepancies, the purpose of this work was to understand patterns of tolerance variability and investigate the mechanisms of genetic determination of the tolerance to acid mine drainage in planktonic invertebrates.

1.1. Thesis outline

To achieve the main purpose, the research was organized into three experimental sections (Chapters II, III, and IV), linked by an introductory chapter on the effects of AMD on populations of aquatic planktonic invertebrates, and a general conclusion, highlighting the most important findings and future perspectives. Each experimental chapter provides a detailed introduction on the respective issue, methodological support, obtained results and discussion. A final section lists the scientific works referenced throughout the dissertation.

1.1.1. Experimental structure

In Chapter II, the issue of genetic erosion, previously discussed by Lopes et al. (2004a) and Martins et al. (2009), is retaken with methodological modifications. The copepod *Copidodiaptomus numidicus* (Calanoida) — a planktonic invertebrate with obligatory sexual reproduction — was used instead of the parthenogenic

D. longispina (Cladocera), to minimize the presence of individuals originated from the same clone. The internal transcribed spacer regions (ITS) of ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) were used as target regions to look at DNA polymorphisms related with metal stress response, by PCR-RFLP analysis.

In Chapter III, effects of AMD at the cellular level were assessed to look for evidences of mutagenic effects that, even at (pertinent) low concentrations, could be responsible for variations on DNA structure underestimating a real decrease in genetic variability by the loss of sensitive genotypes. The *Allium cepa* genotoxicity test, which has a discriminative skill for mutagenicty, was applied. Structure alterations and DNA damages during mitosis in meristematic root cells were observed using an optical microscope. The effects of mutagenic stress after AMD exposure (24 and 48 hours) were assessed.

Chapter IV focuses on metal exposure effects at the gene level. Key metabolic pathways (glycoysis and electron transport, cell signaling and metal transport) were evaluated. The gene expression variation during a copper exposure, as well as natural variation patterns in the stages of postnatal development, growth and sexual maturation of a planktonic invertebrate, were assessed by quantitative PCR. *Daphnia magna* was chosen due to its role as model organism in aquatic ecotoxicology studies and to the already acquired knowledge on its stress responses.

1.1.2. Specific objectives

Chapter II

i) To categorize the tolerance to copper — a selectable marker — of a reference natural population of a planktonic invertebrate;

ii) To obtain patterns of variability in ribosomal DNA and mitochondrial DNA, using neutral markers with PCR-RFLP;

iii) To characterize "contaminant-indicative bands" (CIB), aiming at contributing to the identification of physiological mechanisms responsible for differences between sensitive and tolerant organisms to copper lethal concentrations.

Chapter III

i) To characterize cytotoxic and genotoxic effects of AMD in somatic cells;

ii) To assess the AMD mutagenicity as enabler of increasing diversity of genetic profiles of a natural population submitted to a long-term exposure.

Chapter IV

i) To profile expression of genes responsive to metal exposure, belonging to different metabolic pathways, during growth (neonate, juvenile and sexual maturation stages) of *D. magna*;

ii) To interrelate the gene expression pattern with *D. magna* life cycle events;

iii) To interrelate the gene expression profile with dafnids ecophysiological responses to copper exposure.

Chapter II

Copper contamination indicative bands by PCR-RFLP in a freshwater copepod natural population – a failed attempt

Abstract

Copper has a significant role in vital processes as a cofactor for a wide range of enzymes. Its ionic form is easily interchangeable between cupric and cuprous ions and this redox reaction is important for the activation of certain proteins with essential biological functions, such as cytochrome-c-oxidase (COI) and Cu/Zn superoxide dismutase (SOD), wich are the final acceptor in the electron transport chain in mitochondrial oxidative phosphorilation and the catalyst of the superoxide dismutation into oxygen and hydrogen peroxide, respectively. However, the existence of copper in the environment is an issue of concern, and has been reported as responsible for effects on growth, reproduction and survival. Differences on copper tolerance have been found among contaminated and reference populations of aquatic invertebrates', metal-stressed populations exhibiting higher tolerance levels. *Daphnia logispina* from a population impacted by metal-rich acid mine drainage (AMD) have been used to study the effects of contaminant driven genetic erosion. Following this *D. longispina* case-study, this work aimed at categorizing the range of copper tolerance, using selectable markers, of a freshwater copepod population (*Copidodiaptomus numidicus*), from a reference site, and obtaining patterns of variability in rDNA and mtDNA regions, through PCR-RFLP. The organisms exhibited a similar pattern in tolerance categories in accordance to *D. longispina*, with a LT_{50} of 28.5 hours (95% confidence limits of 23.8 – 34.2). The most sensitive (decease until 2 hours) and the most tolerant (alive at 64 hours) were used at PCR-RFLP analysis. Only the rDNA ITS 1 region was amplified. No differences were found among tolerant and sensitive organisms in the band pattern resulted from endonucleases' digestion. Consequently, no putative contaminant-indicative band (CIB) related to copper tolerance were found. This

work confirms some limitations of neutral markers, without a solid knowledge on gene sequence, to assess environmental contamination driven genetic variability. The use of alternative methods to assess the formerly found inconsistency between selectable and neutral markers results (Martins et al. 2009, Silva et al. 2010) is discussed.

Keywords:

contaminant indicative band (CIB), PCR-RFLP, copepod, cooper resistance, rDNA, mtDNA, time to dead assay

2.1. Introduction

Copper is an essential element and has an important role in vital processes by acting as cofactor[1] for a wide range of enzymes, including those involved in DNA synthesis, mitochondrial oxidative phosphorylation and oxidative stress protection (Chang and Fink 1994, Horn and Barrientos 2008). Copper change its redox state between the oxidized Cu^{2+} (cupric ion) and the reduced Cu^{+} (cuprous ion). In contrast to the reduced form, the cupric ion has high solubility and it is the most present form inside the cell, because the cuprous ion is readly oxidized by oxygen (Arredondo and Núñez 2005, Horn and Barrientos 2008, Turski and Thiele 2009). This redox reaction is important for the activation of proteins that have essential biological functions, such as the enzymes cytochrome c oxidase – the final acceptor in the electron transport chain in mitochondrial oxidative phosphorilation – and Cu/Zn superoxide dismutase – the catalyst of the superoxide dismutation into oxygen and hydrogen peroxide (Horn and Barrientos 2008). However, redox reactions generate hydroxyl radicals, through the Fenton-like[2] reaction, which can seriously damage all types of cell molecules (Sies 1993, Arredondo and Núñez 2005).

Despite being essential to life, copper poses environmental problems. Its toxicity is well documented for different types of ecologically relevant effects (survival, growth, reproduction) (Winner and Farrell 1976, Nor 1987, Flemming and Trevors 1989, Altenburger et al. 2003, Bossuyt and Janssen 2003, Bossuyt et al. 2005, Canli 2006). Tolerance to copper, at the population level, can be acquired by natural selection, through the elimination of the most sensitive genotypes, and by

[1] A chemical compound that is bound to a protein (usually an enzyme) and it is necessary to activate it.
[2] During the Fenton-like, reaction Cu^{1+} loses an electron, changing to the cupric form (Cu^{2+}), and hydrogen peroxide (H_2O_2) is transformed into hydroxyl radical ($HO^{\bullet}$) and water (H_2O).

phenotypic plasticity (including acclimation). Differences on copper tolerance have been found among stressed and reference populations. Regarding invertebrates, most of the revised studies by Klerks and Weis (1987) found organisms from contaminated sites to be more tolerant than those from reference sites and reported this tolerance to be genetically determined. Lavie and Nevo (1982) analyzed the effects of copper (and zinc) in the marine gastropods *Monodonta turbinata* and *M. turbiformis* and concluded that at least a part of the allozyme polymorphism was subject to natural selection. Lopes et al. (2004a) made use of fully acclimated *D. longispina*, originated from natural populations, and reported a lower tolerance in organisms from a reference site than those from an impacted site when exposed to different copper concentrations (60, 80, 150, 250, and 350 $\mu gL^{-1}$of copper). They also found that lineages categorized as Very Tolerant (VT) and Extremely Tolerant (ET) were present both in reference and stressed populations with identical percentages; Tolerant lineages (T) formed the largest group of the impacted population, comprising 80% of the lineages exposed to copper; and Extremely Sensitive (ES) and Very Sensitive (VS) lineages were only present in the reference population. Later studies reported the occurrence of differences in genetically-determined sensitivity to lethal levels of copper rich acid mine drainage among *Ceriopahnia pulchella* (Cladocera) (Lopes et al. 2005a), and *D. longispina* (Lopes et al. 2009) from reference and historically metal-stressed populations after laboratorial acclimatation, supporting the “genetic erosion hypothesis” by the elimination of sensitive genotypes (Ribeiro et al. 2012).

These studies used an approach presented by Hoffmann and Parsons (1991) to assess genetic variation for stress resistance: the use of selectable markers based on known data about organisms’ phenotypic variance in the stress response (e.g.

sensitive traits), enabling the separation of genetic and environmental components. The alternative approach uses neutral markers and is based on known genetic polymorphisms to achieve correlations with genetic variance in stress response (e.g. tolerance, avoidance). The first approach does not require previous knowledge about the genetic structure (and is considered as the standard approach on quantitative genetics), in opposite to the second approach, which is mainly used on enzyme polymorphisms studies. Martins et al. (2009) used both approaches (standard and alternative). First, they followed a similar procedure to Lopes et al. (2004a) with a stressed population and two reference populations of *D. longispina*. A higher frequency of VT and T, as well as a lower frequency of S and VS organisms, were found at the impacted population, contrasting with the reference populations, where VS and S organisms were more frequent. Second, they used Amplified Fragment Length Polymorphisms (AFLP) markers to determine the genetic diversity of the populations. Despite the lack of differences found in genetic diversity indices (Nei's genetic diversity, observed heterozigoty and Shannon's information) among populations, the impacted population stood out from the reference ones when analyzed with Nei's unbiased genetic distance. Genetic distances among tolerance categories were evaluated and three partitions were established: an initial separation of VT, a second split in T, and lastly the categories with the highest similarity, S and VS. They also compared bands frequency among tolerance categories. A particular band (the E-ACT-M-CAG-0091 band) was significantly more frequent in T and VT than in S and VS organisms. This was, thus, considered a putative "contaminant-indicative band" (CIB) (Theodorakis and Shugart 1997). However, band sequencing was not carried out.

Contaminant-indicative bands (CIB) are a concept introduced by Theororakis and Shugart (1997, 1998) and were defined as bands present at higher frequency in organisms from a contaminated site. They studied radionuclide contaminated mosquitofish (*Gambusia affinis*) populations and found significant differences in DNA breakage relatively to reference sites. Organisms from the contaminated site presented a higher frequency of some bands obtained by Random Amplification of Polymorphic DNA (RAPD), which were designated as contaminant indicative bands. Later on, Theororakis and Bickman (2004) proceeded to the nucleotide sequencing of these CIB. No homologies were found between band sequences and sequences previously associated with this type of contamination in databases. Therefore, the authors could not go further in pursue on CIB relationship with radionuclide mechanism of action. Nevertheless, they find that CIB sequences presented a high degree of conservation when compared to and aligned with nucleotide sequences of other organisms (vertebrates and invertebrates). This high degree of similarities among different organisms' sequences means that this particular region of DNA had a high functional value (Weiss and Buchanan 2004).

The present study aimed to characterize the CIB and, thus, contribute to the identification of physiological mechanisms responsible for the differences between organisms sensitive and tolerant to lethal levels of copper. The above mentioned quantitative genetics' standard approach, sensu Hoffmann and Parsons (1991), was followed, continuing the path of Martins et al. (2009) work. However, a sexually reproducing organism was used – the freshwater copepod *Copidodiaptomus numidicus*. Daphnids reproduce mainly by parthenogenesis, thereby enlarging the chances that the organisms analyzed by Martins et al. (2009) belonged to a narrow range of clones, and consequently, presented a lower genetic variability in a natural

population of sexually reproducing organisms which explain a smaller number of CIB than expected. The freshwater copepod *C. numidicus* has a compulsory sexual reproduction, which makes it a realistic model for sexually reproducing organisms (Bron et al. 2011). Besides the target organism, another modification was adopted in the present study. Instead of AFLP, an alternative technique to obtain the banding pattern was used. The advantage of AFLP is that it allows polymorphisms' detection in the entire genome, without a prior knowledge of the nucleotide sequence for primers design, (Vos et al. 1995, Mueller and Wolfenberger 1999). Restriction enzymes of well-known cutting sites (such as EcoRI and MseI) and double-stranded adapters provide spots in DNA chain for primer annealing and ensuing amplification by Polymerase Chain Reaction (PCR) (Vos et al. 1995). As a dominant molecular marker, AFLP do not allow distinguishing by band pattern heterozygotes from homozygotes. Therefore, a sample without or with just few null homozygotes[3] became a statistical problem when estimating alleles' frequencies (Zhivotovsky 1999). The AFLP technique presents some other disadvantages that may reduce the reproducibility of band patterns. Jones et al. (1997) have shown that AFLP is a technique that requires training to obtain reliable results. A test among different laboratories showed significant differences in the profiles of AFLP (about 50% of the bands were not detected) at the beginning of calibration tests. These differences were eliminated with increased training. But the main disadvantage of AFLP is that it requires an especially pure DNA, since impurities will inhibit subsequent enzymatic processes (Savelkoul et al. 1999, Rådström et al. 2008). This is very important when dealing with DNA provided by a whole organism, such as zooplankton wherein the exoskeleton and lipid content are critical factors for DNA contamination. To

[3] A band is present in plus-allele cases (AA or Aa). Null-allele (aa) don't present a band because they are unamplifiable with PCR

overcome the drawbacks of the AFLP technique, PCR-RFLP (Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments) was selected. With this technique, only a region of the genome is amplified and followed by digestion with a battery of endonucleases. Besides being a highly reproducible technique, it can be performed with small quantities of DNA, allowing the analysis of individual small-sized organisms, such as daphnids and copepods, without the need of expensive reagents and advanced instruments (Rasmussen et al. 2012). As the fragments are separated in agarose gel electrophoresis, the isolation of bands for further analysis is much easier. The major disadvantage of PCR-RFLP is the need of specific primers for the target DNA region, which requires a prior knowledge about that specific DNA region (Muller and Wolfenbarger 1999).

Based on information gathered by Theodorakis and Bickham (2004) about the conserved DNA sequences inside CIB and its potential functional value, the target DNA regions in the present study were mitochondrial (mtDNA) and ribosomal DNA (rDNA). The former is not as conserved as the nuclear DNA, but it encodes genes of great functional interest such as the NADH dehydrogenase and cytochrome c oxidase enzymes, respectively the first and final electron acceptors in the respiratory chain. As these genes have been associated with sensitivity to metal exposure (Machida et al. 2004, Achard-Joris et al. 2006, Poynton et al. 2007), this makes mtDNA a region of potential interest for CIB. The rDNA contain the genes for ribosome proteins, and as this organelle is responsible for the synthesis of all other proteins it is, therefore, indirectly linked to the organisms' viability (resilience, growth, reproduction) (Kupriyanova 2000). The coding regions have remained highly conserved but are separated by internal transcribed spacers regions (ITS 1 and ITS 2) containing multiple repeats with high level of variation, working as useful

targets for polymorphism analysis (Kupriyanova 2000, Chu et al. 2001, Ki et al. 2009).

2.2. Materials and Methods

2.2.1. Test organisms

Egg-bearing *Copidodiaptomus numidicus* Gurney females were sampled at a reference pond within the same aquatic system studied by Martins et al. (2009) (37°40'N 7°30'W, Tapada Grande, São Domingos mine, Southeast Portugal), which has no anthropogenic pressures (Ribeiro et al. 2012). Until nauplii eclosion, organisms were kept individually in 40 mL of ASTM Moderately Hard Water (Lewis et al. 2002, ASTM 2010), enriched with the organic supplement Marinure (Baird et al. 1989b). They were fed daily with $3x10^{3}$ $cell.mL^{-1}$ of a 1:4 mixture of the green algae *Chlamydomonas pseudocostata* Pascher and Jahoda and *Pseudokirchneriella subcapitata* (Koršhikov) Hindak, based in Caramujo and Boavida (1999), and cultured at 20±1 C and 14:10h light:dark cycle. Offspring of each female were kept separately. Nauplii were fed when culture medium was renewed (3 times a week) with $2.5x10^{4}$ $cell.mL^{-1}$ of the algae mixture (per 20 nauplii). Copepodites were fed with the total food ration ($5x10^{4}$ $cell.mL^{-1}$ per 20 copepodites) when culture medium was renewed, and half ration in intermediate days, to prevent the trapping of copepodites into algae clusters. Once females and males reached sexual maturity, the sensitivity to copper of both was evaluated. Mature males were visibly recognized by the hinge on the right antenna (Lowry 1999, Bradford-Grieve 2002) and mature females by oviducts density (dark bands along the digestive tract) and visible egg-sac.

