Universidade de Aveiro Departamento Biologia
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Alterações climáticas: efeito de salinização secundária em organismos dulçaquícolas

Climate changes: effects of secondary salinisation in freshwater organisms

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, professor Catedrático do Departamento de Biologia da Universidade de Aveiro e da Doutora Isabel Lopes, Investigadora Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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resumo À medida que os padrões climáticos se alteram também a disponibilidade de água doce se irá alterar. Como tal, a salinização de ecossistemas costeiros, principalmente os dulçaquicolas, torna-se um ponto de preocupação fundamental. Quer devido ao aumento de intrusões de água do mar por inundação ou por intrusões salinas através dos lençóis freáticos, quer devido à diminuição de entrada de água doce, provocada por períodos mais prolongados de seca, evaporação e aumento do uso de água para actividades antropogénicas.

De acordo com o exposto, o presente trabalho pretendeu avaliar as respostas de organismos dulçaquicolas a alterações provocadas pelo aumento de salinidade. Para atingir este objectivo principal foram delineados dois objectivos específicos: (i) comparar a toxicidade de água do mar com a do sal NaCl, comummente usado em laboratório como substituto de água do mar e (ii) averiguar uma possível correlação entre a resistência a contaminação química (cobre) e a aumento de salinidade; uma vez que muitas das populações que se prevê virem a ser afectadas por salinização estão, presentemente, já expostas a contaminação química. Para abordar o primeiro objectivo, a alga verde Pseudokirchneriella subcapitata (Korshikov) F. Hindák e o cladócero Daphnia magna Straus foram expostos a dois gradientes crescentes de salinidade estabelecidos com água do mar natural e com NaCl dissolvido num meio artificial.. No ensaio com a alga verde unicelular foi avaliada a inibição do crescimento; no ensaio com D. magna foram avaliados os seguintes parâmetros: mortalidade, tempo decorrido até libertar a primeira ninhada, comprimento corporal, reprodução total, taxa de crescimento intrínseco.

Para atingir o segundo objectivo, foram seleccionadas quatro linhagens do cladócero Daphnia longispina O.F Müller com sensibilidades diferentes a níveis letais de cobre. As quatro linhagens foram expostas a um gradiente de concentrações, letais e sub-letais, de NaCl. Neste ensaio foram analisados os mesmo parâmetros descritos anteriormente para o ensaio com D. magna.

Os resultados demonstram que o sal NaCl apresentou maior toxicidade do que a água do mar natural, quer para P. subcapitata (LOEC de 5.9mS/cm e de 9.6mS/cm, respectivamente para NaCl e água do mar), quer para D. magna (LC50,48h de 9.88mS/cm e LC50,48h= 11.32mS/cm; e EC50, para reprodução total, de 8.9mS/cm e 10.4mS/cm, respectivamente para NaCl e água do mar). Estes dados sugerem que o uso de NaCl, em laboratório, como um substituto de água do mar deve ser considerado como uma abordagem protectora, uma vez que simula um cenário de maior toxicidade. Não foi observada uma associação significativa entre maior resistência a cobre e a NaCl nas linhagens de D. longispina testadas ($r < 0.92$ and $p \geq 0.08$), apesar de as duas linhagens mais resistentes a cobre apresentarem as maiores sensibilidades a níveis subletais (para reprodução total) de NaCl. Finalmente, os dados obtidos demonstram que D. longispina é mais sensível ao aumento de salinidade (o intervalo de valores de LC50,48h calculados foi de 2.85g/l a 2.48g/l de NaCl, correspondente a valores de conductividade de 5.50mS/cm e 4.57mS/cm, respectivamente) que a espécie padrão (D. magna), salientando a importância do uso de espécies autóctones na avaliação de risco ecológico em situações de intrusões salinas.

keywords Climate changes; increased salinity; NaCl; Daphnia longispina; Daphnia magna; co/multiple-tolerance; cladoceran; plankton; Pseudokirchneriella subcapitata

abstract As global climate patterns change, so will freshwater availability. Specially, salinisation of freshwater costal ecosystem is a major point of concern; either by surface flooding or by groundwater intrusions of seawater. This may be potentiated by the decrease of freshwater availability provoked by longer drought periods, evaporation, and increased freshwater extraction (for example for agriculture and other human uses).

According, the present work aimed at evaluating how freshwater organisms responded to an increase in salinity. To achieve this main objective two specific goals were delineated: (i) to compare the toxicity of seawater with a surrogate (NaCl), commonly used in laboratory toxicity assays, to two standard freshwater species, and (ii) to assess if an association exist between resistance to chemical contamination and to increased salinity; since many populations, predicted to experience future increased salinity, are presently exposed to chemical contamination. To accomplish the first objective the sensitivity of the green algae Pseudokirchneriella subcapitata (Korshikov) F. Hindák and of the cladoceran Daphnia magna Straus to NaCl and to natural seawater was evaluated. Growth rate for P. subcapitata, and mortality, time to release the first brood, body size, total reproduction, and intrinsic rate of natural increase for D. magna, were monitored after exposing these species to two series of solutions with an increasing gradient of salinity. One series of solutions was established with a natural seawater sample and the other with NaCl dissolved artificial media. To address the second objective, four cloned lineages of Daphnia longispina O.F. Müller, exhibiting different sensitivities to lethal levels of copper, were exposed to a gradient of lethal and sublethal levels of salinity, established with the salt NaCl. The same endpoins described for D. magna were also monitored for D. longispina.

The obtained results showed that NaCl exerted a higher toxicity to P. subcapitata (LOEC of 5.9mS/cm and 9.6mS/cm, respectively for NaCl and seawater) and to D. magna (LC50,48h of 9.88mS/cm and 11.32mS/cm; and EC50 for total reproduction of 8.9mS/cm and 10.4mS/cm, respectively for NaCl and seawater) than the natural seawater. These data suggest that the use of NaCl as a surrogate for seawater to predict, in laboratory, the effects of seawater intrusion in freshwater is a protective approach as it simulates a “Worst Case Scenario” of exposure. An association between resistance to copper and to NaCl was not observed for the tested cloned lineages of D. longispina ($r < 0.92$ and $p > 0.08$), though the two clonal lineages most resistant to copper also exhibited the highest sensitivity to sublethal levels of NaCl (determined as the EC20 for total reproduction). Finally, obtained data demonstrated that D. longispina was more sensitive to increased salinity (LC50,48h of 2.85g/L to 2.48g/L or, conductivity values of LC50,48h of 5.50mS/cm to LC50,48h= 4.57mS/cm which correspond respectively to the highest and lowest recorded values in these assays) than the standard species (D. magna), highlighting the importance of using autochthonous species for the ecological risk assessment of secondary salinisation.

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Chapter 1

Introduction:

As referred in the series of five papers “Understanding Changes in Weather and Climate Extremes”, the period in which we are living is one of extreme changes (Changnon et al. 2000; Easterling et al. 2000; Meehl et al. 2000a 2000b; Parmesan et al. 2000). In fact, there is an evident awaking to the fact that climate change and global warming are in progress (Meehl et al. 2000a; Bindoff et al. 2007). Recent projections for future decades, related with the impacts of climate change, include, among others, awareness regarding global sea level rise (Nerem et al. 2006; Parry et al., 2007; Rahmstorf, 2007). Actually, in the last century a global average in sea level rise of 12 to 22 cm was already registered, and, at present, sea level is rising, in average, at a rate of approximately 1.7 ±0.5mm/yr (Bindoff et al. 2007). But, the expected increase in global mean temperature, due to global warming, foresee a further raise in sea level, which will mainly be caused by the expansion of oceans water (thermal expansion) and to a lesser extent by melting glaciers and small ice caps, and by a combination of melting and collapse of portions of coastal sections of the Greenland and Antarctic glaciers into the ocean (Cubasch et al. 1992; Meehl et al. 2005; Nerem et al., 2006; IPPC, 2007; Rahmstorf, 2007). In line with this, the projections of the Intergovernmental Panel on Climate Change (IPCC) estimates a global mean sea level rise of approximately 60-330 mm by 2050 and of 90 to 880 mm by 2100 (Parry et al., 2007). Such global sea level rise emerges as a major threat to low-lying coastal ecosystems worldwide as it may cause its salinisation, either through the occurrence of floodings (sea water intrusions at the surface) in coastal regions or through seawater intrusions into coastal aquifers due to increased evapotranspiration (due to temperature increase) and lower groundwater recharge rates (lower renewable groundwater resource and groundwater levels) (Parry et al., 2007). In addition, groundwater salinisation may be exacerbated by human overconsumption or unregulated extraction of groundwater (Cincotta et al. 2000; Vörösmarty et al. 2000; Bindoff et al. 2007). As a consequence of this, low lying freshwater costal ecosystems are expected to be threatened in a near future due

to such secondary salinisation. These low-lying coastal ecosystems (e.g. coastal lagoons, wetlands), are usually highly productive, harboring a large and unique biodiversity (many constituting biodiversity hotspots). Furthermore, they support a range of natural services highly valuable for society, providing both commercial and recreational functions (e.g. fisheries productivity, storm protection) (Bobbink et al., 2006 and references therein; Anthony et al., 2009). It is, then, important to develop efficient and sustainable protection schemes for these regions, and to attain this, a good understanding of how increased levels of salinity will affect these ecosystems is needed.

Seawater differs mainly from freshwater in its ionic composition and concentrations, holding higher level of salts (higher salinity). Since freshwater organisms are adapted to low osmotic pressures, as salinity increases this biota will become osmotically stressed (James et al. 2003; Ghazy et al. 2009). In fact, salt can be very toxic to freshwater, as it interferes with basic ecological and physiological functions (e.g. affects the capacity of organisms to osmoregulate), adversely affecting species life histories and fitness, food supply, available habitat or breeding grounds (James et al., 2003; Grzesiuk et al., 2006; Gonçalves et al., 2007). Furthermore, several authors observed a strict correlation between increasing salinity values and reduction of species diversity (Greenwald and Hurlbert, 1993; Brock et al., 2005; Nielsen et al., 2008). However, most of the works that have been carried out in order to understand the effects of increased salinity in freshwater biota involve exposure to a unique salt (usually NaCl is used as a surrogate of seawater) (Cowgill et al. 1991; Sarma et al., 2002, 2006; Gama-Flores et al., 2005; Gonçalves et al., 2007; Martínez-Jerónimo and Martínez-Jerónimo; 2007). But, it has been shown that the ionic composition of the media greatly influences the tolerance of freshwater biota to salinity. Usually, media with more than one salt have been reported to induce a lower toxicity to biota than media with a single salt (Mount et al., 1997; Kefford et al., 2004; Zalizniak et al., 2006; 2009). Also, other authors compared the toxicity of artificial seawater with that of NaCl, and found that the latter was more toxic than the former one (Kefford et al., 2004a). But, artificial seawater has also been reported to under-estimate the toxicity of natural salt waters (Kefford et al., 2000). Therefore, it is important to

understand if the commonly used salt NaCl is a protective surrogate to evaluate the adverse effects that increased salinity may pose to freshwater biota.