2.2.2. Lethal time test

A lethal time test was performed with a single concentration of 0.5mgL^{-1} of copper, as $CuSO_4.5H_2O$ (Merck, Darmstadt, Germany). 1122 organisms (687 females and 435 males) were exposed individually in 8 mL of test solution at 10mL glass containers. A control was also performed with 114 organisms (70 females and 44 males) in ASTM moderately hard water. Mortality (no movement 15 seconds after gentle prodding) was registered at 1, 2, 4, 8, 16, 32, and 64 hours of exposure. Dead organisms were collected and preserved in ethanol (70%) for DNA analysis.

2.2.3. Genetic analysis

Genetical analisys were performed with all organisms that died up to 2 hours of exposure (17 organisms: 10 females and 7 males) and equal number of resistant organisms that remained alive after 64 hours of exposure. Genomic DNA of each individual was extracted with REDExtract-N-AmpTM Tissue Kit (Sigma-Aldrich) according to the manufacturer instructions for DNA extraction from Mouse Tails, Animal Tissues, Hair, or Saliva. Polymerase chain reaction (PCR) conditions were selected and optimized to each set of primers and a GeneAmp® PCR System 2700 thermal-cycler (Applied Biosystems, Cheshire, UK) was used. Primers were selected following previous studies on copepod rDNA (ITS region) and mtDNA, (Tab. 2.1) and 9 restriction enzymes were selected: Alu I, Rsa I (Invitrogen), Hinf I, Msp I, Hha I, Hae III (GE Healthcare), EcoR I, Pst I (Promega), and Dde I (Qbiogen). Amplicons were digested according to the manufacturer instructions for each enzyme. Digested products and a DNA Ladder (100 bp) were submitted to electrophoresis in 1.5% agarose gel (100V; 1h) with ethidium bromide incorporated on gel (4 µL:100mL gel) and observed on UV transilluminator (E CX-20.M-Vilbert Lourmat, Marne-la-Vallée, France).

2.2.4. Data analysis

Cumulative mortality during the lethal time test was used to determine median lethal times (LT_{50}) and respective 95% confidence limits using QCal Software (Lozano-Fuentes et al. 2012) after data correction with Abbott's formula (Abbott 1925) used to adjust mortality not associated with copper exposure.

Agarose gels from genomic DNA, PCR amplification and PCR-RFLP digestion were photographed with Bio-Print Mega (Vilbert Lourmat, Marne-la-Vallée, France) and DNA fragments were analyzed using Mega-Capt version 15.12 software (Vilbert Lourmat, Marne-la-Vallée, France).

2.3. Results

2.3.1. Lethal time assays

Cumulative mortality on the controls of the lethal time test reached 13% at the last observation moment. Only 18.5% of the 1122 organisms exposed to copper remained alive after 64 hours. The frequency of organisms in each category of sensibility is presented in Figure 2.1. The median lethal time (95% confidence limits within brackets) was 28.5 hours (23.8 – 34.2), being similar for females (n = 687) and males (n = 435): 25.3 (21.4 – 29.9) and 24.6 hours (21.2 – 28.6), respectively.

Table 2.1: Primers sequences, and respective sources, used for amplification of ITS 1 and ITS2 regions and mitochondrial genomes of *Copidodiaptomus numidicus*.

Code		5'-……………………………Primer Sequence…………………………-3'	Reference
A	ITS 1P1	CACACCGCCCGTCGCTACTA	Copepoda: Harpacticoida
	ITS 1P2 *	TCGACSCACGAGCCRAGTGATC	(Denis et al. 2009)
B	ITS 1 P1	GTAAAAGTCGTAACAAGG	
	ITS 1 P2 *	TCCTCCGCTWAWTGATATGC	Decapoda: Cambaridae
C	ITS 2 P1 *	TGYGAACTGCAGGACACA	(Harris and Crandall 2000)
	ITS 2 P2 *	TGTGTCCTGCAGTTCRCA	
D	srRNA-L	GTAGCATGAATCGGAGTTGTTCC	Copepoda: Calanoida; Harpacticoida
	srRNA-H	CACACAAACAATTTAAAAAATCGACAATACGGGGGATTGGCC	(Machida et al. 2002)
E	H26-Met	TCCTATCAAAGAGTTATGAGCTCT	Copepoda: Calanoida; Harpacticoida
	H13842-12S *	TGTGCCAGCASCTGCGGTTAKAC	(Machida et al. 2004)
F	H13842-12S *		Copepoda: Calanoida; Harpacticoida
	L12168-16S *	CGTCGATTTKAACTCAAATCATGT	(Machida et al. 2006)

* Degenerate primers (mixture of similar primers that have different bases at the variable position: K=GT, R=AG, S=CG, W=AT, Y=CT)

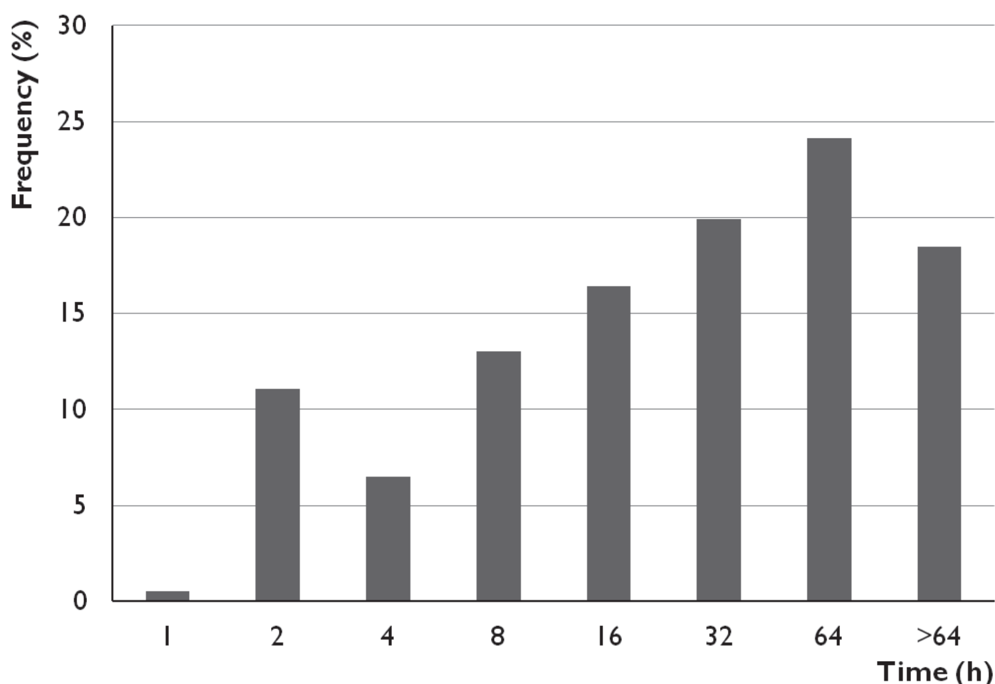

Figure 2.2- Frequency distribution of mortality of *Copidodiaptomus numidicus* exposed to 0.5mg.L^{-1} of copper during lethal time assays. The ">64" category correspond to organisms that remained alive after 64 hours and were scarified for genetic analysis.

Genetic analysis

Only two out of the six pairs of primers were able to amplify the respective target, both for ITS I region, resulting on a aproximately 550 bp (coded as A) and 700 bp (coded as B) amplicon, respectively (Tab 2.2). The gel image analysis, after digestion with every endonuclease to each ITS I amplicon (A and B), shown that Rsa I and EcoR I were not able to cleave both amplicons (Tab. 2.2). Enzymes Hinf I, Hae III and Pst I cuted the longest amplicon (B) but not the shorter amplicon (A). However the sum of fragment size is about 60 and 70bp higher than the amplicon size for Hinf I and Pst I respectively (Tab. 2.2). Msp I, Alu I, Hha I and Dde I clived both amplicons. Despite varying the size of digested fragments in each amplicom (A

and B) no differences were found between sensitive and resistant organisms through band pattern after each enzyme digestion.

Table 2.2. Amplicons size and respective fragments after digestion with restriction enzymes. PCR performed with primers coded as C, D, E and F were not effective for *C. numidicus* DNA and no amplification occurred (NA). Enzymes marked with asterisk (*) did not recognize a cut site at the amplified DNA.

Amplicon		Restriction Enzymes								
Code	Size (bp)	MspI	HinfI	AluI	RsaI	HaeIII	HhaI	EcoRI	DdeI	PstI
		Fragment size (bp)								
A	≈ 550	360 130 80	*	350 200	*	*	230 180 120	*	350 100	*
B	≈ 700	450 250	620 150	300 250 150	*	320 250 120	480 200	*	400 300	420 340
C, D, E, F	NA	---	---	---	---	---	---	---	---	---

2.3. Discussion

Of the three proposed objectives, it was only possible to achieve the first one. The tolerance to copper was not the same for all organisms tested. While some succumbed in a short-time exposure (2 hours), others maintained their vital functions after 64 hours. Despite the failed attempts to genetically distinguich toterant and sensitive organisms and to find CBI, further research towards the same goals should not be disencouraged. The methodological innovations introduced in the present study, in comparison to the work of Martins et al. (2009), need to be addressed aiming at understanding the lack of results. The use of PCR-RFLP technique and the target regions (rDNA and mtDNA) were totally in accordance with the main aims and were justified by their power analysis and encoded genes.

Due to the lack of DNA sequences to *C. numidicus*, the primers here used belong to other species and were chosen by their availability on literature (Harris and Crandall 2000, Machida et al. 2002, 2004, 2006, Denis

et al. 2009). Amplification of the ITS 1 region occurred for two set of primers. This rDNA region is characterized by the existence of highly variable tandem repeats (Kupriyanova 2000, Chu et al. 2001, Ki et al. 2009) which increases the polymorphisms probability. Therefore, theoretically, the set of restriction enzymes here used, with different cutting sites, should have been able to detect those polymorphisms. More restriction enzymes and multiple combinations of enzymes could be used to enlarge the fragment pattern. Nonetheless, the main issue would persist: the lack of solid information for specific primer design and amplification of complete DNA segments of target regions. To solve this problem, a complete research, including genes sequencing, on the target regions of *C. numidicus* would be needed.

Chapter III

Could contaminant induced mutations lead to a genetic diversity overestimation?

Abstract

Contaminant driven genetic erosion reported through the inspection of selectable traits can be underestimated using neutral markers. This divergence was previously reported in the aquatic system of an abandoned pyrite mine. The most sensitive genotypes of the microcrustacean cladoceran *Daphnia longispina* were found to be lacking in the impacted reservoir near the entrance of the metal rich acid mine drainage (AMD). Since that divergence could be, at least partially, accounted for by mutagenicity and genotoxicity of the AMD, the present study aimed at providing such a characterization. The *Allium cepa* chromosomal aberration assay, using root meristematic cells, was carried out, by exposing seeds to 100, 10, 1, and 0.1 % of the local AMD. Chromosomal aberrations, cell division phases and cell death were quantified after the AMD exposure and after 24 and 48 h recovery periods. The AMD revealed to be mutagenic and genotoxic, even after diluting it to 1 and 0.1 %. Dilutions within this range were previously found to be below the lethality threshold and to elicit sublethal effects on reproduction of locally collected *D. longispina* clonal lineages Significant mutagenic effects (micronuclei and chromosomal breaks) were also found at 0.1 % AMD, supporting that exposure may induce permanent genetic alterations. Recovery tests showed that AMD genotoxic effects persisted after the exposure.

Keywords:

Chromosomal aberrations, Micronuclei, *Allium cepa*, Acid mine drainage; Recovery

3.1. Introduction

The loss of genetic diversity by contaminant driven directional selection has been reported through the inspection of adequate selectable traits (Ribeiro and Lopes 2013). In large populations, where genetic drift is negligible, this loss would be undetected through the use of neutral markers, which are suited to evaluate microevolutionary events other than directional selection (Pfrender et al. 2000, Ribeiro and Lopes 2013). Furthermore, if contaminant induced mutations occur then genetic diversity could be increased and/or overestimated. Ribeiro et al. (2012) reviewed a case-study of contaminant driven genetic erosion of a zooplankton population — the crustacean cladoceran *Daphnia longispina* — impacted with a metal rich acid mine drainage (AMD), using tolerance to lethal levels of AMD and of copper as the selectable markers (Lopes et al. 2004a, Agra et al. 2011). This genetic diversity loss was undetected using neutral markers, with some results pointing to the opposite direction (Martins et al. 2005, 2009, Silva et al. 2010). This could have been due to an ineffective genetic drift, possibly masked with the incursion of new alleles by gene flow, and/or by the increase of profiles diversity due to an increased mutation rate (Ribeiro et al. 2012). Therefore, the present study aimed at evaluating the mutagenicity and genotoxicity of that particular AMD effluent. The median effective dilution, using artificial pond water, of this AMD effluent on *D. longispina* reproduction was previously found to range between 0.1 and 1.1 % (Saro et al. 2012). An exposure to a 3 % dilution would reduce the population size down to at least 10 %, with a median survival time lower than 24 h (Lopes et al. 2005, Martins et al. 2007). Therefore, mutagenic effects of this AMD would only be relevant for microevolution of the population at the impacted site if occurring at percentages close to or lower than around 1 %.

Houk (1992) considered three classes of DNA damage: point mutations on DNA sequence, loss or gain of chromosome fragments (clastogenesis), and alterations on chromosome number by loss or gain of whole chromosomes (aneugenesis). Genotoxicity includes a wide range of effects such as lesions in the DNA strand, DNA adducts, sister chromatide exchange, additional DNA synthesis, and also effects on cellular components related with the cellular cycle, such as protein growth factors, spindle fibers and enzymes (e.g. topoisomerase) (Dearfield et al. 2002, Whysner et al. 2004). Repair mechanisms can recuperate temporary genotoxic effects, but mutagenic effects are persistent (Dearfield et al. 2002). With cytogenetic assays, it is possible to identify harmful effects of a stressor at different concentrations and exposure times (Au et al. 1990, Heddle et al. 1991, Ulsh et al. 2004, Pérez-Cadahía et al. 2008). There are hundreds of short-term cytogenetic assays, using a wide range of test organisms (from prokaryotic to mammal cells), which can be applied to study potential mutagenicity and genotoxicity. Assays with plants can be used to assess genotoxicity, providing accurate information and being easier than with animal cells or embryos (Fiskesjö 1985, 1993, Rank and Nielsen 1993, Grant 1994, 1998, 1999, Chauhan et al. 1999, Patra and Sharma 2002, Ma et al. 2005, Leme and Marin-Morales 2009, Mazzeo et al. 2011). In the present study, the *A. cepa* chromosomal aberration assay, using root meristematic cells, was carried out to evaluate the possibility of AMD induced mutations.

3.2. Materials and Methods

3.2.1. Acid mine drainage

The AMD effluent was collected in the water system of a copper mine located in southern Portugal (Mina de São Domingos, 37'37°N, 07°30'W). Even though being abandoned since 1967, the effluent is highly acidic (pH ≈2) and contaminated with metals (Table 1.1) as the result of continuous oxidation of mine tailings (Pereira et al. 1995, Lopes et al. 1999, Pereira 2000, De Bisthoven et al. 2004, Moreira-Santos et al. 2004, Gerhardt et al. 2005, Lopes et al. 2005, Moreira-Santos et al. 2008, Agra et al. 2010). No other sources of contamination are present in this system, neither agriculture and industrial activities nor urban runoff (Ribeiro et al. 2012).

Metal concentrations were quantified by graphite furnace atomic absorption (Cd, Co, Ni, and Pb), by inductively coupled plasma atomic emission (Al, Cu, Fe, Mn, and Zn), and by hydride generation atomic absorption (As), in a certified laboratory (Instituto Superior Técnico, Lisbon, Portugal).

Table 3.1. Metal concentrations in the tested dilutions of the acid mine drainage (AMD) used in the *A. cepa* genotoxicity test.

	Metal ($\mu g L^{-1}$)									
AMD	Al	Fe	Cu	Mn	Zn	Co	Ni	Cd	Pb	As
100 %	440×10^3	353×10^3	41×10^3	28×10^3	21×10^3	2.5×10^3	800	308	24	1.5
10%	46×10^3	33×10^3	4.0×10^3	2.9×10^3	2.1×10^3	2.5×10^2	72	7.3	3	< 1.0
1%	4.7×10^3	3.3×10^3	410	280	220	24	7	< 1.0	< 3	< 1.0
0.1%	450	300	< 50	28	< 50	3	< 5	< 1.0	< 3	< 1.0

The sign < means that the value is below the detection limit of the method used in the analysis.