Another issue that should be considered, within the context of predicting the effects that increased salinity may pose to freshwater communities inhabiting low-lying coastal ecosystems, is that some of these ecosystems are already exposed to chemical contamination (Zu et al 1994. Ghazy et al. 2009; Gómez-Díaz et al 2009). In such a situation, the input of seawater will function as an additional stressor to the biota inhabiting those regions. Natural populations are genetically variable, i.e. hold a number of several genotypes that will respond differently to chemical contamination. If the intensity of such chemical contamination is strong enough it may lead to the disappearance of the most sensitive genotypes, to that particular contamination, from the initial population, causing its genetic erosion (e.g. van Straalen and Timmermans, 2002; Lopes et al., 2009; Agra et al., 2010; Ungherese et al., 2010). Whether the remaining resistant genotypes are also resistant to other type of contamination (namely increased salinity) will determine the survival and persistence of the genetically-eroded population under a situation of future contaminant's inputs. Three scenarios may occur: (i) if a positive association occur between resistance to the different chemicals, then the individuals surviving the input of the first chemical, will be able to cope with the second input of a different chemical; (ii) if a negative association exists between the two chemicals, then the individuals remaining in the population (resistant to the first chemical) may die after exposure to a second chemical (to which they are sensitive); and (iii) if no association exist between resistance to the different chemicals, then exposure to the first chemical will lead to the disappearance of the most sensitive individuals to this chemical, and exposure to a second chemical will lead to the disappearance of the most sensitive individuals to it (within this scenario, the intermediately or highly resistant individuals to both chemicals will remain in the population) (Vinebrooke et al., 2004). The association between resistance to more than one chemical, namely for several metals, has already been reported by several authors (e.g. Soldo and Behra, 2000; Gonnelli et al., 2001). However, an association between NaCl and other chemicals has rarely been addressed, and existing published works were mostly carried out with plant

species (Hodson et al. 1981; Shah et al., 1993, 2002; Kobayashi et al., 2004). For example, Hodson et al. (1981) compared the sensitivity of clones of the grass *Agrostis stolonifera*, from a salt marsh and an inland ecosystem to several ions (e.g. lithium, potassium, rubidium, caesium, magnesium, and calcium), and observed that the former was always more tolerant than the inland one. Thus, suggesting that the tolerance of the salt march clone to NaCl, conferred it an increased resistance to the other tested ions. But, independency or inverse relationship between tolerance to NaCl and other chemicals has also been reported. As an example, Wu et al. (1991) reported independency in tolerance to NaCl and selenium in tall fescue lines (*Festuca arundinacea* Schreb).

According to the above mentioned, the present study intends to:

1- Compare the toxicity of freshwater organisms to increased salinity established with natural seawater and with NaCl. To attain this objective two standard freshwater species (the algae *Pseudokirchneriella subcapitata* and the cladoceran *Daphnia magna*), representative of different taxonomic and functional groups, were exposed to lethal and sublethal levels of the natural seawater and of NaCl solutions. This objective is addressed in chapter 2 of this thesis.

2- Evaluate if an association between copper contamination and salinity exists. To attain this objective, the lethal and sublethal sensitivity of four clonal lineages of the cladocera *Daphnia longispina* to NaCl was determined and compared with their sensitivity to lethal levels of copper. This objective is addressed in chapter 3 of this thesis.

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Chapter 2

Comparative effects of NaCl and Seawater on freshwater organisms

Abstract

The use of NaCl as a surrogate to evaluate the adverse effects that increased salinity, due to sea level rise, may pose to freshwater organisms, has been generalized in scientific investigation. This practice does not take into account the complex mixture of salts and other elements that occur in natural seawater. According, to assess if the use of NaCl has been inflicting some kind of inaccuracy or under-protection of freshwater ecosystems when predicting the effects of salinisation, this work aimed at comparing the sensitivity of freshwater biota to increased salinity established with NaCl and with natural seawater. To attain this objective, lethal and sublethal toxicity assays were carried out by exposing the green algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (producer) and the cladoceran *Daphnia magna* Straus (primary consumer) to two gradients of salinity: one established with a natural seawater sample and the other with NaCl dissolved in an artificial media. For *P. subcapitata*, the effects of increased salinity on the growth rate were monitored, while for *D. magna* lethal and sublethal effects (time to first brood, body size, total reproduction, and intrinsic rate of natural increase were monitored.

The obtained results showed that both species were more sensitive to NaCl than to the natural seawater, thus, NaCl exerting a higher toxicity to these freshwater species. The LOEC computed for growth inhibition of *P. subcapitata* were 5.9mS/cm and 9.6mS/cm, respectively for NaCl and seawater; the $LC_{50,48h}$ for *D. magna* were 9.88 mS/cm and 11.32 mS/cm, respectively for NaCl and seawater; and the EC_{50} computed for the total number of neonates released per female, at the end of the assay, were 8.9 and 10.4mS/cm, respectively for NaCl

and seawater. The body size of *Daphnia magna* was significantly smaller, comparatively to the control, at lower conductivities in NaCl (4.6mS/cm) than in seawater (7.4 mS/cm). The obtained results suggest that the use of NaCl as a surrogate to predict, in laboratory, the effects of seawater intrusion in freshwater is a protective approach as it simulates a “Worst Case Scenario” of exposure.

Keywords: Natural seawater; Increased salinity; NaCl; *Pseudokirchneriella subcapitata, Daphnia magna*

Introduction

The salinisation of costal freshwater ecosystems is emerging as a major problem within the context of global sea level rise due to the predicted climate changes (IPPC, 2007). In fact, in the last century, a global average in sea level rise of 12-22 cm was already witnessed. At present, and according to the Intergovernmental Panel on Climate Change (IPPC) reports, sea level is rising, in average, at a rate of approximately 1.7 ± 0.5 mm/yr (Bindoff et al. 2007). But, the expected increase in global mean temperature foresee a further raise in sea level, which will mainly be caused by the expansion of oceans water and to a lesser extent by melting glaciers and small ice caps, and by a combination of melting and collapse of portions of coastal sections of the Greenland and Antarctic glaciers into the ocean (Nerem et al., 2006; IPPC, 2007; Rahmstorf, 2007). Within this scenario, the IPPC estimate a global mean sea level rise of approximately 60-330mm by 2050 and of 90 to 880mm by 2100 (IPPC, 2007).

Costal freshwater ecosystems are by definition near the sea and at a low altitude, as such, it is expected that sea level rise will cause their salinisation, either by the gradual intrusion of seawater at the surface (floodings) or through infiltration in depleted aquifers (IPPC, 2007). In addition, the concomitant increase of overconsumption by human society, the unregulated extraction of groundwater and the decrease of freshwater influx in aquifers and major waterways, will intensify the predicted scenarios of salinisation (IPPC, 2007). These low-lying freshwater coastal ecosystems (e.g. coastal lagoons, wetlands), are usually highly productive, harboring a large and unique biodiversity (many constituting biodiversity hotspots), and provide commercial and recreational functions (Bobbink et al., 2006 and references therein). It is, then, of major importance to develop efficient and sustainable protection schemes for these regions. To efficiently accomplish this, a good understanding of how increased levels of salinity will affect such ecosystems is needed. Actually, a number of works already addressed this issue. But, most of them used the salt NaCl to simulate the increase in salinity (Cowgill et al. 1991; James et al. 2003; Sarma et al., 2006; Gonçalves et al. 2007; Martínez-Jerónimo et al. 2007; Ghazy et al. 2009; Goméz-Díaz et al. 2009). As the

natural seawater is a complex mixture of salts and minerals, the data obtained in these studies may lead to an over or under estimation of the real effects of increased salinity due to sea level rise. Within this perspective, some researchers already compared the sensitivity of freshwater biota to increased salinity caused by NaCl, by a mixture of salts and by artificial seawater. In general, these works reported that freshwater biota is more sensitive to NaCl (Kefford et al., 2004; Roache et al., 2006; Zalizniak et al., 2006, 2009). As an example, Kefford et al. (2004), simulated, in mesocosms, non-rheophilic riverine communities and exposed them to solutions of NaCl and to artificial seawater. At the end of the experiment, these authors observed that, in general, the taxa exposed in the mesocosms exhibited a higher lethal sensitivity to NaCl than to the artificial seawater.

According to the above mentioned, the present study intended to compare the toxicity of NaCl and of a natural seawater to freshwater biota. Though some authors already compared the toxicity of NaCl with that of artificial seawater, a more ecologically relevant comparison would be attained by using natural seawater. Furthermore, though accurate concentrations of salts are added to artificial salt water to simulate the exact composition of natural seawater, some differences in their compositions occur which may influence the toxicity to freshwater biota. For example, the concentration of strontium is usually much lower in artificial salt waters. Also, depending on the reagent grade of the used salts, the ionic concentration of the artificial salt water may be different from the natural seawater (e.g. if NaCl reagent grade contains the maximum impurities of PO_4^- and Fe, then artificial water will contain levels of this ions ten and four times more, respectively, than the natural water) (Kester et al., 1967).

To attain the main objective of this study, the lethal and sublethal sensitivity of two aquatic species, representative of different taxonomic and functional groups (*Pseudokirchneriella subcapitata* (Korshikov) F. Hindák as a producer and *Daphnia magna* Straus as a primary consumer), to solutions of NaCl and to a sample of natural seawater from Northern Atlantic Ocean was assessed.

Materials and Methods

Test solutions

For the toxicity assays, a natural seawater sample was collected at the north Atlantic Ocean on a site located far from any anthropogenic activity (between Praia Quiaios and Praia da Tocha: +40°16'11.02", -8°52'15.20"). This seawater (conductivity = 50.6mS/cm, salinity = 33.1, pH = 8.05) was prepared for the toxicity assays by filtering it trough Cellulose Nitrate Membranes of 0.20µm (ALBET-Hahnemuehle S.L., Barcelona, Spain), to remove particles in suspension and possible organisms.

The NaCl (Sigma-Aldrich, St Louis, MO, USA) solutions were prepared by adding this salt to the culture media of each tested species: for algae, to the Woods Hole Marine Biological Laboratory (Woods Hole, MA, USA) growth medium (hereafter referred to as MBL), prepared in accordance to Stein (1973), and for the cladoceran, to the American Society for Testing and Materials hardwater (hereafter referred to as ASTM; ASTM, 2000).

Test species

Two standard species, representing different trophic levels, were selected to carry out this study: the green microalgae *Pseudokirchenriella subcapitata* (Korshikov) F. Hindák (formerly known as *Selenastrum capricornutum* Printz; producer) and the cladoceran *Daphnia magna* Straus (primary consumer). These species are easily available (from laboratory culture) and maintained in laboratory under reproducible culture conditions; and are recommended for toxicity testing by several guidelines (EC, 1992; USEPA, 1994; OECD, 2004; OECD, 2006).

Cultures of *P. subcapitata* were maintained in nonaxenic batch cultures, in 5L glass flasks, with 4L of MBL (Stein,1973), with continuous aeration and under a controlled temperature of 19 to 21 °C and continuous light. For the maintenance of the laboratory cultures and the start of new cultures, algae were harvested while

still in the exponential growth phase (5–7 days old) and inoculated in fresh medium.