3.2.2. Citotoxicity, mutagenicity and genotoxicity testing

Seeds of *A. cepa* (2n = 16 chromosomes) from a same strain and variety (baia periform) were used to evaluate genotoxic and mutagenic effects of the effluent. The AMD effluent was serially diluted with milli-Q water and 100, 10, 1, 0.1, and 0 % AMD were tested, the latter being the negative control. Conductivity of AMD dilutions was 4780, 849, 149, and 18.9 μS/cm, respectively, and pH was 1.8, 2.3, 2.9, and 4.1, respectively. Two positive controls were used: methyl methanesulfonate (MMS, CAS 66-27-3) at 4×10^{-4} M, as a clastogenic agent, and trifluralin (2,6-dinitro-N,N-dipropyl-4-trifluoro-methylaniline) (CAS 1582-09-8) at 0.84 ppm as an aneugenic agent (Fernandes et al. 2007).

Onion seeds were germinated at 19–21 °C in Petri plates, internally covered with filter paper wetted with the respective treatment. Four replicates per treatment, each one with 100 seeds were used. The test ended when roots from the negative control were 1-cm long, which took 4–5 days. Then, a third of the germinated seeds was collected and fixed in Carnoy I (3:1 alcohol:acetic acid; v:v) from 6 to 24 h. The remaining germinated seeds were transferred to other Petri plates wetted with milli-Q water. This recovery treatment was used to assess the conduct of the injured cells after some cycles of mitotic division. After 24 and 48 h, one third of the seeds was fixed as described above. Fixed roots were hydrolyzed in HCl 1 N, in a bath at 60 °C for 8–11 min, stained with Schiff's reagent for 2 h in the dark, and then washed with distilled water. To prepare the slides, the meristematic regions were cut onto a slide into a drop of 2 % acetic carmine solution to increase cytoplasm contrast, covered with a cover slip and carefully squashed. The cover slip was removed with liquid nitrogen and the slides mounted in synthetic resin (Enthellan®, Merck) to further analysis.

At the end of the test, the germination index, in %, was calculated as the proportion of seeds with visible radicle protrusion. Over 40 randomly picked roots from each treatment were measured. Ten slides of each treatment were observed at an optical microscope (1000x) and a minimum of 500 intact cells per slide (5000 per treatment) were counted, distinguishing the number of cells in each stage of mitosis, interphase and cell death. Chromosome aberrations and micronuclei were recorded in all phases of the cell cycle. Cytotoxic effects were analyzed by quantifying both mitotic cells and cells in death process. Cell death was identified by morphological alterations of the nucleus and/or cell (Kroemer et al. 2009). Mainly at the undiluted AMD, fragmented cells with loss of cellular and nuclear contents were observed, but these cells were not counted due to the difficulty of distinguishing cytotoxic effects (cell in death process) from technique effects (cell rupture during slide preparation). Despite the soft squash of the root tip being identical in all treatments, cells exposed to 100 % AMD were less resistant. Dead cells were those showing vacuolated and swollen cytoplasm or heteropycnotic, condensed and/or fragmented nuclei (Majno and Joris 1995). Four types of morphological change of the nucleus were here considered: (i) reduction of the nuclear volume (pyknosis), (ii) nuclear fragmentation (karyorrhexis), (iii) vacuolated cytoplasm and nucleus displaced to a peripheral position, and (iv) chromatin dispersion (karyolysis) (Kroemer et al. 2009). Genotoxic effects were quantified by micronuclei and chromosomal aberrations, such as C-metaphases, multipolar anaphases and telophases, chromosome adherences, chromosome bridges, polyploidy, bi-nucleated cells, and chromosome losses and breaks. Mutagenicity was quantified by micronuclei and chromosome breaks.

3.2.3. Data analysis

Interphase, mitotic, cellular death, micronuclei, and chromosomal aberrations indexes were normalized by calculating the number of cells in each category if exactly 500 cells would have been observed per slide. These indexes are the proportion of cells in each category relatively to the total number of intact cells (500).

Root length values were log transformed and proportional data were arcsine transformed using the Freeman and Tukey modification (Zar 2010). Significant differences against the negative control were checked with one-way ANOVA, followed by the one-tailed Dunnett's multiple comparisons test. A back transformation with correction (Zar 2010) was applied to estimate means of proportional data. Assays were validated through the comparison of the three controls using a one-way ANOVA. The software STATISTICA 8.0 was used.

3.3. Results

The germination rate per Petri plate ranged between 64 and 99 % with no significant differences among treatments ($p = 0.209$) (Fig. 3.1). The 10 and 100 % AMD reduced root growth ($p < 0.0001$) (Fig. 3.2). Interphase, mitotic, chromosomal aberrations, and cellular death indexes were significantly altered by AMD dilutions (Fig. 3.3). The 0.1 % AMD presented significantly less cells in mitosis and more cells with chromosomal aberrations than the negative control (Fig. 3.3). The undiluted AMD showed a comparatively low number of chromosomal aberrations, which was due to the very low number of live cells in mitosis and interphase (Fig. 3.3). Results on chromosomal aberrations, nuclear abnormalities and cellular death types are shown in Table 3.2. The frequency of multipolarity, C-

metaphase and adherence after the exposure to 1 % AMD was significantly higher than in the negative control. Adherence was also significantly higher in the 0.1 % AMD. Mutagenicity was found for 0.1 % AMD (Fig. 3.4).

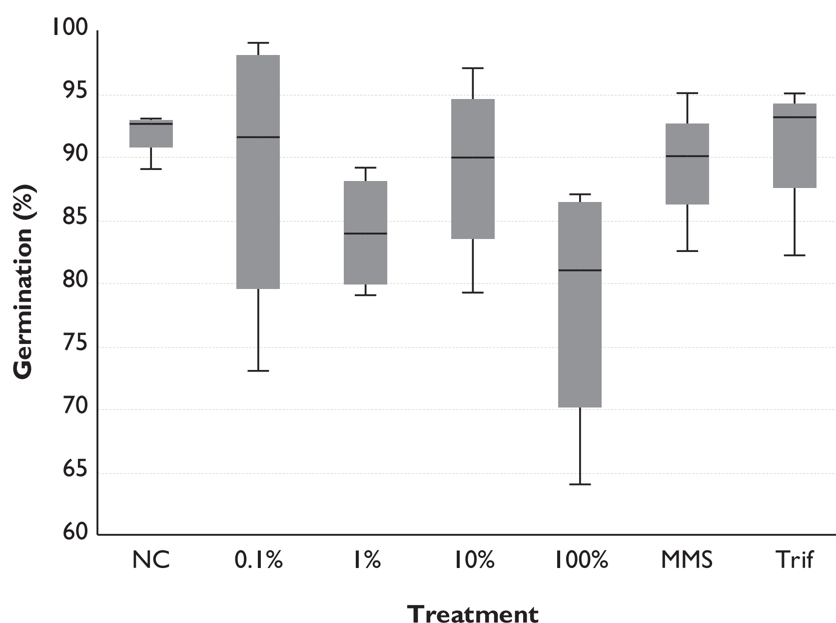

Figure 3.1. Box-and-whiskers plot of the *A. cepa* seed germination index after an exposure to 100, 10, 1, and 0.1 % acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively.

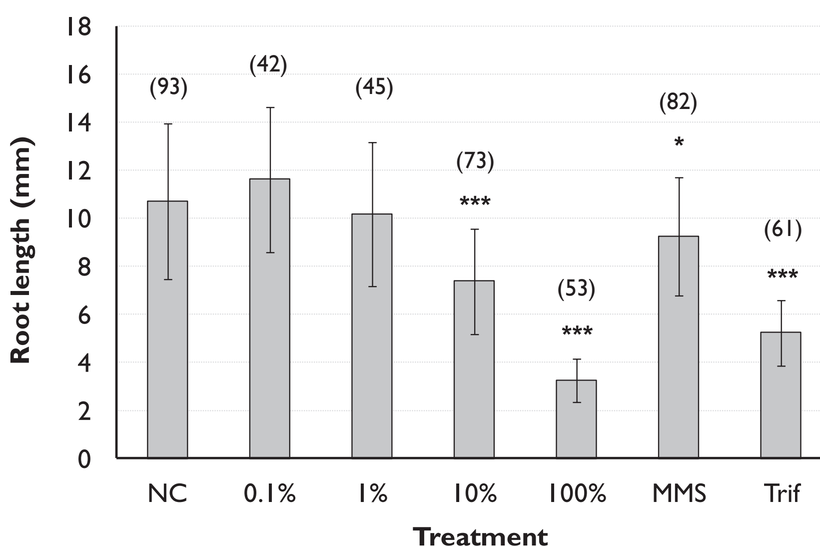

Figure 3.2. Mean and SD of *A. cepa* root length (in mm) after an exposure to 100, 10, 1, and 0.1 % acid mine drainage. The abbreviations *NC*, *MMS* and *Trif* indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively. Sample size is indicated inside brackets. Asterisks indicate significant differences relatively to the negative control (* $p \leq 0.05$; ** $p \leq 0.001$; *** $p \leq 0.0001$).

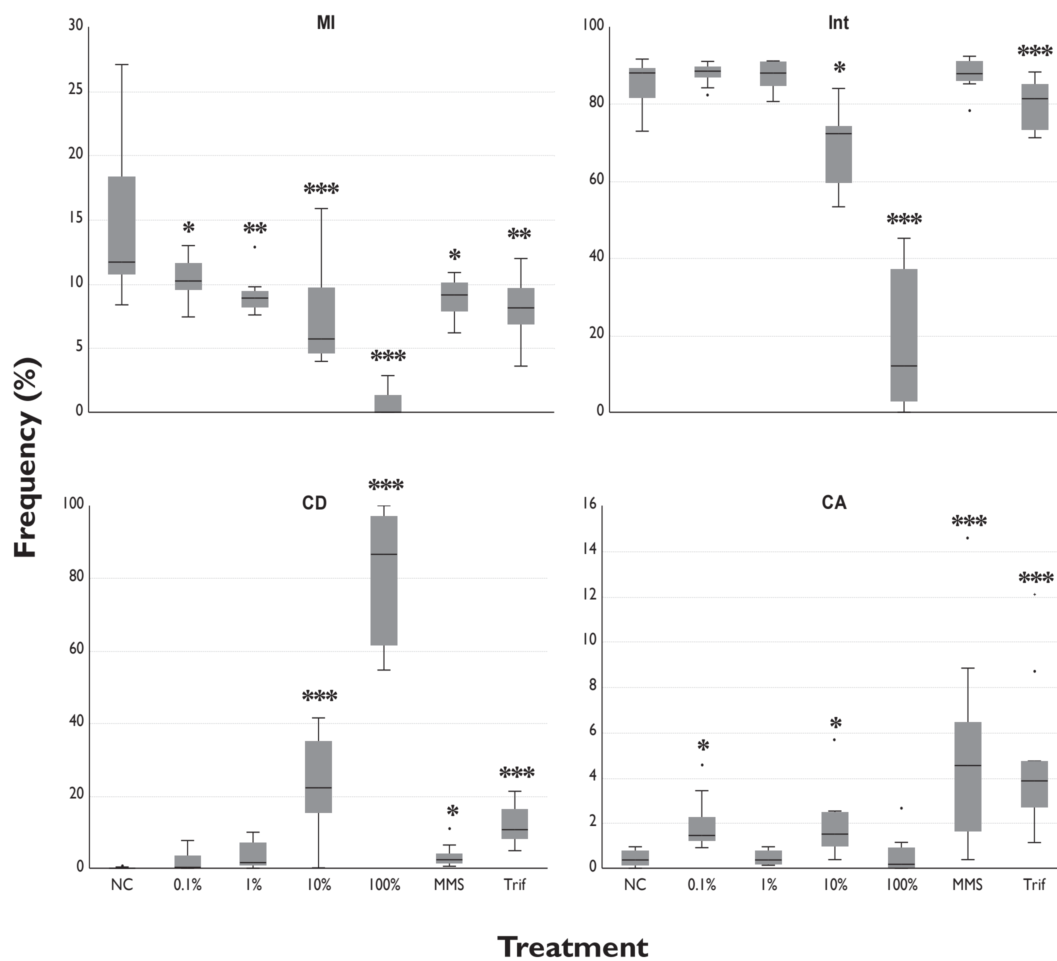

Figure 3.3. Box-and-whiskers plots of mitotic (MI), interphase (Int), cellular death (CD), and chromosomal aberrations (CA) indexes indexes of *A. cepa* root meristematic cells after an exposure to 100, 10, 1, and 0.1 % acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively. Asterisks indicate significant differences relatively to the negative control (* $p \leq 0.05$; ** $p \leq 0.001$; *** $p \leq 0.0001$). Outliers are represented by black dots.

Table 3.2. Back transformed means and coefficients of variation of arcsin transformed values (in %, inside brackets) of frequencies of chromosomal aberrations, nuclear abnormalities and cellular death types in *A. cepa* root meristematic cells after an exposure to 100, 10, 1, and 0.1% acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively.

	NC	0.1% AMD	1% AMD	10% AMD	100% AMD	MMS	Trif
Micronuclei	0.128 (3)	0.428 (3)	0.376(5)	0.879(6)	2.150(12)*	4.122(8)*	2.017(7)*
Chromosomal break	0.021 (1)	0.027 (2)	0.020 (1)	0.020 (1)	0.063 (2)	0.139 (3)*	0.000 (0)
Chromosomal loss	0.021 (1)	0.052 (2)	0.042 (2)	0.059 (2)	0.000 (0)	0.133 (2)*	0.076 (2)
Chromosomal bridge	0.000 (0)	0.017 (1)	0.060 (2)	0.120 (3)*	0.000 (0)	0.040 (2)	0.075 (2)*
C-metaphase	0.000 (0)	0.051 (2)	0.115 (3)*	0.020 (1)	0.000 (0)	0.000 (0)	0.593 (6)*
Adherence	0.192 (3)	0.911 (2)*	0.882 (3)*	0.561 (3)*	0.049 (2)	0.403 (4)	0.301 (3)
Multipolarity	0.020 (1)	0.118 (3)	0.256 (2)*	0.235 (4)	0.020 (1)	0.061 (2)	0.300 (3)*
Laggard	0.000 (0)	0.030 (2)	0.028 (2)	0.036 (2)	0.000 (0)	0.041 (2)	0.076 (2)*
Nuclear buds	0.108 (3)	0.010 (1)	0.114 (3)	0.066 (2)	0.063 (2)	0.136 (3)	0.555 (3)*
Lobulated nuclei	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.828 (4)*
Polynucleated cell	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.057 (2)*
				Cellular death			
Peripherical nuclei	0.000 (0)	0.196 (4)	0.050 (2)	0.734 (7)	3.980 (10)*	0.114 (3)	0.170 (3)*
Karyolysis	0.000 (0)	0.032 (2)	0.029 (2)	0.000 (0)	0.617 (6)*	0.821 (8)	1.847 (7)*
Pyknosis	0.057 (2)	0.861 (8)	1.971 (10)	6.681 (11)*	33.91 (21)*	1.288 (6)*	3.702 (7)*
Karyorrhexis	0.082 (3)	0.263 (4)	1.545 (8)	16.35 (14)*	34.17 (23)*	1.338 (8)	6.471 (13)*

* Value significantly larger than negative control ($p < 0.05$).

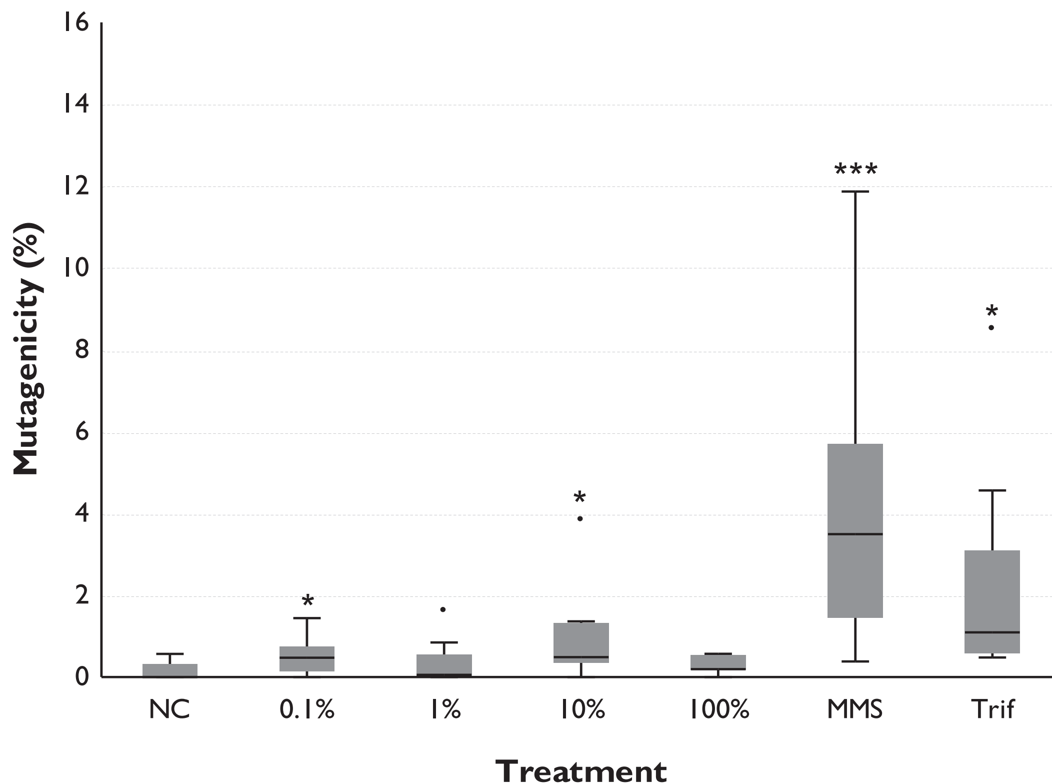

Figure 3.4. Box-and-whiskers plot of the mutagenic index of *A. cepa* root meristematic cells after an exposure to 100, 10, 1, and 0.1% acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively. Asterisks indicate significant differences relatively to the negative control.

After 24 and 48 h of recovery, in the absence of contaminants, cytotoxic and genotoxic effects of AMD on the meristematic cells were still present (Fig. 3.5). Among the interphase, mitotic, chromosomal aberrations, and cellular death indexes, only the former revealed a recovery. Worth noting is that the significantly higher amount of cells with chromosomal aberrations in the 0.1 % AMD, relatively to the negative control, persisted throughout the recovery period of 48 h. Mutagenicity was reduced when ended the exposure to AMD, with AMD dilutions losing their effects at 24 h of recovery and the undiluted AMD at 48 h (Fig. 3.6).

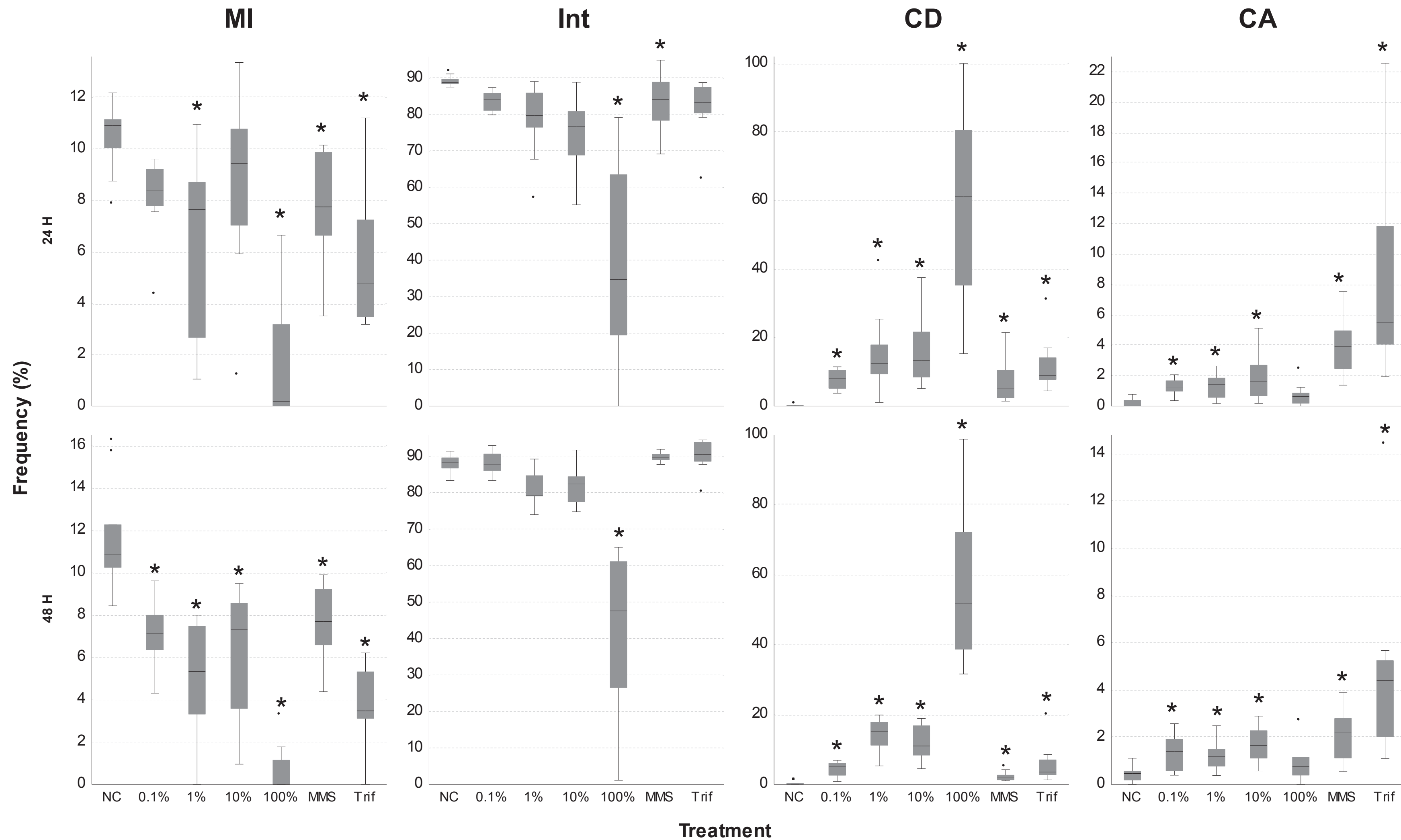

Figure 3.5. Box-and-whiskers plots of mitotic (MI), interphase (Int), cellular death (CD), and chromosomal aberrations (CA) indexes of A. cepa root meristematic cells at 24 and 48 hours after an exposure to 100, 10, 1, and 0.1% acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively. Asterisks indicate significant differences relatively to the negative control.

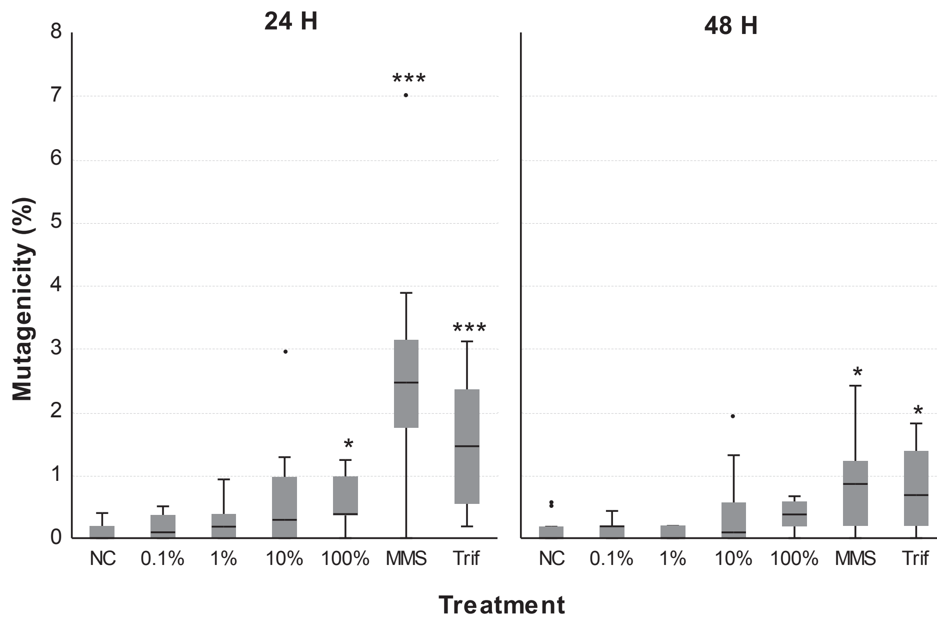

Figure 3.6. Box-and-whiskers plot of the mutagenic index of *A. cepa* root meristematic cells at 24 and 48 hours after an exposure to 100, 10, 1, and 0.1% acid mine drainage. The abbreviations NC, MMS and Trif indicate the negative control and the positive controls with methyl methanesulfonate and trifluralin, respectively. Asterisks indicate significant differences relatively to the negative control.

3.4. Discussion

Due to the extreme acidity of undiluted AMD (pH 1.8), a significant inhibition of germination was expectable as reported by other works studying the process of seed germination under acidic conditions. For instance, Salter and McIlvaine (1920) promoted the germination of wheat, corn, soybean, red clover, and alfalfa in culture media with pH ranging from 2 to 7.5 and found that germination occurred at pH 3, but at pH 2, although the germination process started (swelling of the seeds), only two species had germination rates above 50 % and all seedlings were dead after 7 days. Turner et al. (1988) exposed *Paulownia tomentosa* seeds to pH between 1.5 and 7.0, to address the possibility to colonize affected soil in charcoal mines and verified that no sprout take place below pH 4. Shoemaker and Carlson (1990) only obtained normal levels of germination at pH 5 when using a filter paper culture. *Passiflora alata* seeds showed higher germination rates and initial normal

development at pH 3 (Wagner Jr et al. 2006). *Pinus contorta* and *Picea glauca* seeds presented normal germination rates at pH 2.2 (Redmann and Abouguendia 1979). Germination occurrence does not imply that the remaining steps of root development may proceed without interference of acidity, as germination and development are two different processes (Bewley 1997). During root growth, energetic resources stored in the seed are used and molecular and physiological processes (DNA synthesis, protein, cell division) are activated, so growth is affected by mode of action and/or intensity of stressors over those processes (Cho et al. 2003; John et al. 2009). Root growth assays have been considered a good indicator for metal sensitivity since their design in 1957 by Wilkins (Baker 1987). Growth was inhibited at the two highest AMD concentrations, agreeing with other studies where seeds of *A. cepa* (Fiskesjö 1985, Lerda 1992) and other plant species were exposed to metals that are present in the AMD effluent (Wong and Bradshaw 1982, Symeonidis and Karataglis 1992, Lin et al. 2003, Liu et al. 2007, Aydinalp and Marinova 2009, Soudek et al. 2011).

Alterations in the mitotic index have been considered good evaluators of cytotoxic effects of environmental contaminants (Smaka-Kincl et al. 1996, Fernandes et al. 2007, Caritá and Marin-Morales 2008, Leme et al. 2008). Cell division only completes the cycle if all signaling pathways are functional. If chemical requirements are disrupted (e.g. the production of cyclins regulators of G1 phase to S phase and G2 to mitosis; activation of cyclin-dependent protein kinases), cell division check-points are not transposed, and the process is discontinued. Significant decreases in the mitotic index mean that external factors had interfered with the cell division process. In the present study, even the lowest AMD concentration acted as a stressor at the cellular level. According to Patra et al. (2004), metals, mainly from

groups IV and VII, have implications on the mitotic index (Cd, Cu, Hg, Cr, Co, Ni, Be > Zn, Al, Mn, Fe, Se, Sr, Sb, Ca, Ti, Mg > V, As, Mo, Ba, Pb) and this AMD have at least 4 metals belonging to the strongest effectors group (Cu, Ni, Cd, and Cr).

Cell death is also an endpoint for cytotoxicity. Death is induced when a contaminant promotes permanent damages in DNA molecules and/or when some other cell processes occur, such as changes in enzyme activity, osmotic deregulation and loss of membrane integrity. Cell death can be identified by morphological alterations of the nucleus and/or cell (Kroemer et al. 2009). Among the morphological changes of the nucleus leading to cell death, AMD induced pyknosis and karyorrhexis with a dose–response pattern.

The presence of chromosomal aberrations provides a measure of genotoxicity. They allow the discrimination between clastogenic and aneugenic effects (Leme and Marin-Morales 2009), and contribute to understand the cell death process and effects persistence after exposure to contaminants (Fernandes et al. 2009, Leme and Marin-Morales 2009). Even with 0.1 % AMD, an increased frequency of chromosomal aberrations occurred. Only at the undiluted AMD, no significant differences were found, but this was most probably due to the low number of live cells and consequent reduction in the relative occurrence of chromosomal aberrations.

Chromosomal adherences were observed in all AMD dilutions, occurring mainly during metaphase with some chromosomal compression on the equatorial plate (not complete compression). Chromosomal abnormalities induced by AMD included also micronuclei and multipolar anaphases and telophases. Adherences and multipolarity are considered aneugenic effects, while micronuclei can be clastogenic

or aneugenic, depending if they are originated by chromosomal breaks or losses of whole chromosomes, respectively (Fernandes et al. 2007). In the present study, the frequency of chromosomal breaks and chromosomal losses was similar, such as bridges and C-metaphases, disallowing a clear categorization of AMD as clastogenic or aneugenic. Chromosomal losses are not considered as mutagenic effects. First, because if nuclei lose a whole chromosome, cell will be unviable and death process will begin. Second, because those chromosomes can return to nuclei, which is the case of synchronous micronuclei (Huang et al. 2012). These micronuclei will respond to the cell signaling as the main nuclei and during the cell division process they will be incorporated again (Gustavino et al. 2001). Chromosomal breaks and asynchronous micronuclei are the chromosomal aberrations used to evaluate mutagenicity. The significant occurrence of micronuclei and chromosomal breaks combined revealed AMD to be mutagenic even at a 0.1 % concentration. At the highest AMD concentrations, the reduced number of live cells masked possible dose–response trends which were undetected in most chromosomal aberrations.

Recovery assays revealed the so-called "cell cycle delay", which leads to late cell responses; even though cells are no longer subjected to direct toxic exposure, they continue to express genotoxic effects (Kirkland 1998, Komissarova et al. 2005). Longer recovery periods would be needed to quantify the temporal extension of AMD genotoxic effects (Aaron et al. 1995, Kirkland 1998; HERAG 2007).

3.5. Conclusions

In conclusion, data gathered in the present study revealed the AMD effluent of the abandoned São Domingos mine to be mutagenic and genotoxic, even after diluting it to 1 and 0.1 %. Although transferability of these laboratory results to the complex field scenario is not straightforward, dilutions within this range were previously found to be below the lethality threshold and to elicit sublethal effects on reproduction of locally collected *D. longispina* clonal lineages (Lopes et al. 2005, Martins et al. 2007, Saro et al. 2012). Therefore, when using neutral markers, as Martins et al. (2009) and Silva et al. (2010) did, a possible overestimation and/or increase in genetic diversity due to mutations cannot be ruled out.

Chapter IV

Gene expression variation caused by contamination: profiling *Daphnia magna* growth and ecophysiological responses to copper with quantitative-PCR

Abstract

Daphnia magna gene expression was analyzed by q-PCR, to determine neonate to 1st adult instar profiles, under standard laboratorial conditions and after exposures to 6µgL^{-1} of copper. Eight target genes [metallotionein (Mt), ferritin (Fer), citocrome-c-oxidase (COI), NADH dehydrogenase (ND2), lactate dehydrogenase (LDH), inositol monophosphatase (IMP), vitellogenin (Vtg), and ecdysone receptor (EcR)] were chosen due to their association to copper tolerance. GeNorm and BestKepper were used to select the most stable reference gene among actin (Act), glyceraldehyde-3P-dehydrogenase (GAPDH), and ubiquitin conjugating enzyme (UbC) for each exposure time. Under laboratorial conditions, *D. magna* exhibited a regular profile until the development of ovaries (72 h). Then, a general increase followed by sharp decrease at 168 hours, were observed for almost genes. The fact that Vtg also decreased, suggests that the presence of eggs in brood pouch biased the results. The gene expression pattern of EcR was higher at 144 and 168 hours than during previous ecdysis, revealing that this isoform has a more significant role in *D. magna* reproduction than in growth. Under copper exposure, gene expression was analyzed with Rest© software. Fer and Mt were affected by copper, down and up-regulated at 12 and 96 hours, respectively. Despite some significant results of COI, ND2 and LHD, their high levels of variance led to very large confidence intervals. The most responsive gene was IMP, up-regulated at 6, 24, 48, 72, and 168 hours and down-regulated at 120 hours. EcR was expected to be more reactive nearby ecdysis occurrences but down-regulation was observed only at 6 hours. Vitellogenin gene expression was significantly down-regulated at 72 hours. A relationship between copper exposure and changes in gene expression was

observed, mainly in the cell signaling pathway, which could be responsible for growth and reproduction stress responses.