Daphnia magna were continuously reared under semi-static conditions, controlled photoperiod (16:8 h light:dark) and temperature (19 to 21ºC), in ASTM hardwater medium (ASTM, 2000). This ASTM media was supplied with a supplement of vitamins and a standard organic extract “Marinure 25”, an extract from the algae *Ascophylum nodosum* (Baird et al., 1989), (Pann Britannica Industries Ltd., Waltham Abbey, UK) (7.5mL/L of a suspension with an absorbance of 620 units at 400 nm) to provide essential microelements to daphnids. Cultures were renewed every 2 days and the organisms were fed daily with the green algae *P. subcapitata* at a rate of 3.0×10^5 cells/mL/day).

Growth inhibition assay with Pseudokirchneriella subcapitata

The growth inhibition assays, with the green algae *P. subcapitata*, were carried out in sterile 24-well microplates (with 1mL of medium per well) (EC, 1992; Blaise et al., 1998; Moreira-Santos et al., 2004). Algae were exposed, for 72h, to a control (consisting of plain MBL culture media), to a range of five dilutions of the natural seawater (salinity measured as the electrical conductivity: 8.2, 9.6, 11, 13, and 16mS/cm) and to a serial of five concentrations of NaCl (with salinities values of 5.9, 6.9, 8.0, 9.4, and 11mS/cm; corresponding to the following NaCl concentrations: 3.0, 3.4, 4.0, 4.5, 5.2g/l), at 24 to 26ºC and with a constant luminous intensity (60–120µE/m^2/s, equivalent to 6,000–10,000lx). For natural seawater, dilutions were made directly from the water sample. For NaCl the tested solutions were obtained by diluting the highest concentration of 5.2g/L with MBL. Algae were exposed in 24-well microplates (VWR Tissue Culture Plates – 24 Wells - Sterile, Leuven, Belgium), where each well was filled with 900µL of test water and inoculated with 100µL of the correspondent algal-inoculum solution (10^5 cells/mL), so that the nominal initial cell concentration in the test was 10^4 cells/mL (the absorbance of this solution was measured in a spectrophotometer at 440nm). Three replicates were set up randomly for each treatment and a control (MBL

medium) per microplate. The peripheral wells were filled with 500μL of distilled water, to minimize evaporation in the test wells. During the exposure period, each well was shacked manually twice per day. At the start of each assay, conductivity, salinity, PPS (HANNA Instruments Seawater Refractometer HI 96822;Woonsocket, RI, USA), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, Brüssel, Belgium), and DO (Wissenschaftlich Technische Werkstätten OXI92 oxygen meter, Brüssel, Belgium) were measured.

After 72h of exposure the concentration of algae was computed at each replicate by measuring absorbance at 440nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, USA) and converting it to the number of cells per milliliter, using the following formula:

$$\text{Conc (cells/mL)} = -17107.5 + (\text{ABS} * 7925350)$$

where ABS is the absorbance measured at 440nm and C is the concentration of algae (in cells per milliliter).

For each concentration, the average specific growth rate (for exponentially growing cultures) and the percentage of reduction in average growth rate compared to the control value were calculated, after a period of 72h of exposure (OECD, 2006).

Lethal assays with D. magna

The lethal assays with *D. magna* were performed according to OECD (2004). Test organisms were obtained from females of the laboratory cultures. Neonates (>6h and < 12h old), born between the 3rd and 5th broods, were exposed to a control (consisting of the culture media ASTM), to five dilutions of seawater (8.1, 9.6, 11.2, 13.35 and 14.9mS/cm), and to five concentrations of NaCl (7.64, 9.05, 10.52, 12.65 and 14.37mS/cm; corresponding to the following NaCl concentrations: 4.0, 4.8, 5.7, 7.0, 8.0g/l). The assays were performed using a static design, where 20 neonates were exposed per concentration and per control (5 neonates per each

replicate). Neonates were introduced, after placing 50ml of the test solutions (or control) in each glass vessel. Organisms were exposed for a period of 48h under the same environmental conditions as described for culture maintenance. Survival of the neonates was monitored after 24 and 48h of exposure, an organism being considered dead when it remained immobile during 15s after gentle prodding. During the test, organisms were not fed.

Salinity (HANNA Instruments Seawater Refractometer HI 96822, Woonsocket – RI – USA, Romania), conductivity (Wissenschaftlich Technische Werkstätten LF92 conductivity meter, Brüssel, Belgium), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, Brüssel, Belgium), and DO (Wissenschaftlich Technische Werkstätten OXI92 oxygen meter, Brüssel, Belgium) were measured at the start and at the end of the assay.

Sublethal toxicity assays with D. magna*:*

To assess the comparative effects of NaCl and seawater on life-history parameters of *Daphnia magna* the standard protocol OECD 211 (1998) was followed up. Ten neonates (> 6 and < 24h old), from the third to the fifth brood were exposed individually, under the same controlled conditions of temperature and photoperiod as for the laboratory cultures, to a range of five NaCl concentrations and a control (ASTM hardwater) and another group to five seawater dilutions and a control (ASTM hardwater). Test salinities ranged between 4.6mS/cm and 9mS/cm for NaCl (corresponding to the following NaCl concentrations: 2.9, 3.2, 2.5, 4.0, 4.5g/l) and 5.4mS/cm to 10mS/cm for seawater (using a dilution factor of 1.2x). This gradient of conductivity was prepared by dissolving NaCl and filtered seawater in ASTM hardwater. Each individual was introduced in 50-mL glass vessels filled with 50 mL of test solution with the addition of “Marinure 25” and of the green algae *P. subcapitata* ($3x10^5$cells/mL/d). Organisms were fed every day, medium being renewed every other day. Time to release the first brood, total number of neonates released per female, and body size were monitored and the intrinsic rate of increase was computed at each

treatment. Body length was recorded in all tested females at the end of each test. The assay ended after the stipulated 21 days time period for sub lethal assays with *D. magna*.

Salinity (HANNA Instruments Seawater Refractometer HI 96822, Woonsocket – RI – USA, Romania), conductivity (Wissenschaftlich Technische Werkstätten LF92 conductivity meter, Brüssel, Belgium), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, Brüssel, Belgium), and DO (Wissenschaftlich Technische Werkstätten OXI92 oxygen meter, Brüssel, Belgium) were measured at new and old sampling water.

Data analysis

The average specific growth rate of *P. subcapitata* was computed as the logarithmic increase of the number of algal cells by using the following equation:

$$Growth\ rate = \frac{Ln(C3) - Ln(C0)}{3}$$

where C0 was the value of the number of algal cells per milliliter at the beginning of the assay (10^4cell/ml in this case) and C3 the number of algal cells measured at the end of the test (after 72h of exposure, corresponding to day 3). The number 3 corresponds to the period of exposure in days.

The inhibition of algal growth was calculated as the percentage of reduction in growth rate comparatively to the respective control using the equation:

$$Growth\ Inhibition\ (\%) = \left(\frac{(\mu c) - (\mu s)}{\mu c}\right) * 100\%$$

where μ_c is the mean growth in the control and μ_s the mean growth in the water samples.

To analyse algae growth data and life-history parameters of *D. magna*, and determine which treatments differed significantly from the control, a one-way

analysis of variance was carried out, followed by post hoc comparisons using Dunnett's test (to determine significant difference relatively to the control and the no observed effect concentration-NOEC and the lowest observed effect concentration-LOEC), when significant differences were found ($p \leq 0.05$). Assumptions of normality and homoscedasticity of data were checked with the Shapiro-Wilks and Bartlett's tests, respectively.

Survival data of *D. magna* was used to compute the median lethal concentrations (causing 50% of immobility; LC50) with the respective 95% confidence limits, for NaCl and seawater through the probit analysis, using the software Priprobit (Sakuma, 1998).

The intrinsic rate of natural increase (r) was computed for each clone, after the life history experiment, using Lotka's equation:

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

with *lx* being the age-specific survivorship; m_x the number of neonates at day *x* and x the age in days. The estimation of the pseudovalues and standard errors for *r* was done through the jackknifing technique (Meyer et al. 1986).

Concentration-response curves and effective concentrations promoting 50% and 20% (EC50 and EC20, respectively) of effect, with the respective 95% confidence intervals, were computed through a logistic curve model, using the software Statistica 8.0 (StaSoft, Tulsa, OK, USA) (OECD, 1997, 1998).

Results

Growth inhibition assay with P. subcapitata

During the72-h assay with NaCl, the measured pH values ranged from 7.5 to 7.6 and dissolved oxygen was always above 8.3mg/L. The highest variability observed in conductivity and salinity during the assay was 0.5mS/cm and 0.2, respectively. For the seawater solutions the pH values ranged from 7.5 to 7.6 and dissolved oxygen was always above 7.1mg/L. The highest variability observed in conductivity and salinity during the assay was 0.46mS/cm and 0.3, respectively.

A significant decrease in the growth rate was observed for algae exposed to all tested concentrations of NaCl, comparatively to the respective control ($F_{5,14}$=62.2; $p<10^{-5}$; $\text{Dunnett's: } p \leq 0.0002$) (Fig. 2.1). The lowest salinity exhibiting a significant decrease (LOEC) in growth rate was 5.9mS/cm. The percentage of growth inhibition registered for each tested salinity was: 25% for 11.18mS/cm, 23% for 9.44mS/cm, 35% for 8.04mS/cm, 17% for 6.85mS/cm, and 14% for 5.9mS/cm.

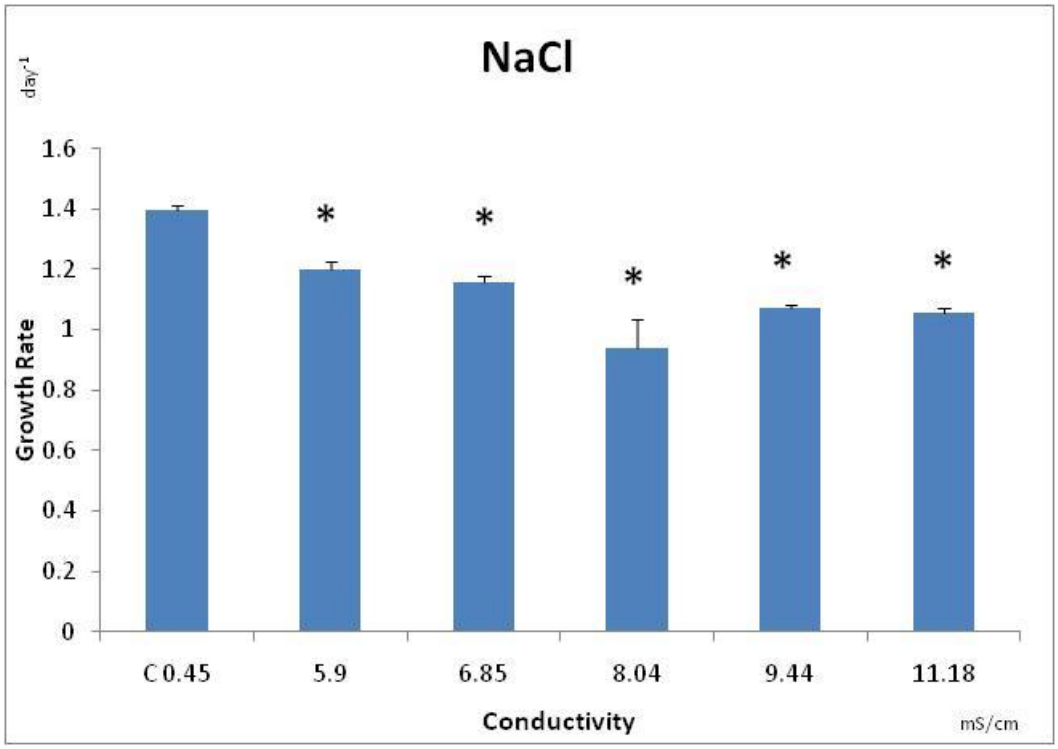

Figure 2.1 – Average of the growth rate (day^{-1}), with the corresponding standard deviation (error bars), of *Pseudokirchneriella subcapitata* after being exposed for 72 h to NaCl. * - symbolizes significant differences from the Control (Dunnett's test: p≤0.000021).

For *P. subcapitata* exposed to natural seawater, a significant decrease in growth, comparatively with the respective control, was observed at 13.1 and 15.5mS/cm ($F_{5,14}$= 328; $p<10^{-5}$ followed by Dunnett´s: $p \leq 7x10^{-5}$) (Fig. 2.2). The determined NOEC was 8.2mS/cm and the LOEC was 9.6mS/cm (Fig. 2.2). The percentage of growth inhibition, registered for each tested salinity, was: 60% for 15.51mS/cm, 25% for 13.14mS/cm, 10% for 11.24mS/cm, 5% for 9.6mS/cm, and 2% for 8.2mS/cm.

The median effect concentration of seawater causing 50% and 20% of growth inhibition in *P. subcapitata* were, respectively: 14.44mS/cm (confidence limits: 14.26 - 14.62mS/cm) and 12.28mS/cm (confidence limits: 12.01 - 12.55mS/cm), respectively.

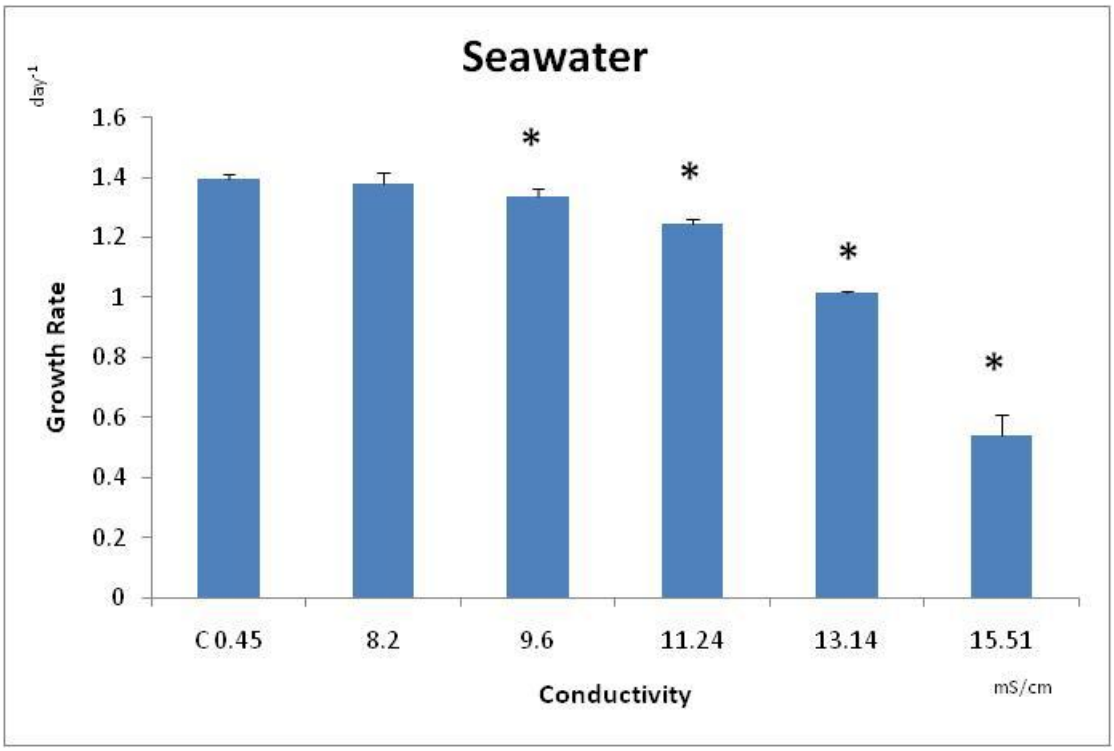

Figure 2.2 - Average of the growth rate (day^{-1}), with the respective standard deviation (error bars), of *Pseudokirchenriella subcapitata* after being exposed for 72 h to natural seawater. * - symbolizes significant differences from the control (Dunnett's test: $p \leq 7x10^{-5}$)

***Lethal assay with* D. magna**

During the lethal assay with *D. magna* exposed to NaCl, the values of pH ranged from 7.8 and 8.6 and dissolved oxygen was always above 8.2mg/L. The highest variability observed in conductivity and salinity during the assay was 0.6mS/cm and 0.4, respectively.

For the lethal assay with *D. magna* exposed to seawater, the values of pH ranged from 7.8 and 8.6 and dissolved oxygen was always above 7.9mg/L. The highest variability observed in conductivity and salinity during the assay was 0.4mS/cm and 0.2, respectively.

The $LC_{50,24h}$ computed for NaCl and seawater were 10.21mS/cm and 14.84mS/cm, respectively; and the $LC_{50,48h}$ values computed for NaCl and seawater were 9.88mS/cm and 11.32mS/cm, respectively (Fig. 2.3).

The results of this assay reinforce the data obtained for *P. subcapitata*, that NaCl exerts a higher toxicity comparatively to seawater.

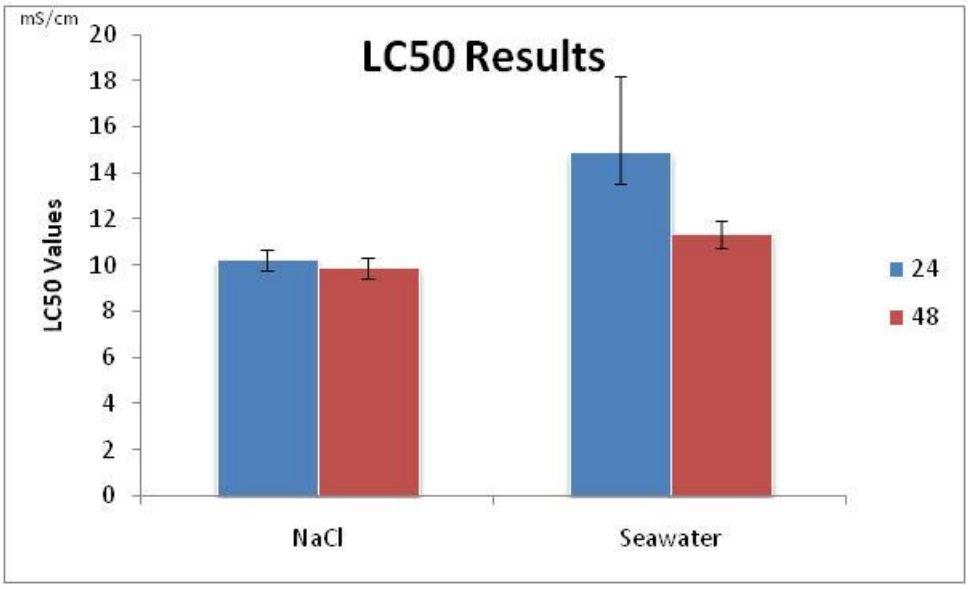

Figure 2.3 – Values of the median lethal salinity (LC50 in mS/cm) for *Daphnia magna* after being exposed for 24 and 48h to NaCl and natural seawater, with the respective 95% Confidence limits (error bars).

Sub lethal assay of* D. magna *exposed to NaCl

During the assay with NaCl, the pH values ranged from 7.77 to 7.80 and dissolved oxygen was always above 8.0mg/L. The highest variability observed in conductivity and salinity during the assay was 0.7mS/cm and 0.4, respectively.
A high mortality was observed at the end of the assay in the highest salinity treatment (9mS/cm), where 80% of daphnids died.

A significant decrease in age at first brood, comparatively with the control, was observed at a salinity of 7.5mS/cm ($F_{5,39}$=4.72; p=0.0007; followed by Dunnett's: p≤0.016) (Fig. 2.4). The determined NOEC was 6.5mS/cm and the LOEC was 7.5mS/cm of NaCl.

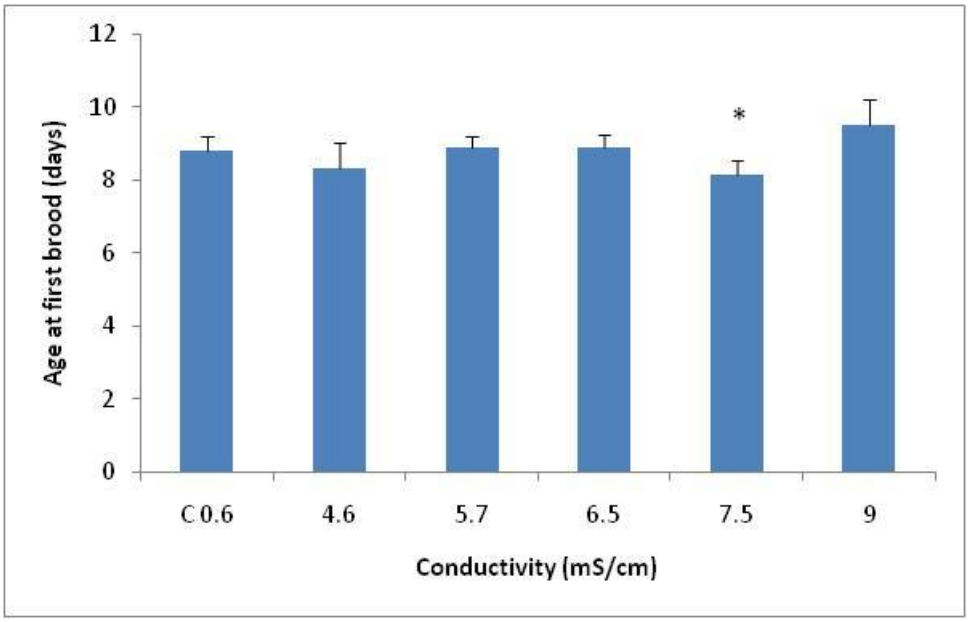

Figure 2.4 – Average of age at first brood (days), with the respective standard deviation (error bars), of females of *Daphnia magna* exposed to serial dilutions of NaCl. * symbolizes significant differences relatively to the Control (p≤0.016).