Keywords:

Gene expression profile, copper, quantitative PCR, metabolic pathways, *Daphnia magna* growth

4.1. Introduction

Long-term exposure to contaminants has been identified as responsible for genetic changes in populations (Van Straalen and Timmermans 2002, Medina et al. 2007, Ribeiro et al. 2012, Ribeiro and Lopes 2013). Both selectable markers (Lopes et al. 2004b, Agra et al. 2010) and neutral markers (Theodorakis and Shugart 1997, Martins et al. 2009, Silva et al. 2010) related with contaminant tolerance, have been used to evaluate those changes. The acquisition of tolerance, at population level, depends of the amount of genetic variability available at the time, and, in turn, genetic variability depends on the cumulative increased tolerance at individual level and/or selective pressure (Hoffmann and Parsons 1991). In an equilibrium population, natural selection tends to maximize survivorship and fecundity and minimize the development time, however, when a stressor affects negatively one life-cycle stage this could imply fitness costs to another (Forbes and Calow 1997). Such responses can be triggered by physiological and/or biochemical mechanisms or even alternative genetic programs activated during development (Hoffmann and Parsons 1991). With gene expression studies it is possible to detect which genes have an active response, (up-regulated or down-regulated) under a certain factor of stress, and when compared to a non-stressed situation, the gene expression profiling may improve the knowledge of contaminants mode of action (van Straalen and Roelofs 2006, Piña et al. 2007).Gene expression studies are based on the cells ability to change their processes of protein synthesis according to the external signals. The regulatory process of protein synthesis is complex, with several control points (inside and outside the nucleus), but were transcription is the first step. It is possible to associate the physiological response of the cell to stress level trough the amounts of mRNA transcript; and to check the occurrence of mutations between

lineages by mRNA quantification. Also, as some genes are only expressed in certain cells, tissues or organs, or in specific developmental stages (e.g. growth or sexual maturation stages), it is possible to use gene expression profiling to analyze different types of stress responses during an organism life cycle.

Microarray hybridization and quantitative PCR are the most used methods for gene expression assessment[4] into ecotoxicogenomics (Neumann and Galvez 2002, Heckmann et al. 2006, Vetillard and Bailhache 2006, Watanabe et al. 2007, Connon et al. 2008, Nota et al. 2008, Banni et al. 2009, Garcia-Reyero et al. 2009, Nota et al. 2010, Pereira et al. 2010, Spanier et al. 2010). This technique can be used to screen, simultaneously, a large number of gene transcripts with relatively low cost (Gracey and Cossins 2003). However, when genes have similar sequences, cDNA probes can exhibit a low specificity, compromising data analysis. The complexity of data analysis and some variability of differential expression have also been referred asdisadvantages of Microarray hybridization (Kothapalli et al. 2002, Morey et al. 2006). Quantitative PCR (qPCR) has some advantages such as the ability to analyze small amounts of RNA and it also allows simultaneous measurement of gene expression with different samples (biological or treatment samples). Furthermore, the most advantageous feature of this technique is the ability to monitor the amplification process in a real time scale. Precise and accurate data for gene expression quantification may be acquired during exponential phase if analysis conditions meet the requirements of equal initial amount of total RNA, similar reaction conditions and efficiencies, a set of reference genes and the right

[4] Other used methods are Subtractive Hybridization, Expressed Sequence Tag Sequencing, and Serial Analysis of Gene Expression

methodology to choose the most stable gene(s) (aka housekeeping genes[5]) to perform data normalization (Dale and van Schantz 2002, Spanier et al. 2010, Chen et al. 2011). Normalization is a technical requirement in gene expression analysis used to decrease the non-specific variation and to allow a comparison of normalized expressions values between samples (Thellin et al. 1999, Bustin 2002, Bustin and Nolan 2004, Dheda et al. 2004, Huggett et al. 2005). However, there are several methods with different statistical approaches perform normalization. Huggett et al. (2005) discuss important considerations and summarizes the mathematical bases of the most used algorithms: "geNorm" (Vandesompele et al. 2002), "BestKeeper" (Pfaffl et al. 2004), and "NormFinder" (Andersen et al. 2004). These programs were developed as Excel-based software tools to provide a friendly data analysis. GeNorm uses a pairwise comparison approach by calculating the arithmetic mean of the pairwise variations between one gene and all others candidate genes to obtain the gene-stability value M (Vandesompele et al. 2002). BestKeeper does a pairwise correlation analysis of all candidate reference genes, and the highly correlated are combined into an index. It also provides the standard deviation, percent covariance, and the power of the candidates values as an adjuvant to choose the most stable genes (Pfaffl et al. 2004). NormFinder calculates a stability value using the variations inter- and intragroup, and the most stable genes are considered the one with the minimal combined intra- and intergroup variation (Andersen et al. 2004). The use of multiple normalization methods is recommended for a strong data analysis and to detect bias due to the potential presence of

[5] Housekeeping genes are genes related with essential functions for cell maintenance and are expressed in all cell types. Their expressions are relatively constant even when experimental conditions are changed.

coregulated genes among the candidates (Infante et al. 2008, Spanier et al. 2010, Chen et al. 2011).

At the complex case-study presented by Ribeiro et al. (2012), with natural populations of *D. longispina* from São Domingos mine, Martins (2007) microarray analysis was used to determine gene expression profiles of two *D. longispina* clones with different resistance to copper and acid mine drainage (AMD). A group of *D. longispina* juveniles, with 2 days old, was exposed to 6 μgL^{-1} of copper and a second group was exposed to 0.125% of metal-rich AMD, during 48 hours. The microarray was a custom made *D. magna* microship, containing genes related to energy metabolism, molting and reproduction. The comparison done between clones showed that the sensitive clone had a higher number of significantly changed genes than the resistant clone. The present study was designed to analyse the gene expression profile under a new perspective. The qPCR technique was chosen to quantify gene expression of a set of genes selected due to their role in metabolic pathways, discussed in the existing literature as related to daphnids growth, reproduction, allocation of energetic resources, and detoxification processes (Castro et al. 2004, Lopes et al. 2004a, Amiard et al. 2006, Prohaska 2008, Hannas et al. 2010, Agra et al. 2011). Genes of three metabolic pathways were selected. The metal-binding proteins ferritin (Fer) and metallothionein (Mt), which are related with metal transport and detoxification processes (Harrison and Arosio 1996), were included in the first metabolic group. Those proteins are capable to bind metals and are considered metal regulators in the cell, by storing and sequestering metal ions (Amiard et al. 2006). These genes have been also pointed as biomarkers for metal contamination studies (Poynton et al. 2008, Soetaert et al. 2007). Genes coding enzymes related with glycolysis and electron transport were selected for the

second group. The NADH dehydrogenase subunit 2 (ND2) enzyme belongs to complex I, the first electron acceptor of the mitochondrial respiratory chain. Cythocrome-c-oxidase (COI), a complex metalloprotein containing iron, copper, zinc and magnesium, works as the final electron acceptor in respiratory chain (Brunori et al. 1987, Weiss et al. 1991). Lactate dehydrogenase (LDH) enzyme is specially required to catalyze the reversible reduction of piruvate to lactate when a fast and additional energy is needed, or when levels of oxygen are low (Vassault 1983). Finally, genes related with cell signaling metabolic pathway were selected for the third group. Inositol monophosphatase (IMP) removes a phosphate, by hydrolyse, from the inositol-phosphate molecule activating the inositol molecule that is an important growth factor (Fallahi et al. 2011). The receptor for ecdysone (EcR) is a ligand-dependent transcription factor that binds to ecdysteroid hormones responsible for the coordination of arthropods growth, molting and metamorphosis (Krishnakumaran and Schneiderman 1970, Kiguchi

and Agui 1981, Riddiford et al. 2000, Nakagawa and Henrich 2009). Vitellogenin (Vtg) was also included as a cell signaling protein. Technically Vtg is not a single protein but a group of similar lipid-proteins (Hagedorn and Kunkel 1979), acting as carriers of lipids and proteins to the egg, to form the vitellin that is the main source of energy during embryonic development (Komatsu and Ando 1992, Lee and Puppione 1988).

To explore how the expression levels of those genes change along dafnid life cycle, how it is affected by copper exposure, and to relate these effects with physiological responses to stress in each stage of development, it would be important to select a widely studied organism, which had an extended frame of information on response to contaminants, including metals. Daphnids are ubiquitous

in occurrence with an important ecological role in aquatic environments (De Bernardi and Peters 1987). They are also highly susceptible to contaminants. Furthermore handling and laboratory culturing are very straightforward making it the most widely used test-organism in aquatic ecotoxicology. The knowledge acquired about their physiological and behavioral responses to environmental contamination, could be analyzed under a genomic perspective. This would lead to a better understanding of the mechanisms of action of contaminants (Feder and Mitchell-Odds 2003, Shaw et al. 2008, Colbourne et al. 2011).

This experimental work aimed to profile *D. magna* gene expression of metal responsive genes, from neonate up to the stage of sexual maturation; to connect gene expression patterns with *D. magna* life-cycle events, and ecophysiological responses to copper exposure.

4.2. Materials and Methods

4.2.1. Daphnia magna assay

Group cultures of *D. magna* adult females (clone B, according to Baird et al. 1989a) were maintained in ASTM hard water medium (ASTM 2010) enriched with *Ascophyllum nodosum* extract (Baird et al. 1989b) and fed daily with $5x10^5$ $cellsmL^{-1}$ of the green algae *Pseudokirchneriella subcapitata*. Cultures were maintained at 20±1°C, with a 14:10 hours light:dark cycle. Few hours before broad release (only 3rd to 5th broods were used in the experiments) females were isolated and observed at intervals of 30 minutes. Neonates born during this interval from all females were transferred to a vial to blend until their randomly retreated for testing. Neonates born outside the 30 minutes interval were discarded in order to keep organisms the most synchronized as possible.

A high concentration of copper stock solution (as copper sulfate pentahydrate, supplied by Merck) was prepared and kept at 4°C. A test-solution, with nominal concentration of $6\mu gL^{-1}$of copper, was made from stock-solution by dilution with culture medium every 48 hours. The test-concentration correspond to 1/10 of copper LC_{50} value for *D. magna*, described in the literature (Martins 2007, Poynton et al. 2008, Garcia-Reyero et al. 2009). *Daphnia magna* growth was monitored by measuring the first exopodite from the second antenna, according to Soares (1989). To avoid handling stress the exopodite measurements were made in the molt released at the end of each instar.

Plastic containers (polyethylene terephthalate - PET) were used as test tanks containing 2 liters of culture medium (ASTM 2010) and correspondent food ration. Initially, a total of 18 tanks were prepared (9 with copper solution and 9 as

controls) each with 40 neonates. Due to *Daphnia* growth and transition to pre-adult stages, at 96 hours each group was divided into two sub-groups with 20 organisms per 2L of culture medium.

At the end of each exposure time, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours, 5 replicates were collected, transferred to properly labeled microtubes and preserved with a RNA stabilization reagent (RNAlater®, Quiagen), for further analysis. Each replicate consisted of five organisms until 48 hours; three organisms at 72 and 96 hours; and one at 120, 144 and 168 hours.

4.2.2. Bioinformatics and genetic analysis

Genebank database (NCBI dbEST: Boguski et al. 1993) was used to find registered sequences for target genes. Conservative regions were checked using Bioedit Software (Hall 1999, 2011) to align CDS fasta files of *D. magna* and other cladocerans and crustaceans phylogenetically related. Eight target genes were analyzed, with predicted function in: i) metal transport: metallothionein (Mt) and ferritin (Fer); ii) glycoysis and electron transport: cytochrome c oxidase subunit I (COI), inositol monophosphatase (IMP) and NADH dehydrogenase subunit 2 (ND2); and iii) cellular signaling: vitellogenin fused with superoxide dismutase (Vtg), lactate dehydrogenase (LDH) and ecdysteroid receptor (EcR). The reference genes actin (Act AJ292554) glyceraldehyde-3P dehydrogenase (GAPDH AJ292555) and ubiquitin conjugating enzyme (UbC WFes0004602) were selected based on Heckmann et al. (2006).

Primers and nested primers (Table 4.1) were designed with Prime3 software (Rozen and Skaletsky 2000) and their viability was assessed “in silico” with FastPCR software (Kalendar et al. 2009).

Total RNA was extracted using TRIzol® Reagent (Invitrogen) and treated with DNAse (Invitrogen). The quantity and quality of the RNA were determined by spectrophotometry (Nanodrop ND1000, Thermo Fisher Scientific). A second evaluation of RNA quality was performed by gel electrophoresis (1.2% agarose) and then, the cDNA was synthesized following the manufacturer instructions (High Capacity RNA-to-cDNA™ Master Mix - Applied Biosystems). Primers and cDNA were tested through a standard PCR (MyCycler Thermal Cycler 170-9711, BioRad), performed with one unit of DNA Taq Polymerase (Fermentas), 0.2 mM of dNTP mix (Fermentas), and 50 ng of template DNA, within a final volume of 25 μL (Table 4.2). A positive control was done using cDNA from the oyster *Crassostrea rhizophorae* and specific primers for actin (F: 5' GCCAACAGAGAAAAGATGACA 3'; R: 5' TTCGTTGCCAATGGTGATGA 3').

Table 4.1. Primers and nested primers used on PCR and qPCR, with respective amplicon size and melting temperatures.

Gene		Primer sequence 5' → 3'	TM (°C)	Fragment size (bp)		Nested primer sequence 5' → 3'	TM (°C)
Mt	*F*	GTGTGGAACCGAATGCAAAT	61	183	106	AATGCCCGTCAGCCACAT	63
	R	TGACGTTGAAAGAGCATAACG	59			TACTTGCAGCAGGCGGACT	62
Fer	*F*	CACTTGGAGGAGCATTTCTTG	60	129	129	CTTGGAGGAGCATTTCTTGG	60
	R	CAAAAAGACTCCTAGGCCATCA	61			AAAGACTCCTAGGCCATCACC	60
COI	*F*	GCCCACGCGTTTGTAATAAT	60	204	129	AATTGGAGGTTTCGGGAAC	60
	R	AGTTCAACCAGTCCCAGCAC	60			GCCCCTCCCACAAGTAAAAG	61
IMP	*F*	TGCACCTCCAGAAGGAAAAC	60	224	153	TGACACCCGTAGTTCAGCAA	60
	R	TTGCAGTCAAAATTGCTTCG	60			CGCATGTGTCCCACTTTTT	60
Vtg	*F*	GCGTGGAAGGAGACAAGAAG	60	212	156	CCTGTTGAACAAGGAGAAAATTG	60
	R	CATGTGGGAGATGGGAGAGT	60			TCAATGAAGTTATCAGCGGTTTT	60
ND2	*F*	TTTGGCCTTTTTGCCTCTAA	60	209	122	TTTTCTTACACAAACTTTGGCATC	60
	R	ATCAAACACGTTTCCCCAAT	59			CCGCCCCCAATTTAATACTT	60
LDH	*F*	ATTATTGGGGAACACGGTGA	60	191	150	CACGAGTGTTCCTGTGTGGT	60
	R	ATTGCCCAAGAAGTGTGTCC	60			TTCAAACGGATAATTTCATAGGC	59
EcR	*F*	CATCAACCGGCTCGTCTATT	60	221	150	CCAGGAAGAATTCGATCAGC	60
	R	CCTTGAGCAACGTGATCTGA	60			AACCGCTTCGAGAATTCCA	61

Reference genes			
Act	*F*	CCACACTGTCCCCATTTATGAA	60
	R	CGCGACCAGCCAAATCC	62
GAPDH	*F*	GGCAAGCTAGTTGTCAATGG	57
	R	TATTCAGCTCCAGCAGTTCC	57
UbC	*F*	TCACCTGCACTCACCATTTC	58
	R	AATCTCCGGAACCAAAGGAT	58

Melting temperature was calculated to 50mM Na^+

Primers were synthesized by Invitrogen

Table 4.2. Reagent concentrations and run conditions for standard PCR. (According Invitrogen Taq polymerase guidelines)

Preparation

Reagent	Initial concentration	Final concentration	Volume (μL)
Taq Buffer	10x	1x	2.5
$MgCl_2$	25mM	1.5mM	1.5
dNTP	10mM	0.2mM	0.5
Taq DNA polymerase	5U/μL	1U/μL	0.2
Primer F	10μM	5μM	0.5
Primer R	10μM	5μM	0.5
Water nuclease-free		Fulfill 25μL[a]	18.8
Template (100ng cDNA)			0.5

Reaction

Step	Temperature (°C)	Time	Cycles
Preparation (lead heating)	95	---	
Initial denaturation	95	2 minutes	1
Denaturation	95	30 seconds	
Annealing	56	30 seconds	30
Extension[b]	72	20 seconds	
Final extension	72	5 minutes	1

a. Final volume 25μL for one reaction. Reaction-Mix were prepared as (n+10%) reactions to minimize pippeting error. Microtubes were submitted to a short spin at 4°C before start run.
b. 10 seconds per 100bp

4.2.3. Cloning and sequencing

Amplicons were cloned using a "p-GEM-t Easy" kit (Promega) and DH5α as competent cells. Transformed bacteria were spread onto LB agar plates and were incubated until they form individual colonies (37°C, 250 rpm, 18h). DNA was extracted from colonies according the "QIAprep Spin Miniprep Kit" protocol (Quiagen), and quantified (Nanodrop). To eliminate DNA without the insert, a portion of each colony DNA was analyzed by PCR, followed by agarose gel

electrophoresis. For each gene sequencing reactions were prepared with 50ng of plasmid DNA, 3.2pmol of M13pUC primer (forward or reverse) and nuclease free water in a total volume of 7.5µL. Sequencing was performed in a 48-capilar 3730 DNA Analyzer (Applied Biosystems) provided by PDTIS/FIOCRUZ. The sequenced DNA fragments were analyzed with Bioedit, WebTraceMiner, and NCBI Blastn software (BLASTN 2.2.26+).