The total number of neonates released per female was significantly lower in treatment with a salinity of 9mS/cm, comparatively to the control ($F_{5,39}$=5.59; p=0.00056 followed by Dunnett's: p=0.00031) (Fig. 2.5). Thus, the NOEC computed for total reproduction was 7.5mS/cm and the LOEC was 9mS/cm. The EC50 computed for brood size was 8.9mS/cm (confidence limits: 8.6mS/cm to 9.5mS/cm) and the EC20 was 8.4mS/cm (confidence limits: 7.4mS/cm to 9.3mS/cm).

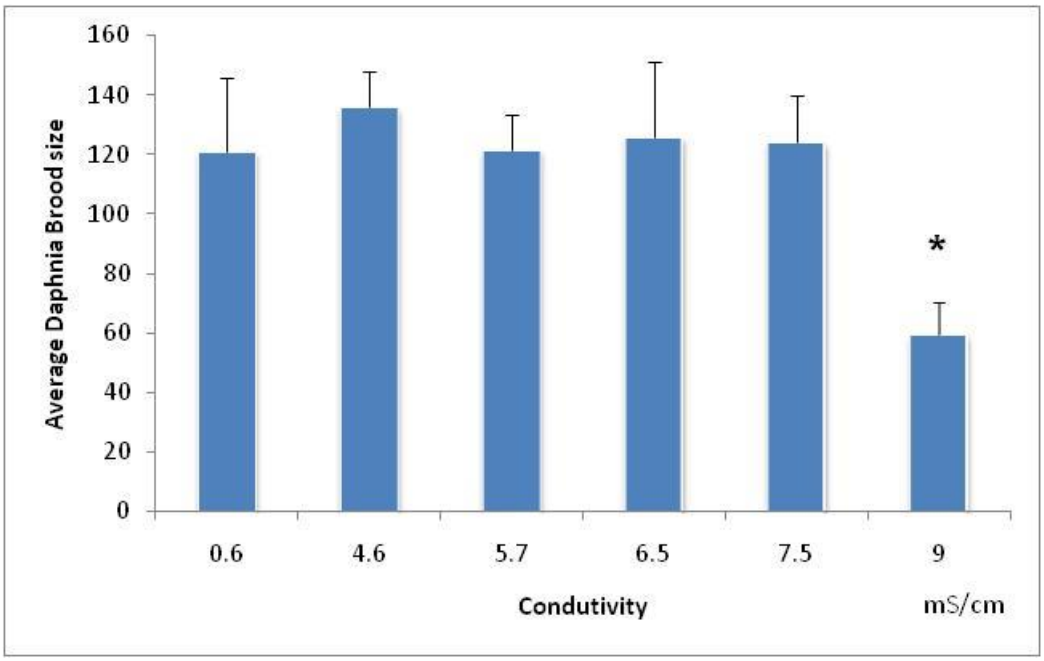

Figure 2.5 – Average of brood size (number of neonates per female), with the respective standard deviation (error bars), of females of *Daphnia magna* exposed to serial dilutions of NaCl. * - symbolizes significant differences from the control (Dunnett's test:p=0.00031).

All tested salinities (NaCl) provoked a significant decrease in the body size of females of *D. magna*, relatively to the control ($F_{5,39}$=9.75; p=0.000004 followed by Dunnett's: p=0.0116) (Fig. 2.6). Thus, the NOEC was lower than the lowest tested salinity, and the LOEC was determined as 4.6mS/cm.

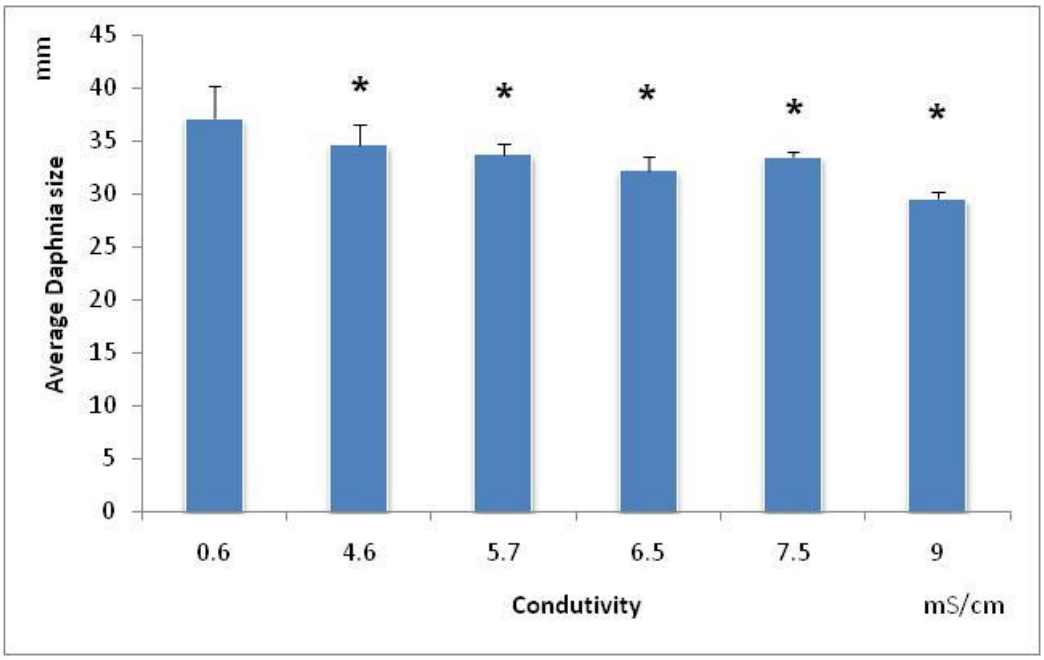

Figure 2.6 – Average body size (mm), with the respective standard deviation (error bars), of *Daphnia magna* exposed to a serial dilution of NaCl. * - symbolizes significant differences from the control (Dunnett's test:p=0.0116).

The salinities 6.5mS/cm and 9mS/cm caused a significant decrease in the intrinsic rate of natural increase, comparatively to the control ($F_{5,54}$=668; p=0.00 followed by Dunnett's: P=0.000283) (Fig. 2.7). Thus, the NOEC was determined as being 5.7mS/cm and the LOEC as 6.5mS/cm.

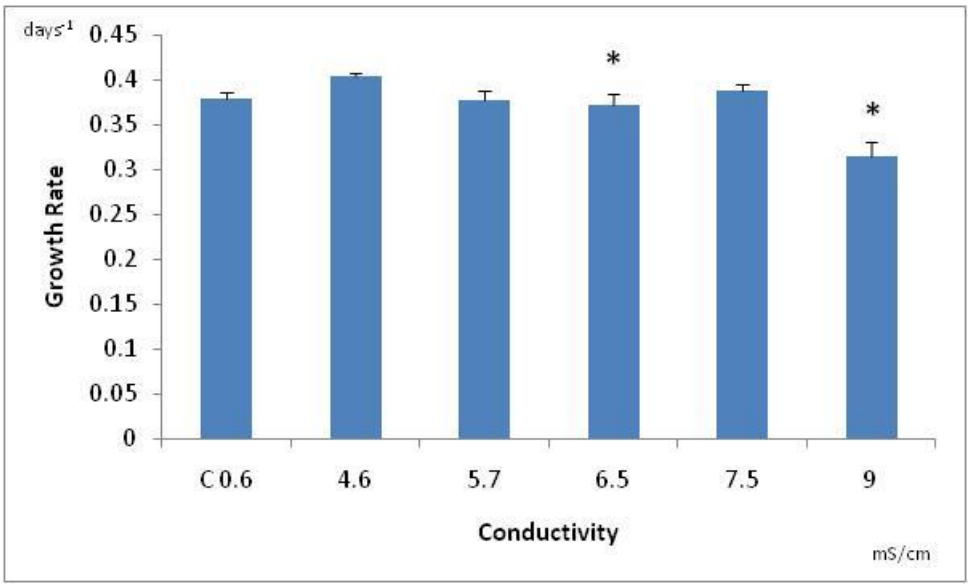

Figure 2.7 – Averages of intrinsic rate of natural increase (r: day^{-1}), with the respective standard deviation (error bars), of *Daphnia magna* exposed to a serial dilution of NaCl. * - symbolizes significant differences from the control (Dunnett's test:p=0.000283)

Sub lethal assay of D. magna *exposed to seawater*

During the assay with seawater, the pH values ranged from 7.77 to 7.8 and dissolved oxygen was always above 8.0mg/L. The highest variability observed in conductivity and salinity during the assay was 1.1mS/cm and 0.6, respectively.

A significant increase in age at first brood, comparatively with the control, was observed at salinities of 8.7 and 1.0mS/cm ($F_{5,50}$=3.11; p=0.016; followed by Dunnett's: p≤0.032) (Fig. 2.8). The determined NOEC was 7.4mS/cm and the LOEC was 8.7mS/cm of seawater.

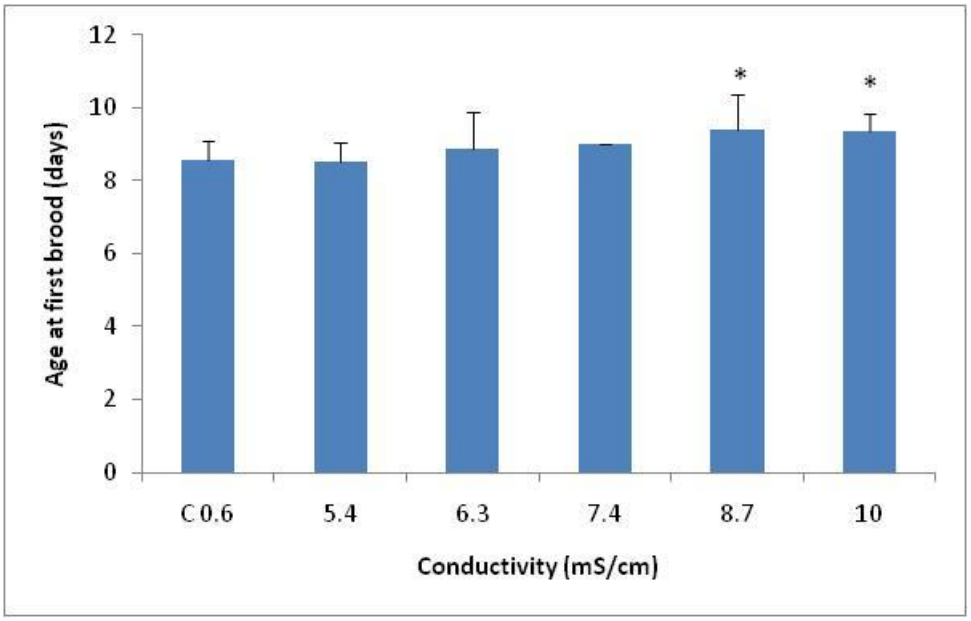

Figure 2.8 – Average of age at first brood (days), with the respective standard deviation (error bars), of females of *Daphnia magna* exposed to serial dilutions of seawater. * symbolizes significant differences relatively to the Control (p≤0.032).

The obtained results showed that only the dilution with a conductivity of 10mS/cm provoked a significant decrease in the number of neonates produced per female, comparatively to the control ($F_{5,50}$=4.44; p=0.0019 followed by Dunnett's: p=0.0078) (Fig. 2.9). Therefore, the NOEC was determined as being 8.7mS/cm and LOEC as 10mS/cm. The EC50 computed for total reproduction was 10.43mS/cm (confidence limits: 9.4 to 11.54mS/cm) and the EC20 was 8.9mS/cm (confidence limits: 7.9 to 10mS/cm).

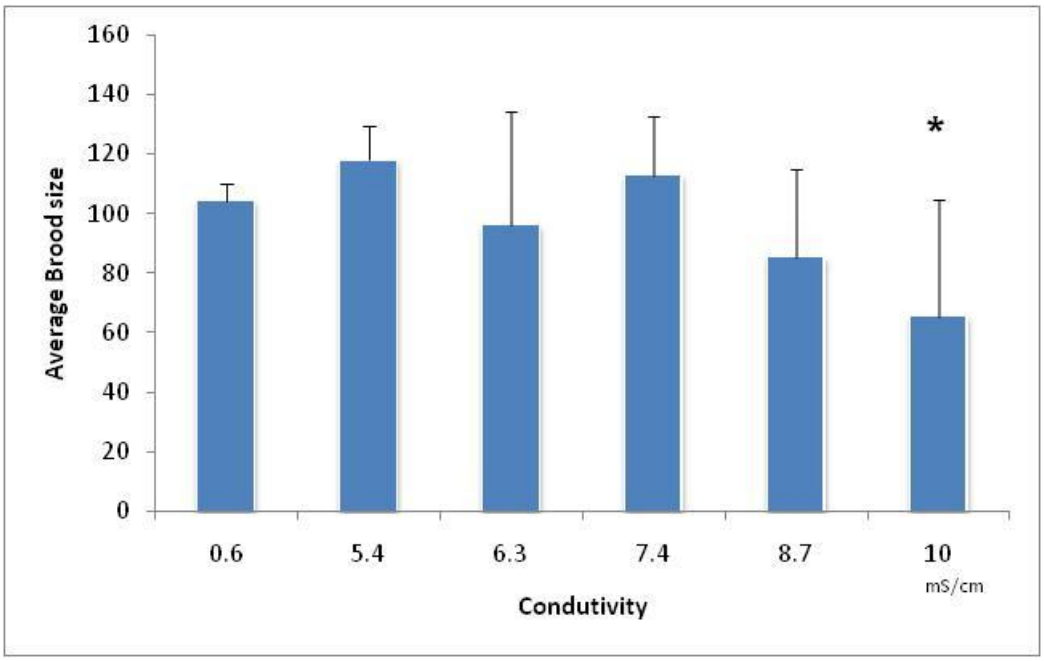

Figure 2.9 – Average brood size (number of neonates per female), with the respective standard deviation (error bars), of females of *Daphnia magna* exposed to serial dilutions of seawater. * - symbolizes significant differences from the control (Dunnett's test: p=0.0078)

Salinities above 7.4 mS/cm caused a significant decrease in the body size of *D. magna*, comparatively with the control ($F_{5,50}$=11.35; p=0.00 followed by Dunnett's: P=0.0095) (Fig. 2.10). Therefore, the determined NOEC was 6.3mS/cm and the LOEC was 7.4mS/cm. The EC20 computed for body size was 9.1mS/cm (confidence limits: 8.03 to 10.2mS/cm).

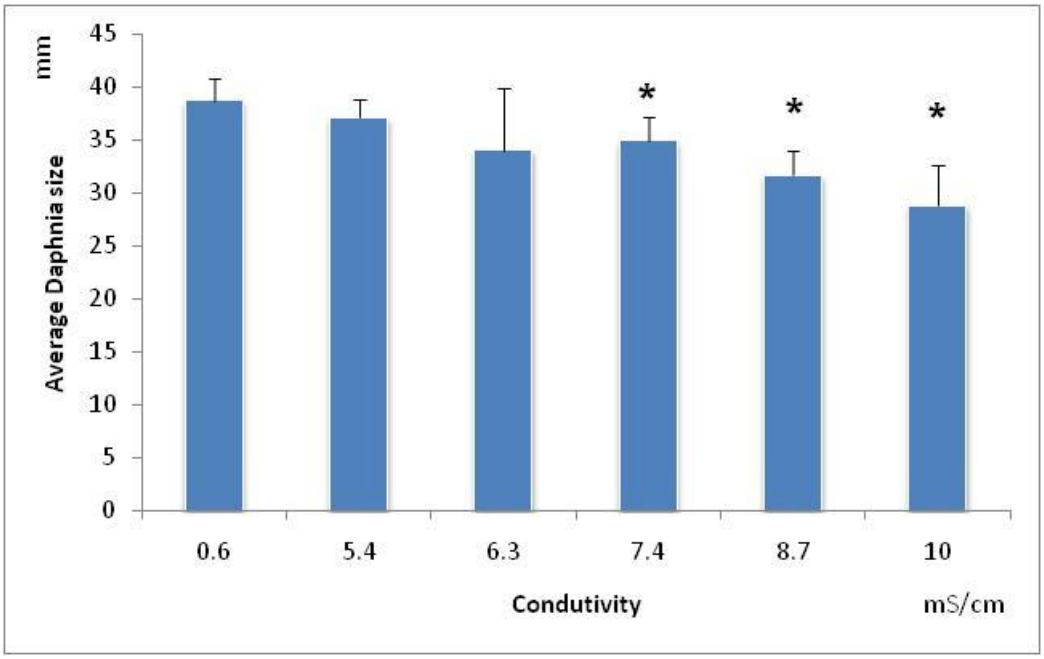

Figure 2.10 – Average body size (mm), with the respective standard deviation (error bars), of *Daphnia magna* exposed to a serial dilution of seawater. * - symbolizes significant differences from the control (Dunnett's test: p=0.0095)

The values of the intrinsic rate of natural increase were significantly lower than in the control at salinities of 6.3mS/cm, 8.7, and 10mS/cm ($F_{5,54}$=294; P=0 and Dunnett's: P=0.000021) (Fig. 2.11). The NOEC being 5.4 mS/cm and LOEC 6.3mS/cm.

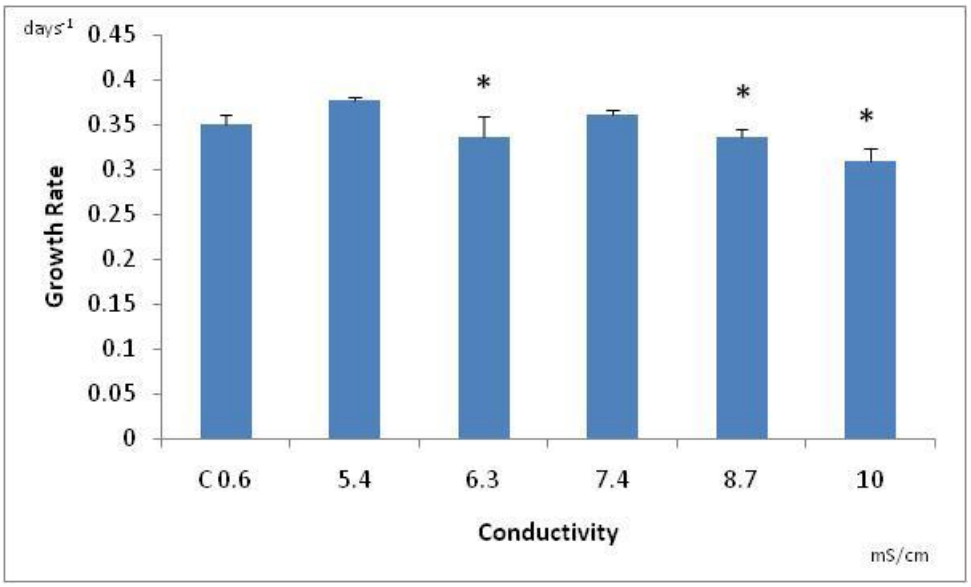

Figure 2.11 – Averages of intrinsic rate of natural increase (r: day^{-1}) of *Daphnia magna* exposed to a serial dilution of seawater with the respective standard deviation (error bars) * - symbolizes significant differences from the control (Dunnett's test:p≤0.000021)

Discussion:

Salinity is a known stress factor in both freshwater invertebrates and algae (Bartolomé et al 2008, Kefford et al. 2004). Values of salinity lower than 2g/L have been reported to exert adverse effects in some freshwater species of microinvertebrates (James et al., 2003 and references therein; Nielsen et al., 2008). However, freshwater biota exhibit a wide range of sensitivities to salinity, and some species may show a very high tolerance to it. For example, some species of charophytes (e.g. *Lamprotnamnium macropogon*) are able to tolerate salinities values of up to 58g/L (James et al., 2003 and references therein). Specifically for the cladoceran *D. magna*, the values of $LC_{50,48h}$, for NaCl, reported in literature range from 2.6 to 7.8g/L (Schuytemaet al., 1997; Gonçalves et al., 2007; Martínez-Jerónimo and Martínez-Jerónimo, 2007; Santos et al., 2007); and salinity values as low as 5.0g/L were shown to cause a significant decrease in the number of neonate produced per female (Gonçalves et al., 2007). The results obtained in the present study are in line with these findings. The lethal and sublethal sensitivities of *D. magna* were within the range reported in the literature: the $LC_{50,48h}$ was 9.88mS/cm (which corresponds to approximately 5g/L of NaCl), and the LOEC for reproduction was 9mS/cm (corresponding to 4.5g/L of NaCl). Interestingly, these results showed that the mortality and reproductive impairment in *D. magna* occurred within a very narrow range of salinities (9 to 9.88mS/cm). These data corroborate the findings of Gonçalves et al. (2007), who also observed mortality and reduction in reproduction within a limited salinity range of 5.0 to 5.5g/L of NaCl. Relatively to the sensitivity of *P. subcapitata* to salinity, Santos et al. (2007) reported and $EC_{50,72h}$ of 0.87g/l of NaCl. In the present study, an $EC_{50,72h}$ could not be computed for NaCl, but a significant decrease in growth rate (14%) was registered at 5.9mS/cm (corresponding to 3g/L of NaCl). These differences in sensitivity could be related with the fact that Santos et al (2007) used a different culture medium for *P. subcapitata*, being constituted by different concentrations of several ions, and, as has been reported in the literature, ion composition of the media may greatly influence the tolerance of biota to salinity (Kefford et al., 2004; Zalziniak et al., 2006, 2009a).