4.2.4. Quantitative PCR and data analysis

Quantitative real time PCR (qPCR) reactions were performed according to the protocol of the "SYBR® Green PCR Master Mix Green" from the StepOnePlus™ Real-Time PCR System (Applied Biosystems) and respective software version 2.1. Amplification parameters are presented in table 4.3.

Each plate with 96 wells was designed with a standard curve created by serial dilution (1:1, 1:10, 1:100 and 1:1000). Samples were analyzed in triplicate. Gene expression was calculated in relation to expression levels in each control group and normalized against the most stable reference gene, using Rest-2009© software (Pfaffl 2001, Pfaffl et al. 2002).

Prior to data normalization, the control and treatment cycle threshold (Ct) values of reference genes were statistically compared using two approaches: i) exposure times as separate groups and ii) all exposure times as a single group. Kruskal-Wallis and one-way ANOVA were used to analyze data. The software STATISTICA 8.0 was used.

GeNorm, BestKeeper, and Normfinder algorithms were used to select the most stable gene or gene combinations to normalize the gene expression data.

Table 4.3. Conditions (temperature, time and number of cycles) at each stage of the qPCR amplification (According Applied Biosystems guidelines).

Stage	Temperature (°C)	Time	Cycles
Holding	95	10 minutes	1
Cycling	95	15 seconds	40
	56	20 seconds	
Melt curve	95	15 seconds	1
	60	1 minutes	
	95*	15 seconds	
Final (conservation)	4	∞	---

* With increments of 0.3°C

4.3. Results

4.3.1. Daphnia magna assay

A similar growth pattern was observed between organisms submitted to copper treatment and control (Fig. 4.1). No significant differences were found by comparing the 1st exopodite from 2nd antennae length, measured at the end of each instar both for control and 6 μgL^{-1} of copper.

4.3.2. Cloning and sequencing

The cloning process performed by DH5α competent cells was successful for all genes and the sequencing results were subjected to minor corrections justified by their respective chromatogram. With exception of IMP R, where no match was found with sequences in GeneBank database, all sequences showed a lower expect value (e-value) (Tab. 4.4). The higher e-value was found for EcR and Vtg, corresponding also to the shortest sequences. Alignment identity values ranged from 94% to 100% for *D. magna* and 84% to 100% for *D. pulex*. The lower *Daphnia* identity value was 69% for *D. cristata* NADH Subunit 2 sequence (ND2). *Daphnia*

ubiquitin (UbC) was compared with other crustacean sequences presenting the lowest identity value as no sequences were found in NCBI blast database (Table 4.4). The alignment of the fragment obtained with three crustacean different species is showed in Table 4.5. Most of the differences were of a single base and the larger mismatches found were four bases for *Eriocheir sinensis* and *Litopenaeus vannamei*.

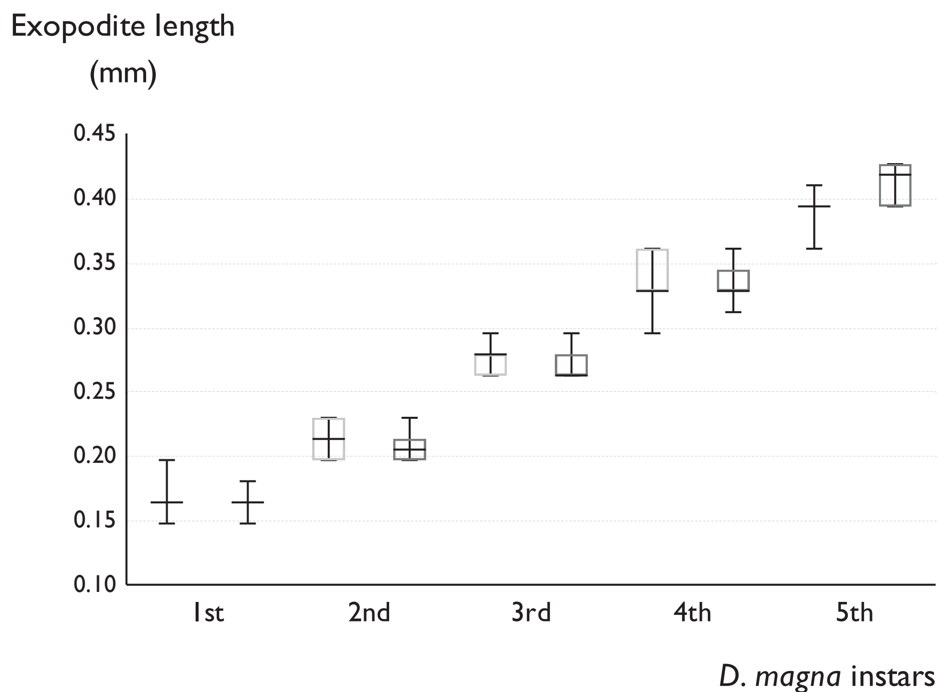

Figure 4.1. Box-and-whiskers plot of *D. magna* exopodite length registered at the end of each instar under control conditions (light boxes) and under exposure of 6 $\mu g L^{-1}$ of copper (dark boxes). A two-tailed t-test for independent samples was used and data normality was previously tested (1st instar n=40; other instars n=20).

Table 4.4. Evaluation of sequences by NCBI Blast (software BLASTN 2.2.26+), using as quality parameters maxima identity and e-value by comparison with sequences registered at nucleotides collection, using "crustaceans (taxid:6657)" as organism filter.

Blast/Gene	Max. Identity	e value	Identities	Accession n°	Organism
Act F	99%	$2e^{-30}$	71/72	AJ2952554.1	*Daphnia magna*
				AJ245732.1	*D. pulex*
Act R	100%	$5e^{-19}$	44/44	AJ2952554.1	*D. magna*
				AJ245732.1	*D. pulex*
GAPDH F	98%	$2e^{-36}$	88/90	AJ2952555.1	*D. magna*
	98%	$1e^{-17}$	52/53	JN994375.1	*D. parvula*
	90%	$1e^{-26}$	81/90	FJ668111.1	*D. obtusa*
	90%	$1e^{-26}$	81/90	EF077787.1	
	88%	$6e^{-24}$	79/90	FJ668132.1 to FJ668125.1	*D. pulex*
GAPDH R	100%	$8e^{-28}$	63/63	AJ2952555.1	*D. magna*
		$3e^{-22}$	53/53	FJ668049.1 to FJ668040.1	
UbC F	77%	$6e^{-11}$	66/86	HQ436509.1	*Eriocheir sinensis*
	77%	$3e^{-08}$	57/74	BT121173.1	*Lepeophtheirus salmonis*
	75%	$4e^{-07}$	58/77	DQ923723.1	*Litopenaeus vannamei*
UbC R	76%	$2e^{-10}$	65/85	HQ436509.1	*E. sinensis*
	77%	$3e^{-08}$	57/74	BT121173.1	*L. salmonis*
	75%	$1e^{-06}$	57/76	DQ923723.1	*L. vannamei*
Mt F	85%	$8e^{-29}$	99/117	EU307302.1	*D. pulex*
Mt R	86%	$3e^{-32}$	102/118	EU307302.1	*D. pulex*
Fer F	96%	$2e^{-37}$	97/101	AJ292556.1	*D. magna*
	84%	$3e^{-21}$	85/101	AJ245734.1	*D. pulex*
Fer R	89%	$1e^{-12}$	62/70	AJ292556.1	*D. magna*
	86%	$4e^{-06}$	43/50	AJ245734.1	*D. pulex*

Table 4.4. (Cont.) Evaluation of sequences by NCBI Blast (software BLASTN 2.2.26+), using as quality parameters maxima identity and e-value by comparison with sequences registered at nucleotides collection, using "crustaceans (taxid: 6657)" as organism filter.

Blast/Gene	Max Identity	e value	Identities	Accession nº	Organism
COI F	99%	$7e^{-62}$	127/128	EU702138.1	*D. magna*
				AY803078.1	
	98%	$3e^{-60}$	126/128	AY803076.1	
COI R	100%	$7e^{-51}$	105/105	EU702138.1	*D. magna*
				AY803078.1	
	99%	$3e^{-49}$	104/105	GQ457333.1	
IMP F [a]	99%	$3e^{-79}$	153/154	DV437806.1	*D. magna*
IMP R [b]	---	---	---	---	---
Vtg F				AB252738.1	
	94%	$5e^{-07}$	32/34	AB252737.1	*D. magna*
				AB114859.1	
	88%	$2e^{-04}$	30/34	EF077809.1	*D. pulex*
				EF077794.1	
Vtg R	97%	$1e^{-06}$	30/31	AB252738.1	*D. magna*
				AB252737.1	
				AB114859.1	
ND2 F	99%	$7e^{-56}$	121/122	DQ132627.1	*D. magna*
	69%	0.14	87/127	DQ132613.1	*D. cristata*
ND2 R	99%	$7e^{-56}$	121/122	DQ132627.1	*D. magna*
	69%	0.14	87/127	DQ132613.1	*D. cristata*
LDH F	100%	$1e^{-16}$	43/43	FJ668069.1 to FJ668060.1	*D. magna*
LDH R	98%	$3e^{-56}$	117/119	FJ668069.1	*D. magna*
	97%	$4e^{-55}$	116/119	FJ668068.1	
EcR F	100%	$2e^{-3}$	20/20	EF363705.1; AB274824.1 to AB274820.1	*D. magna*
EcR R	100%	$4e^{-4}$	21/21	EF363705.1; AB274824.1 to AB274820.1	*D. magna*

[a] The accession number corresponds to the sequence used to prime design (Poynton et al. 2007).
[b] No match was found.

Table 4.5. Alignment of *D. magna* nucleotide sequenced amplicon for UbC gene amongst most similar organisms in NCBI Genbank database (*Eriocheir sinensis*, *Lepeophtheirus salmonis*, *Litopenaeus vannamei*, respectively). Identities are shaded in grey.

Organism	Start	Sequence	End
D. magna	1	TCTCCGGAACCAAAGGATCATCCGGGTTTGGGTCGCATAGCAG	43
E. sinensis	430	TCTCTGGCACCAAGGGATCCTCGGGATTGGGGTCCGTGAGGAG	387
(Decapoda)	44	AGAACAAATTGACAGCAGCACTTTAGAAATGGTGAGTGCAGG	85
	386	GGAACAGATAGACAGGAGCACTTTAGAAATAGTTAAGGCAGG	346
D. magna	15	GGATCATCCGGGTTTGGGTCGCATAGCAGAGAACAAATTGACA	57
L. salmonis	475	GGGTCATCTGGGTTTGGGTCACATAGCAAAGAACATATGGACA	433
(Copepoda)	58	GCAGCACTTTAGAAATGGTGAGTGCAGGTGA	88
	432	ATAAAACCTTCGAAACTGTAAGTGCTGGGGA	402
D. magna	1	TCTCCGGAACCAAAGGATCATCCGGGTTTGGGTCGCATAGCAG	43
L. vannamei	367	TCTCTGGCACCAATGGGTCATCTGGATTTGGATCCGTGAGGAG	325
(Decapoda)	44	AGAACAAATTGACAGCAGCACTTTAGAAATGGT	76
	324	TGAACAAATTGAGAGGAGAACTTTGGATATTGT	292

The comparison of reference genes Ct values showed a significant difference among treatments, when all exposure times where considered together (Act: $F(8, 50)=32.933$ $p<0.000$; UbC: $F(8, 50)=19.956$ $p<0.000$; GAPDH: $F(8, 50)=9.865$ $p<0.000$). The analysis with separate exposure times just GAPDH at 168 hours showed significant differences between control and copper treatment ($H(1, 10)= 4.8109$ $p=0.028$). Concerning normalization steps, NormFinder was removed from data analysis due to its requirement of the number of samples. GeNorm selected UbC as the most stable gene for almost all the time groups (Table 4.6). When the M value was higher than 0.5, a second analysis was performed without the less stable gene. As the same value of M was obtained, the gene with initially lower M value was selected for normalization. At 48 and 72 hours the analysis presented an M value higher than 0.5 even after the remove of the less stable gene. BestKeeper selected GAPDH as the most stable gene for 6 to 72 and 120 hours, Act to 96 and 168 hours, and UbC to 144 hours.

Table 4.6. Values of geometric mean (GeoMean) and the gene-stability value (M) determined as the average pairwise variation between one gene and remaining control genes calculated by geNorm algorithm at each time for reference genes. The lower value of M correspond to the most steady gene (highlighted in bold) used for normalization of genes of interest expression.

	Act		**GAPDH**		**UBC**	
Time (h)	GeoMean	M value	GeoMean	M value	GeoMean	M
6	10.947	**0.216**	3.602	0.398	5.206	0.251
12	1.383	0.433	0.953	0.519	1.968	**0.317**
24	2.136	0.556	1.215	0.302	3.034	**0.286**
48*	2.512	1.235	1.287	1.079 0.615	6.090	**0.771** **0.615**
72*	0.914	1.556	0.804	**1.188** **1.012**	5.281	1.380 1.012
96	1.774	0.237	1.954	0.307	5.543	**0.221**
120	1.452	0.710	1.914	0.557	3.817	**0.430**
144	0.782	0.379	1.376	**0.296**	3.166	0.477
168	5.759	0.245	0.604	0.183	1.263	**0.169**

* When the value of M was higher than 0.5 was performed a second analysis without the less stable gene. In these cases, since was resulted the same value of M was chosen for normalization the gene with initially lower M value.

4.3.3. Gene expression variation through Daphnia magna growth

Figure 4.2 shows the data distribution of the gene expression values determined for genes of interest in each observation time. A narrow and symmetric data distribution until 48 hours was obtained for all genes with exception of ND2 that presented a wide distribution particularly when compared with 6, 24 and 48h where gene expression values were close to zero. At 72 hours a tendency to higher heterogeneity was observed with the exception of Vtg and EcR. At 168 hours Mt and Fer exhibit a notable decrease of gene expression.

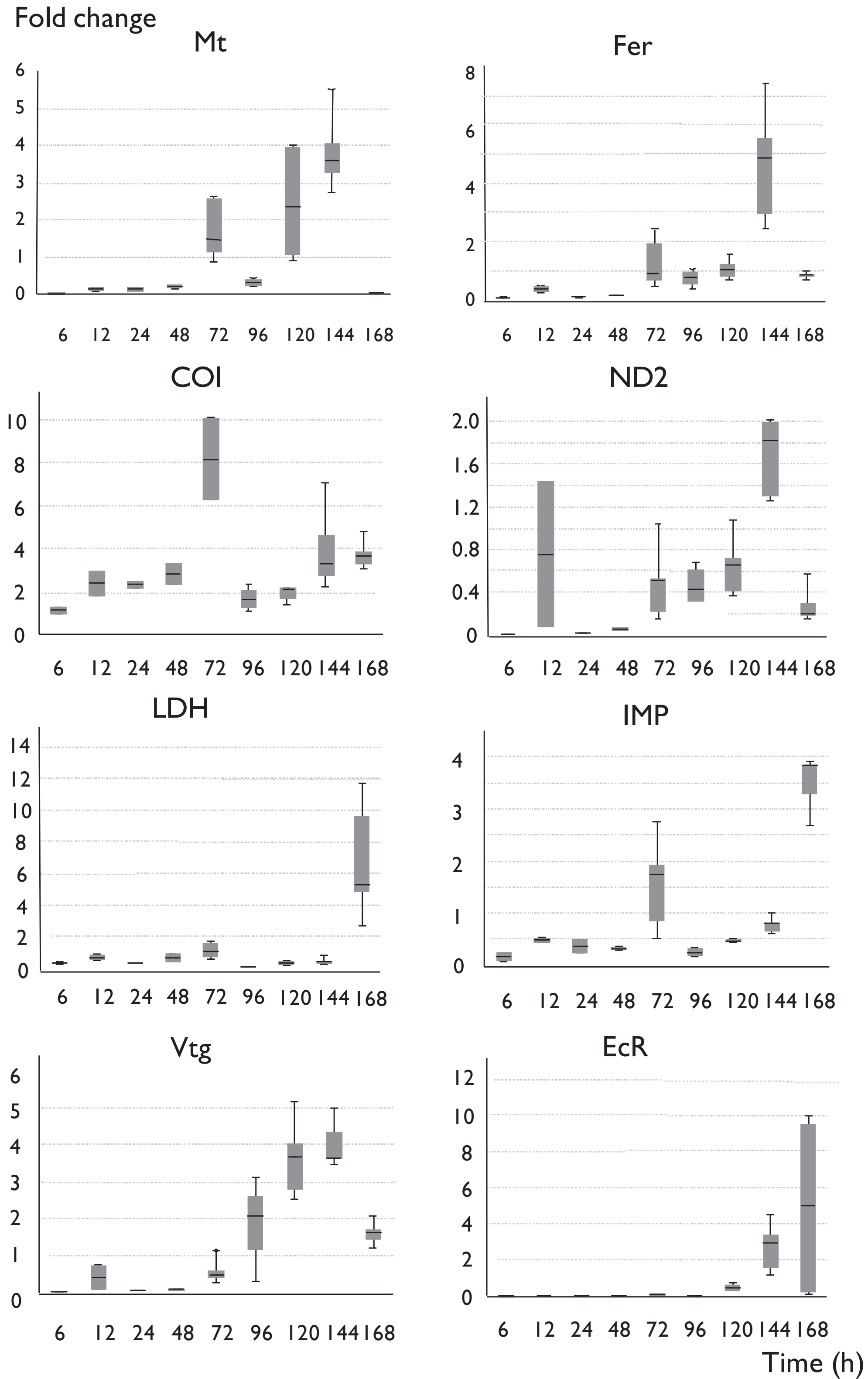

Figure 4.2. Box-and-whiskers plot of the target genes expression (Mt, Fer, COI, ND2, LDH, IMP, Vtg, and EcR) normalized with the most steady reference gene, selected by geNorm algorithm, during *D. magna* growth under control conditions. Outliers are represented by black dots.