The results of sensitivity obtained for *P. subcapitata* and *D. magna* exposed to NaCl and seawater, are in line with the information gathered from the literature, in that NaCl (or other salt) alone, generally, induces a higher toxicity than a mixture of salts (in this study seawater), or than natural seawater (Kefford et al., 2004; Zalizniak et al., 2006, 2009a). The presence of other ions at higher concentrations, in seawater, may be responsible for the observed higher tolerance to salinity. For example, calcium and magnesium ions are known to decrease the permeability of the membranes and increase its integrity. Actually, Dwyer et al. (1992) observed that the tolerance of *D. magna* to salinity increased with increasing hardness (calcium and magnesium). Also, Boulton and Brock (1999) reported that calcium and magnesium comprise approximately 18 and 3 meq%, respectively, of the cations in seawater. This fact may explain the reduction of seawater toxicity relatively to NaCl. Another factor that has been pointed to be responsible for the observed differences in toxicity of NaCl and seawater is the pH. At low pH values an inhibition of Na^+ uptake may occur (Aladin and Potts, 1995). However, within the present study the pH values were always within the neutrality range, thus it was not expected to have such an effect on the tested species. Also, Mount (1997), structured a toxicity level scale of several salts according to their effects on *Ceriodaphnia dubia* and *Daphnia magna*, and fathead minnows (*Pimephales promelas* Rafinesque, 1820); as such $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2+}$; Na^+ and Ca^{2+} were not significant suggesting that the toxicity of Na^+ and Ca^{2+} salts was primarily attributable to the corresponding anion; in this case, Cl^-. As the salinity of the seawater solutions was reached by a greater mix of salts than the salinity of the NaCl solutions it may be suggested that a higher concentration of Cl^- in suspension on the NaCl solution, probably made it more toxic to the test organisms.

The results obtained in the present study shows that the use of NaCl to assess the effects of secondary salinisation in coastal low-lying freshwater ecosystems is a protective approach, as it exerted a higher toxicity to the two tested species comparatively to the natural seawater. The use of NaCl as a worst case scenario is then advised for risk assessment of increased salinity. Furthermore, though artificial sea water was not tested in this study, we suggest

that NaCl should be used as a surrogate, even in detriment of artificial seawater. The use of artificial seawater has already been shown to cause under-protection of biota. Kefford et al. (2000) observed that the determined lethal sensitivity of *D. carinata* to artificial seawater lead to an under-estimation of the toxicity of three saline lakes, though their ionic proportion being similar to that of the artificial seawater.

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Chapter 3

Is there an association between resistance to copper and NaCl in *Daphnia longispina* clones?

Abstract:

It has been observed that populations inhabiting contaminated sites evolve resistance to that particular contamination through the elimination of the most sensitive individuals. In addition, it has been hypothesized that these genetically eroded populations may be at a higher risk of extinction under future exposures to other stressors, if an association between the two stressors does not exist. This work intended to evaluate if resistance to the metal copper is associated with an increased resistance to salinity (predicted to occur due to sea level rise). To address this objective, five clonal lineages of the cladoceran *Daphnia longispina* O.F. Müller, exhibiting different resistance to lethal levels of copper, were selected. Each cloned lineage was exposed to lethal (2.6, 3.0, 3.4, 4.0, 4.5, and 5.2g/l) and sublethal (0.72, 0.86, 1.0, 1.2, and 1.5g/l) concentrations of the salt NaCl. Mortality, time to first reproduction, total number of neonates produced per female, and body size were monitored and the intrinsic rate of increase was computed for each clonal lineage. The data obtained was compared with the data already available on the lethal responses to copper. The results showed no association between the resistance responses to copper and to NaCl. However, it was observed that the two clonal lineages more resistant to copper exhibited the highest sensitivity (EC20 for total reproduction) to NaCl sublethal levels, thus suggesting that in some scenarios, genetically eroded populations of *D. longispina*, due to copper exposure, may be at a higher risk of extinction when impacted with NaCl contamination

Keywords: NaCl; *Daphnia longispina*; multiple/co-tolerance; cladoceran; Copper

Introduction:

The global rising of sea level is recognized as one of the major problems in environmental protection, especially when considering the conservation of biodiversity of low-lying coastal freshwater ecosystems (IPPC, 2007). According, several works have been carried out in order to determine the sensitivity of freshwater species, inhabiting these ecosystems, to increased salinity and understand what ecological changes may occur due to seawater intrusion (e.g. Nielsen et al. 2003; Schallenberg et al. 2003; Martínez-Jerónimo et al. 2007; Mohammed et al. 2007). Most of these works were focused on understanding the intrinsic sensitivity of tested organisms to increased salinity, induced by artificial seawater or by the use of one salt (usually NaCl), under optimal conditions. However, it is pertinent to consider scenarios where coastal ecosystems are already impacted with chemical contamination, and that biota inhabiting these ecosystems will be exposed to an additional stressor under a scenario of future seawater intrusion. If populations of freshwater organisms, inhabiting such impacted sites, were historically exposed to that chemical contamination, then its genetic erosion may have occurred through the elimination of the most sensitive genotypes (e.g. van Straalen and Timmermans, 2002; Lopes et al., 2009; Agra et al., 2010; Ungherese et al., 2010). Whether the remaining resistant genotypes are also resistant to other type of contamination (namely increased salinity) will determine the survival and persistence of the genetically-eroded population under in a situation of future contaminant's inputs. Three scenarios may occur: (i) if a positive association occur between resistance to the different chemicals, then the individuals surviving the input of the first chemical, will be able to cope with the second input of the a different chemical; (ii) if a negative association exists between the two chemicals, then the individuals remaining in the population (resistant to the first chemical) may die after exposure to a second chemical (to which they are sensitive); and (iii) if no association exist between resistance to the different chemicals, then exposure to the first chemical will lead to the disappearance of the most sensitive individuals to this chemical, and exposure to

a second chemical will lead to the disappearance of the most sensitive individuals to it (within this scenario, the intermediately or highly resistant individuals to both chemicals will remain in the population) (Vinebrooke et al., 2004).

The association between resistance to more than one chemical has already been reported, as an example: Soldo and Behra, (2000) carried out experiences with communities of periphyton and reported that long term exposure to copper, also induced an increased resistance to zinc, nickel and silver. However, co- or multiple resistances between NaCl and other chemicals have rarely been addressed, and existing published works were mostly carried out with plant species (Hodson et al. 1981; Shah et al., 1993, 2002; Kobayashi et al., 2004). For example, Hodson et al. (1981) compared the sensitivity of clones of the grass *Agrostis stolonifera*, from a salt marsh and an inland ecosystem to several ions (e.g. lithium, potassium, rubidium, caesium, magnesium, and calcium), and observed that the former was always more tolerant than the inland one. Therefore, the authors suggested that the tolerance of the salt march clone to NaCl, conferred it an increased resistance to the other tested ions. But, independency or inverse relationship between tolerance to NaCl and other chemicals has also been reported by some authors. As an example, Wu et al. (1991) reported independency in tolerance to NaCl and selenium in tall fescue lines (*Festuca arundinacea* Schreb). Furthermore, these authors also reported that selenium and salt tolerance were negatively correlated with tissue Se and salt concentrations.

Accordingly, the present work aimed at evaluating if an association between resistance to copper and to NaCl (considered, in the previous chapter, as a protective surrogate to evaluate the toxicity of increased salinity to freshwater organisms) exists in the freshwater cladoceran *Daphnia longispina*. For this, two specific objectives were delineated: (i) determine the lethal and sublethal sensitivity of clonal lineages of *D. longispina* to NaCl and (ii) determine if and association exist between lethal resistances to copper and lethal or sublethal resistance to NaCl in clonal lineages of *D. longispina*.

Materials and Methods:

Test organisms:

Four clonal lineages of *Daphnia longispina* O.F. Müller were selected to conduct this work: N91, N116, N31, and E89. These lineages derived from two field populations, one inhabiting a reference site and the other inhabiting an acid mine drainage (AMD) historically impacted site, both located at the aquatic system of an abandoned cupric-pyrite mine (Lopes et al., 2004). The sources of contamination of this aquatic system are hydrogen ions and metals present in the AMD (pH ≈ 2.1, contaminated with Fe, Al, Zn, Cu, Mn, Co, Ni, Cd, Pb, Cr, As, in decreasing order), and no other significant contamination sources are present (Lopes et al. 2004). The four clonal lineages were maintained in laboratory, for more than 1000 generations, under controlled conditions of temperature 19 t 21ºC, and 16:8h L:D photoperiod in American Society for Testing and Materials (ASTM) hardwater (ASTM, 2000), with the addition of vitamins and a standard organic extract "Marinure 25" (Pann Britannica Industries Ltd., Waltham Abbey, UK), an extract from the algae *Ascophyllum nodosum* (Baird et al., 1989). Organisms were fed every day with $3x10^5$ cells/mL/d of the green algae *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz), and water medium was renewed every other day. These four clonal lineages were chosen for this study according to their range of genetically determined resistance to lethal levels of copper, which was previously characterized by Venâncio (2010) (Table 1).

Table 1 – Values of the median lethal concentrations (and the respective 95% confidence limits) of copper for the four clonal lineages of *Daphnia longispina*, after being exposed to this metal for 24 and 48h (adapted from Venâncio, 2010).

	LC50,24h	LC50,48h
N91	281 (235-335)	158 (138-175)
N116	221 (196-280)	141 (133-148)
N31	91.5 (80.0-114)	31.2 (?-?)
E89	27.4 (17.7-37.9)	18.2 (13.1-21.1)

Lethal toxicity assays:

Lethal toxicity assays (cumulative mortality) were carried out with the four clonal lineages by following the standard protocol OECD (2004). Neonates (>6 and <24h old) from the third to the fifth brood were exposed to lethal concentrations of sodium chloride (NaCl) and a control (ASTM hardwater). A stock concentration of 5.2g/L of NaCl (Sigma-Aldrich, St Louis, USA) was prepared by dissolving this salt in ASTM hardwater. A gradient of 5 concentrations (4.5, 4.0, 3.4, 3.0, 2.6g/L NaCl) was then achieved by diluting the initial solution with ASTM hardwater. Five individuals were introduced simultaneously in 42-mL glass vessels filled with 20mL of the test solution, with four replicates per treatment. The assays were performed under the same conditions of temperature and photoperiod as those described above for laboratory cultures, and with no addition of “Marinure” or algae. Organisms were exposed for a period of 48h and immobilization (here considered as mortality; organisms remained immobile during 15 s after gentle prodding) being checked at 24 and 48h. Salinity (HANNA Instruments Seawater Refractometer HI 96822, Woonsocket – RI – USA, Romania), dissolved oxygen (Wissenschaftlich Technische Werkstätten OXI92 oxygen meter, Brüssel, Belgium), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, Brüssel, Belgium), and conductivity (Wissenschaftlich Technische Werkstätten LF92 conductivity meter, Brüssel, Belgium) were measured for each treatment.