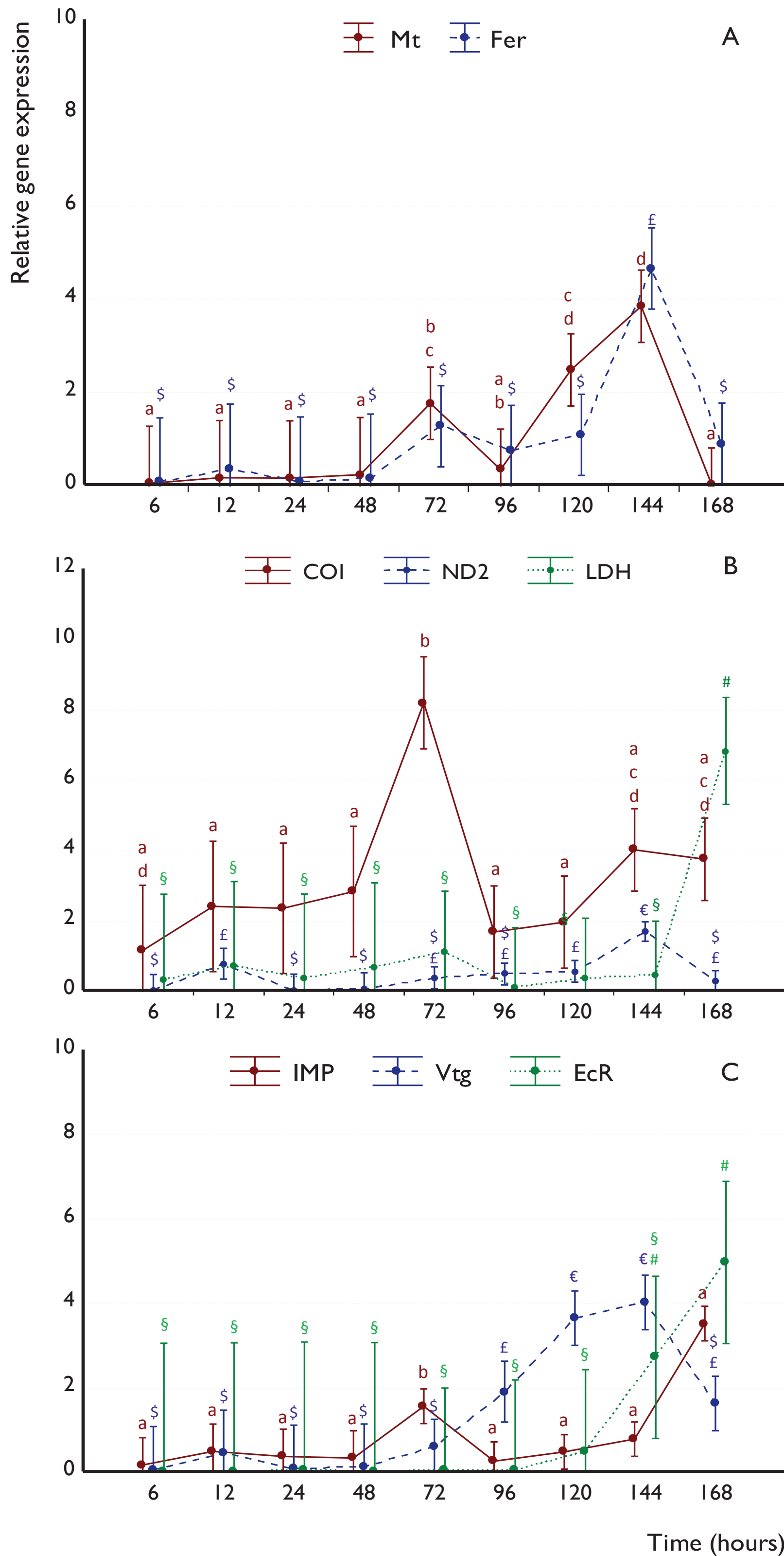

Figure 4.3. Mean variation of *D. magna* gene expression among genes from the same metabolic pathway (A – Metal transport; B – Glicolysis and electron transport; C – Cell singaling). Bars correspond to 95% confidence intervals. For each time, the data were shifted for better visualization. Diferent letres and symbols indicate significant differences ($p<0.05$) within groups for each gene.

The results of gene expression quantification are presented in figure 4.3. A similarity in the profiles among genes belonging to the same metabolic pathway is perceptiple. With the exception of LDH, IMP, and EcR, gene expression decreased at 168 hours, when the eggs from the 1st brood were already in the brood pouch.

One-way ANOVA, followed by the LSD (least significant difference) multi-comparison analysis, revealed significant differences among time groups, however, large confidence limits were registered (fig. 4.3). EcR showed the largest confidence limits followed by COI and LDH.

4.3.4. Gene expression variation of Daphnia magna exposed to copper through growth stages

Data of gene expression were determined using Rest© software. Up and down-regulated genes and respective p values are presented in Table 4.7. The number of interactions run for each gene with Rest algorithm (10000 and 2000)) produced slightly different results.

In the group of metal transporters, Fer was significantly down-regulated by copper at 12 hours and Mt was up-regulated at 96 hours (Fig. 4.4). Regarding the genes of glycolysis and electron transport pathway, significant copper effects were registered only during the early instars. COI was down-regulated at 12 hours, ND2 was up-regulated at 24 and 48 hours, and LDH was down-regulated at 48 hours (Fig. 4.5). Despite the low value of LDH expression registered at 12 hours, no significant down-regulated expression was obtained by Rest analysis (Expression = 0.044; 95% CI = [0.014 - 0.197]; p value = 0.324). The cell signaling group exhibited the more significant effects (Fig. 4.6), mainly for IMP, that was up-regulated at 6, 24,

48, 72 and 168 hours and down-regulated at 120 hours. The highest fold-change was registered at 12 hours but the large spread of 95% confidence limits (5.15 to 62.93) did not allowed the validation of copper effect on gene expression. Vtg and EcR were down-regulated at 48 and 72 hours, and 6 hours of exposure, respectively.

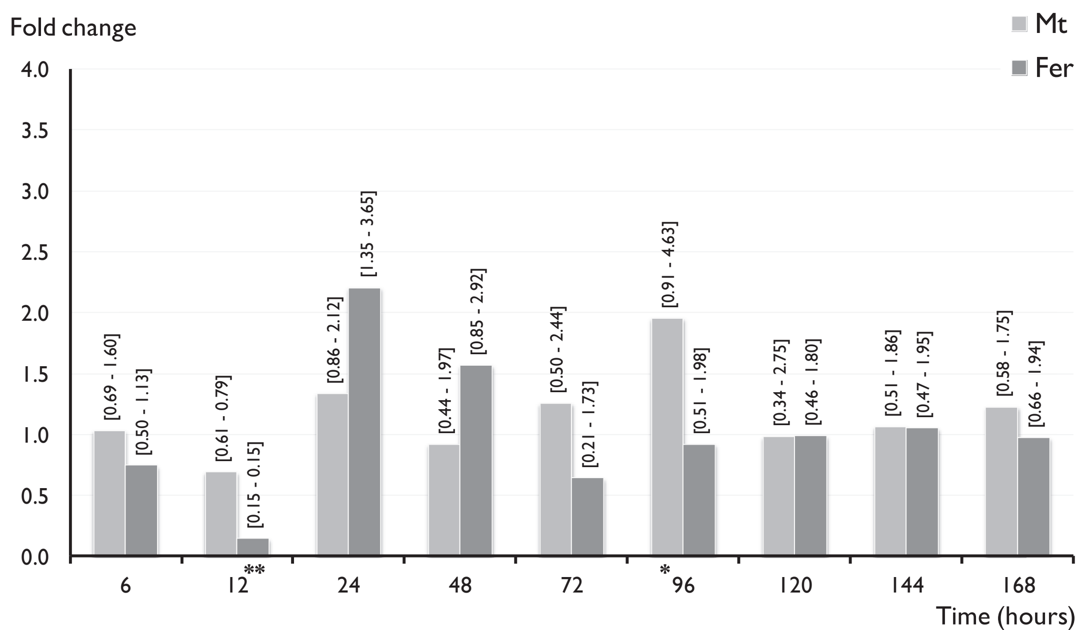

Figure 4.4. Relative gene expression of metallotionein (Mt) and ferritin (Fer) along *D. magna* growth until 1st adult instar, exposed to 6μgL^{-1} of copper. Fer was down-regulated at 12 hours (p=0.000) and Mt was up-regulated at 96 hours (p=0.044). Mt and Fer exhibit a similar trend of gene expression during the 7days period. 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* up-regultation and ** down-regulation).

Table 4.7. Differentially expressed gene, after exposure to 6 µgL[-1] of copper, determined with Rest© sotware. Data normalized with reference genes selected by GeNorm[a] (method 1), Rest[b] (method 2) and BestKeeper[c] (method 3).

		Mt		Fer		COI		ND2		LDH		IMP		Vtg		EcR	
	Hours	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Method 1	6									0.000	0.000	0.000	0.000			0.000	0.000
	12	0.000	0.000	0.000	0.000	0.000	0.000							0.000	0.000		
	24			0.000	0.000			0.000	0.000	0.000	0.000	0.000	0.000				
	48			0.000	0.000			0.000	0.000					0.000	0.000		
	72											0.049		0.017	0.024		
	96	0.018	0.012														
	120																
	144																
	168											0.004	0.002				
Method 2	6											0.000	0.000			0.000	0.000
	12			0.000	0.000	0.000	0.000										
	24				0.000			0.000	0.000	0.000		0.000	0.000				
	48							0.000	0.000		0.000	0.000	0.000	0.000	0.037		
	72											0.012	0.007	0.042	0.037		
	96		0.048														
	120											0.020	0.017				
	144																
	168											0.002	0.004				
Method 3	6											0.000	0.000			0.000	0.000
	12			0.000	0.000	0.000	0.000										
	24				0.000			0.000	0.000			0.000	0.000				
	48							0.000	0.000	0.000	0.000	0.000	0.000				
	72											0.000	0.000	0.044	0.043		
	96	0.044	0.039														
	120											0.017	0.020				
	144																
	168											0.005	0.005				

Values correspond to p values (Rest analysis). The subdivision of each column refers to the number of interactions run with Rest algorithm (A=10000 and B=2000).
Light grey cells: up-regulated genes
Dark grey cells: down-regulated genes
[a] GeNorm most stable gene: 6, 24, 48, 96, 120, 168=UbC; 12h=Act; 72 and 144h=GAPDH
[b] Three reference genes without a previous analysis were used by Rest
[c] BestKeeper index

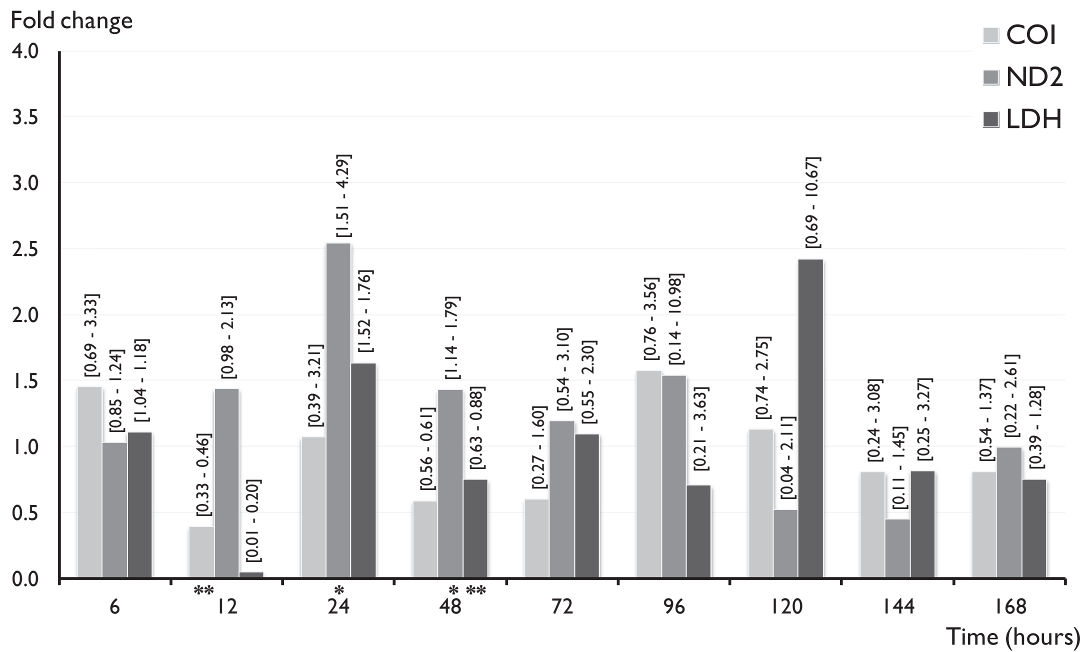

Figure 4.5. Gene expression of cythocrome c oxidase (COI), nicotinamide adenine dinucleotide (ND2) and lactate dehydrogenase (LDH) along *D. magna* growth until 1st adult instar, exposed to 6µgL^{-1} of copper. COI was down-regulated at 12 hours, ND2 was up-regulated at 24 and 48 hours, and LDH was up-regulated at 24 hours ($p < 0.000$). 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* up-regultation and ** down-regulation).

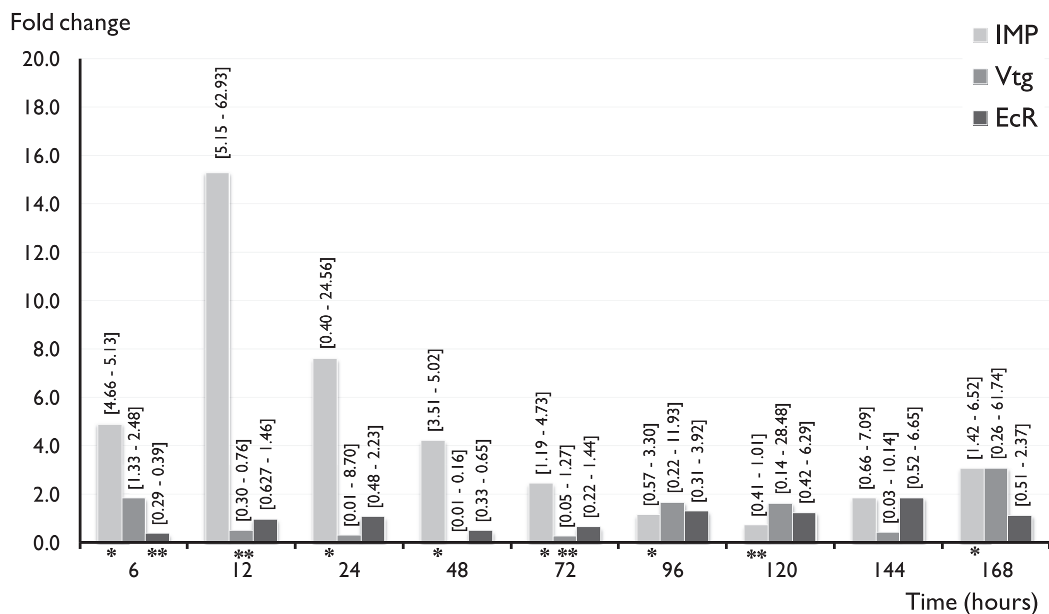

Figure 4.6. Gene expression of Inositol monophosphatase (IMP), Vitellogenin (Vtg) and Ecdysone receptor (EcR) along *D. magna* growth until 1st adult instar, exposed to 6µgL^{-1} of copper. IMP was up-regulated at 6, 24, 48, 72 ($p < 0.000$), 168 hours ($p = 0.012$) and down-regulated at 120 hours ($p = 0.02$). Vtg was down-regulated at 48 and 72 hours ($p < 0.000$ and $p = 0.042$, respectively). EcR was down-regulated at 6 hours ($p < 0.000$). 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* up-regultation and ** down-regulation).

4.4. Discussion

In this study the gene expression profiles of *D. magna* lyfe-cicle stages were identified and quantified. Actin (Act), Glyceraldehyde-3P Dehydrogenase (GAPDH) and Ubiquitin Conjugating Enzyme (UbC) were selected as reference genes due to their general use in *Daphnia* studies (Heckmann et al. 2006, Martin-Creuzburg et al. 2007, Schwarzenberger et al. 2009, Spanier et al. 2010). Furthermore, in the present study a whole organism, at different development stages, with higher variability then a single tissue or organ, was used as RNA source. Thus the use of a reduced number of reference genes would be unsuitable since that only reference genes that have been previously validated for the same organism, tissue and treatment should be considered (Heckmann et al. 2006). The experimental design, involving different developmental stages, presented some constrains for normalization due to the reduced number of samples at 6, 12 and 24 hours. Some samples were discarded due to poor quality of extracted RNA. Therefore, two hypotesis were evaluated to select the best normalization method: i) data for each time were analyzed separately; and ii) data were analyzed simultaneously as expression of reference genes, by definition, would not change significantly over the *D. magna* lifetime. This second hypothesis was rejected after statistical analysis (one-way ANOVA) due to a significant variation among times in each of the three reference genes, mainly detected at 72 and 168 hours. The use of a higher number of reference genes could provide a stronger analysis, since the M values, determined with geNorm, were higher than 0.5 at 48, 72 and 120 hours.

The low levels of Mt and Fer gene expression registered during the early stages (until 48h) were expected due to the specific function of the proteins involved. Metallothioneins are metal-binding proteins, with low molecular weights

and high cysteine content, considered as cell metal regulators by storing or sequestering (Amiard et al. 2006) and have been found in tissues/organs of uncontaminated organisms (Bodar et al. 1990, Roesijadi 1992, Amiard et al. 2006) and are related with other physiological processes such as molting cycle in crustaceans (Pedersen et al. 1997). This can be a constraint to their use as metal exposure biomarkers, requiring for a better characterization, regarding the selection of the organism, organ and/or method of analysis (Amiard et al. 2006, Rebelo et al. 2003). As iron storage protein, Fer acts as an iron regulator during hemoglobin synthesis. In optimal conditions of oxygen and diet, *D. magna* have an increase of hemoglobin synthesis when it reaches sexual maturation with functional ovaries (Smaridge 1956, Green 1956, Dave 1984), approximately 72 hours after the birth (Zaffagnini and Zeni 1986). The obtained results pointed up this tendency since the gene expression of Fer increased at 72 hours (Fig. 4.2), however the wide 95% confidence limits not allowed get stronger conclusions (Fig. 4.3.A). The variation could be due to random heterogeneity in samples, although they were clone organisms rose under controlled conditions, not all organisms were absolultly synchronized. This was a recurrent problem during data analysis, but no replicates were removed from analysis. The higher gene expression values of Mt and Fer were registered at 144 hours, followed by a decrease at 168 hours, corresponding to the period where eggs of the first brood are released to brood chamber, and start their embryonic development with their own gene schedule.

Regarding the second metabolic pathway evaluated a stronger expression of COI and ND2 was expected, due to their essential function in obtaining the energy for life support. The 72 hours peak presented by COI may be related with egg production (Zaffanini 1986). However, the low level of expression is not an atypical

situation taking into account that *D. magna* is able to use lipids as a major source of energy rather than glycogen (Elendt 1989, De Coen and Janssen 2003). Lactate dehydrogenase showed a constant gene expression until 144h, with a notable increase at 168 hours. This protein catalyzes the piruvate to lactate reaction (Vassault 1983) to provide a fast source of energy. The higher level of gene expression could be related with the females' physiological conditions, raising the old debate about the effect of carrying eggs (or embryos) in female metabolic costs. According to some studies (Schindler 1968, Hayward and Gallup 1976, Lynch et al. 1986), *D. magna* brooding females had higher assimilation/feeding/respiration rates than non-ovigerous females. On the other hand, Glazier et al. (1991) with a more specific methodology (removing eggs/embryos from a group of females) found that carrying a brood have negligible costs. Since eggs are provided with lipids but also have a glycogen content, the gene expression of LDH could be biased by the embryonic development. To found an accurate response to this issue a gene expression analysis should be performed removing the eggs before RNA extraction.

A higher value of IMP gene expression was expected nearby the molt periods (24, 48, 96, and 144 hours) since this enzyme is responsible for the activation of inositol, known as a growth factor (Fallahi et al. 2011). The highest values were found at 72 and 168 hours suggesting that in *D. magna* inositol may have a active role not related to growth but with lipid metabolism (Downes and Macphee 1990). Ecdysone receptor showed a constant profile, with low values of gene expression, before 120 hours including the molt periods (approximately at 24, 48 and 96 hours). Since ecdysone plays an important role in arthropods growth and molting high values of gene expression were expected (Krishnakumaran and Schneiderman 1970, Kiguchi and Agui 1981, Nakagawa and Henrich 2009). Nontheless, the increased

value of gene expression observed at 120 hours is probably related with other functions of ecdysone reported for crustaceans and linked to the reproduction process such as the production of male progeny in dafnids and embryo development (Kato et al. 2007, Hirano et al. 2008). The large 95% confidence limits were registered at 168 hours, what should be connected with the beginning of embryogenesis, but could also be related with the high difficulty to normalize data during embryogenesis due to the lack of stable genes (Kato et al. 2007). Another point to considerer is the presence of three EcR isoforms in *Daphnia* (two subtypes similar to *Drosophila* EcR-A and a subtype similar to EcR-B) with temporal changes in expression (Kato et al. 2007). Vtg presented an expected profile, with increasing values until 72 hours. The precursor of yolk protein is synthesized on fat cells, spread along *Daphnia* digestive tract (Zaffagnini and Zeni 1986, Bodar et al. 1990) and, then, is transferred to the oocytes through hemolymph (De Schamphelaere et al. 2004). Given that *Daphnia* has a cyclic reproduction with a continous synthesis of Vtg, the values observed at 168 hours should be higher, or similar to those registered at 140 hours, but, once again, the variability could be due to the embryonic development. During embryogenesis the yolk is consumed and embryos do not synthesize vitellogenin, consequently the low value of gene expression registered at 168 hours is a spontaneous effect due to embryos body mass contribution.

Another objective of this study was to evaluate the expression of the abovementioned genes during *D. magna* growth exposed to copper. The body length results confirmed that the copper concentration used was below detectable sublethal effects, as no significant differences were detected in any of the growth stages between control and treatment.

The relative quantification, among control and copper treatment, was done using REST© software according the model proposed by Pfaffl et al. (2002). Considering the final results, some differences were found among the normalization method. For instance, metal transport and energy metabolism groups showed more significant results with geNorm rather than Bestkeeper choices to "most stable gene. In general, data obtained revealed fluctuations between up and down-regulated genes among *D. magna* developmental stages. An improvement in experimental design, e.g. using a larger number of synchronized organisms per sample in order to obtain higher initial concentrations of total RNA, could elucidate if EcR gene actually have a low and constant expression unaffected by copper exposure, which would reinforce its role in ecdysis processes. Also, the existence of eggs in the brood pouch, may affect results significantly. This is supported by Soetaert et al. (2006) experiments. A microarray library was constructed with pooled adult females and juveniles and the most represented functional class was related to embryonic development (26%), followed by "various cellular processes" and "energy metabolism" (23 and 15% respectively). This also implies that in other studies of gene expression using adult females the influence of active genes at embryonic development could have biased the final results (Poynton et al. 2007, Vandegehuchte et al. 2010, David et al. 2011), even though the methods of normalization could reduce this effect.

Despite the influence of eggs in gene expression, and even if the effect in the first adult instar was in agreement with the early stages and also with an unexpected significant down-regulation at 120 hours (when normalize with BestKeeper), IMP was the gene more significantly affected by copper exposure. This result is in agreement with a recent work were inositol (or myo-inositol) was described as a

responsible for protective effects against copper exposure, when supplied to juveniles of *Cyprinus carpio* (Jiang et al. 2011).

4.5. Conclusions

In this study, it has been shown that the gene expression profile of *D. magna* is not constant along its growth and early amitic reproduction stages. The occurrence of a basal variation in gene expression between individuals, even cloned and synchronized organisms, may affect the profile. This is particularly important for further works with organisms from natural populations so a higher number of replicates should be considered for a more robust analysis.

Daphnids have life stages that are easily recognized by size, ecdysis, and by eggs/embryos in the brood pouch that could be used as key-points for genetic variability analysis. The ecdysis events were not detected by the gene expression pattern of EcR revealing that this isoform does not have an important role in *D. magna* growth. The beginning of ovaries maturity and eggs provisioning is an important phase, were gene expression was higher for the majority of the profiled genes. Eggs or embryos in brood pouch should be removed before RNA extraction to obtain a basal gene profile without the influence of embryonic development. In addition, genes related with lipid metabolism should be included and explored to better understand their role in *D. magna* energetic profile.

Although this analysis need further validation to achieve more powerful results, a relationship between copper exposure and changes in gene expression was established, mainly in the cell signaling pathway, that could be responsible for growth and reproduction stress responses. A similar study with embryonic

developmental stages of *D. magna* could be useful to explore the early effects of metal at the population reproductive level.

Chapter V

General Conclusions

5.1. Final remarks

This study looked at the patterns of variability and how the mechanisms of genetic determination of tolerance work, departing from a long-term research on acid mine drainage effects in planktonic invertebrates.

The existence of well defined categories of tolerance in a *C. numidicus* reference population was in accordance to *D. longispina* previous results achieved with (Lopes et al. 2004a, Martins et al. 2009). No genetic differences associated with copper tolerance were found by the band pattern obtained by PCR-RFLP, but the absence of results highlighted the need of more research on gene sequencing of ecological relevant *taxa*.

Results revealed that the AMD effluent at the São Domingos mine had mutagenic and genotoxic properties, even when very diluted to low percentages (1 and 0.1 %). Data gathered in the present study revealed the AMD effluent of the abandoned São Domingos mine to be mutagenic and genotoxic, even after diluting it to 1 and 0.1 %. Although transferability of these laboratory results to the complex field scenario is not straightforward, dilutions within this range were previously found to be below the lethality threshold and to elicit sublethal effects on reproduction of locally collected *D. longispina* clonal lineages (Lopes et al. 2005b, Martins et al. 2007, Saro et al. 2012). Therefore, when using neutral markers, as Martins et al. (2009) and Silva et al. (2010) did, a possible overestimation and/or increase in genetic diversity due to mutations cannot be ruled out.

Several genes, with functional significance, were analyzed to learn about their differential expression along different stages of planktonic invertebrates life-cycle. The gene expression of the cladoceran *D. magna* growth stages, including the first

reproductive stage, was profiled under normal and copper stressed conditions. The first assay was performed under standard conditions of a laboratorial culture of *D. magna,* to establish basal gene expression and, then, a second assay was performed under copper exposure. The gene expression, after normalizations, presented wide confidence intervals and, therefore, conclusions should be taken with care. Despite of some of this variation could be reduced by increasing the number of replicates, mainly on the initial periods, the variation will be a regular occurrence at gene expression analysis because it reflects physiologically plasticity. Therefore, it is required to define the baseline level of gene expression for successive physiological (De Boer et al. 2011). Daphnids have life stages that are easily recognized by body size, ecdysis, and the presence of eggs or embryos in the brood pouch, which are usually used as endpoints in classical ecotoxicology assays. Here, the gene expression profile was analyzed relating their respective physiological processes. The fact that ecdysis events had not been detected on the EcR expression pattern revealed that the isolated EcR isoform should have a more important role in embryonic development than in juvenil growth. Most of the analyzed genes had and an increased expression at 72 and 144 hours, corresponding to the egg provisioning to the first and second brood, meaning that the ovaries maturity and the beginning of eggs provisioning are associated to a peak of several genes regulation. The sharp decrease at 168 hours revealed the importance of the embryonic development. Therefore, in future studies, the eggs or embryos inside the brood pouch should be removed before RNA extraction. The profiles of COI, ND2 and LDH showed lower levels of expression than expected for genes related with the cell energetic balance. However, since *D. magna* may use lipids rather than glycogen (Elendt 1989, De Coen and Janssen 2003), those values were not entirely

unexpected. In future studies, genes related with lipid metabolism should be included to explore and comprehend they role in *D. magna* "energetics" profile.

After copper exposures, gene expression data was analyzed with two different methods of normalization: geNorm and BestKeeper; some differences were found between them. GeNorm detected more significant results in the metal transport and energy metabolism groups than Bestkeeper, but, for cell signaling group, BestKeeper was more efficient. Those differences underline the need to invest in the development of strong normalization methods that can be applied in studies with more complex designs. The transcript more affected by copper exposure was IMP (up-regulated at 6, 24, 48, 72, and 168 hours and down-regulated at 120 hours). Once growth was not affected, the IMP up-regulated expression might be related with the inositol protective action, as revealed recently by Jiang et al. (2011) what recognized *myo*-inositol protective action against copper-induced oxidative damage in *Cyprinus carpio* juveniles. Regardless of the variability of the some results and the need of more accurate levels of confidence, this work was useful for the construction of a knowledge base for future environmental genomics research.

5.2. Future work

The growing concern about the effect of contaminants on the genetics of natural populations has been subject a large number of studies. How individuals respond to stress and how the responses affect populations' viability is an important basis of knowledge for the global economy due to the chemical support of our society (medical drugs, agrochemicals, food preservatives, and general industry). It is expected that genomics methodologies, that contribute to characterize chemicals mode of action, could be helpful for setting limits to chemical emissions, used in risk

assessment and risk management (Snape et al. 2004). In 2002, van Straalen and Timmermans after a review on the "genetic erosion hypothesis" alleged that ecotoxicogenomics had not yet a solid base of knowledge to be incorporated into decisions of risk assessment. In 2011, van Straalen and Feder did a re-evaluation of the situation concluding that gene expression is able to be used for risk assessment purposes if certain conditions are met. Those conditions include a previous database of gene expression profiles generated in organisms under standard conditions, to have a solid knowledge about how genes work in optimal physiological conditions. Then, to get the gene expression profiles generated under the same stress conditions than classical ecotoxicology tests (species, age, gender, contaminant, concentrations, exposure-time), to connect gene expression profiles, ecophysiological endpoints and chemicals mode of action. So, as future work is intend to continue this approach of gene expression profiles in two ways:

i) First, in the continuity of the work here presented to seek answers for some questions that were unclear; like the role of EcR in daphnids growth, by evaluate the gene expression profile of the three existent isoforms. According to Kato et al. (2007) EcR-B is the isoform with an expression pattern more closely related to molting. Daphnids growth is a complex process with a periodically disposal of their old exoskeleton and the formation of a new one, involving ecdysteroids. Reproduction is also cyclic and the eclosion of a new brood is closely followed by ecdysis. Ecdysteroid hormones seem to be involved in growth and reproduction (Subramoniam 2000, LeBlanc 2007) and also affecting male production (Kato et al. 2007). These are three important endpoints in ecotoxicological studies that can be transferd to functional genomics studies.

ii) The other point is a broader vision and long-term work: enlarge the contribution of ecotoxicogenomics to risk assessment. The idea of gene expression analysis to be used as a biomarker, is not new, but still is not considered appropriate, because it is stronger to recognize exposure than to identify the effects or the degree of effect (van Straalen and Feder 2011). The biggest challenge is to establish a clear link between the contaminant, the biochemical effects in which it intervenes, and the regulation process of the cell, considering the levels of exposure (contaminant concentration and time-exposure) and physiologic stages, towards to risk assessment for complex mixtures of chemicals as AMD into field populations with transcriptome studies.

Chapter VI

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