Sublethal toxicity assays:

To assess the effects of increased salinity on sublethal responses of the four clonal lineages of *Daphnia longispina* the standard protocol OECD 211 (1998) was followed up. Ten neonates (> 6 and < 24 h old), from the third to the fifth brood, of each clonal lineage were exposed individually, under the same controlled conditions of temperature and photoperiod as for the laboratory cultures, to a range of five NaCl concentrations and a control (ASTM hardwater). Test concentrations ranged between 0.72 and 1.5g/L NaCl (using a dilution factor of 1.2x). These test concentrations were prepared by dissolving NaCl in ASTM hardwater. Each individual was introduced in 42-mL glass vessels filled with 20mL of test solution with the addition of "Marinure 25" and the green algae *P. subcapitata* ($3x10^5$cells/mL/d). Organisms were fed daily, medium being renewed every other day. The following endpoints were monitored: time to first reproduction, total number of neonates produced per female, body size, and the intrinsic rate of natural increase. The assay ended when all the ten individuals in the control released the third brood.
Salinity (HANNA Instruments Seawater Refractometer HI 96822, Woonsocket – RI – USA, Romania), Conductivity (Wissenschaftlich Technische Werkstätten LF92 conductivity meter, Brüssel, Belgium), pH (Wissenschaftlich Technische Werkstätten 537 pH meter, Brüssel, Belgium) and DO (Wissenschaftlich Technische Werkstätten OXI92 oxygen meter, Brüssel, Belgium) were measured at new and old medium during renewal.

Data analysis:

Concentration-response (median lethal concentration; LC50), after 24 and 48 hours of exposure, and the corresponding 95% confidence limits were computed, for each cloned lineage, through probit analysis (Finney, 1971), using the software Priprobit (Sakuma, 1998).

To test the significance of the effects of NaCl on sublethal responses, a one-way analysis of variance was carried out, after testing the normality and homoscedasticity of data, with the Shapiro-Wilks and the Barttlet's tests, respectively. Whenever significant differences were registered, a Dunnett test was applied to determine which concentrations provoked responses significantly different from the control. As well, the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined after applying the Dunnett test. The concentration of NaCl inducing 20 and 50% of reduction in total reproduction was computed using a logistic curve model, using the software Statistica 8.0 (StaSoft, Tulsa, OK, USA).

The intrinsic rate of natural increase (r) was computed for each clonal lineage, after the life history experiment, using Lotka's equation:

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

with l_x being the age-specific survivorship; m_x the number of neonates at day x; and x the age in days. The estimation of the pseudovalues and standard errors for r was done through the jackknifing technique (Meyer et al. 1986).

Finally, to analyze the association between resistance to copper an NaCl, correlation coefficients between lethal responses to copper and lethal and sublethal (EC20 for reproduction; for the clonal lineage E89 the value 1.6g/L was used in the correlation analysis, as no EC20 could be computed) responses to NaCl were calculated using the software STATISTICA version 8.0 (Statsoft).

Results:

Lethal assays

During the lethal assay with the four clonal lineages of *D. longispina* exposed to NaCl, the values of pH ranged from 7.7 and 8.4 and dissolved oxygen was always above 7.4mg/L. The highest variability observed in conductivity, during the assay, was 0.7mS/cm (this variation occurred at the concentration of 5.2g/l NaCl in the assay with clonal lineage E89).

The computed median lethal concentrations (LC50) after 24 and 48h of exposure to NaCl were similar between the four clonal lineages: ranging from 2.87g/L (E89) to 3.61g/L (N91) after 24h and from 2.85g/L (N116) to 2.48g/L (N31) after 48h of exposure (Fig. 3.1.).

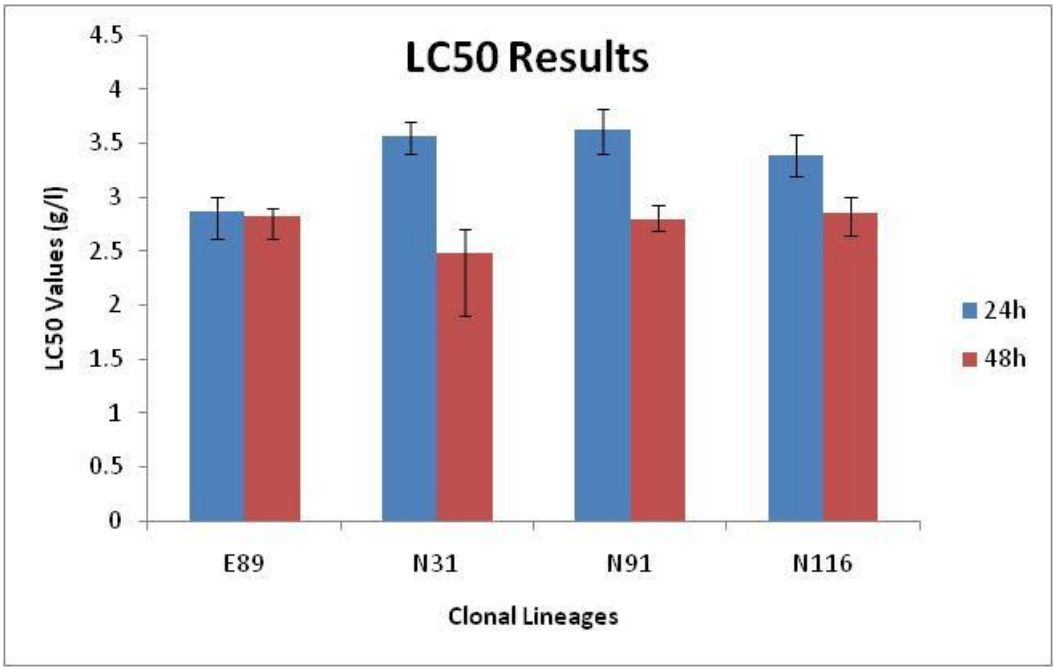

Figure 3.1 – Median lethal concentrations (LC50), with the respective 95% confidence limits (error bars), for clonal lineages of *Daphnia longispina* after being exposed for 24h and for 48h to NaCl concentrations.

Sublethal assays

During the sublethal assays only slightly changes were registered in the physico-chemical parameters that were monitored. The pH values ranged from 8.0 to 8.3; dissolved oxygen was always above 6.9mg/L. The highest variation registered for conductivity was 0.11mS/cm (concentration 1.5g/l of NaCl in assay with clonal lineage N31).

Clonal lineage N31

A significant increase in age at first brood, comparatively with the control, was only observed at the highest concentration of NaCl ($F_{5,53}$=4.0; p = 0.004; followed by Dunnett's: p=0.005) (Fig. 3.2.). The determined no observed effect concentration (NOEC) was 1.2g/L and the lowest effect concentration (LOEC) was 1.5g/L of NaCl.

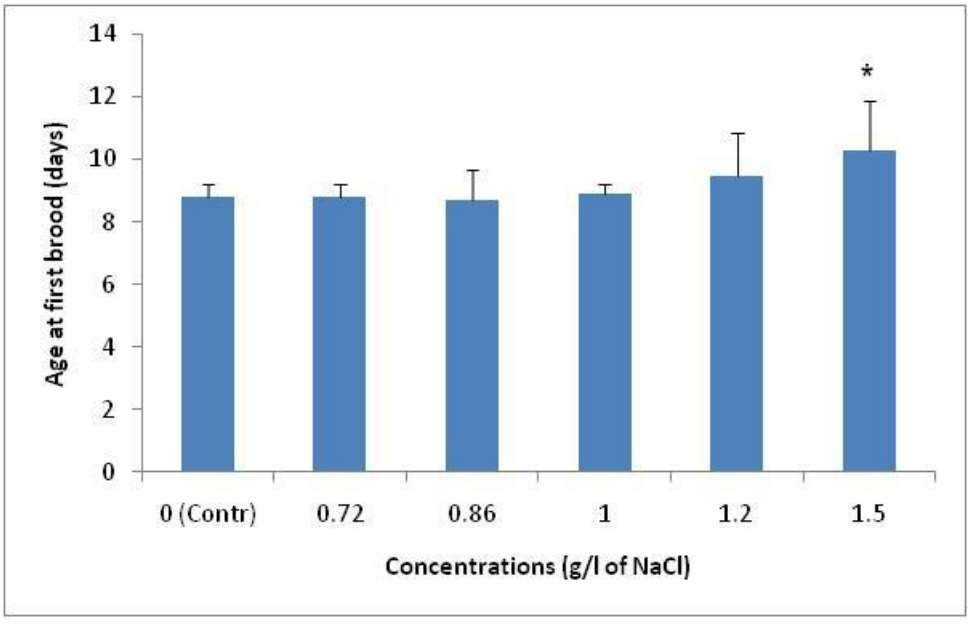

Figure 3.2 – Average of age at first brood (days), with the respective standard deviation (error bars), of clonal lineage N31 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p=0.005).

For clonal lineage N31 a significant decrease in the total number of neonates produced per female was observed at concentrations higher than 1.0g/L of NaCl ($F_{5,53}$=15.001; $p<10^{-5}$ followed by Dunnett's: p≤0.002) (Fig. 3.3). Thus, for this parameter, the NOEC was 1.0 g/l and LOEC was 1.2g/l of NaCl. The EC50 and EC20 computed at the end of the assay for total reproduction were 1.41 g/l (confidence limits: 0.96 to 1.27g/l) and 1.11g/l (confidence limits: 1.30 to 1.5g/l), respectively.

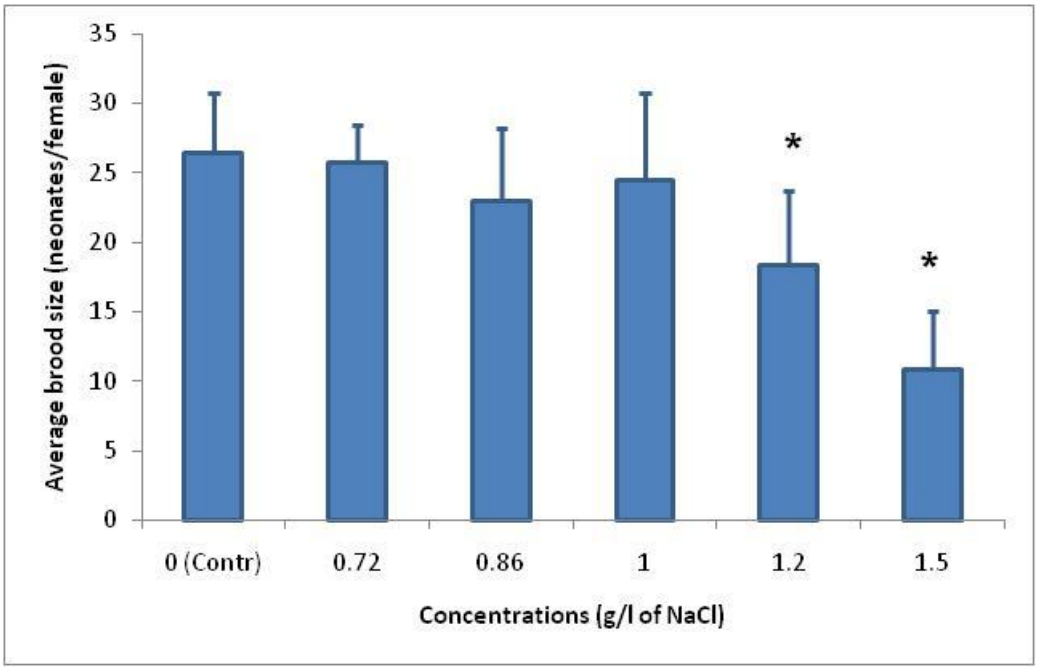

Figure 3.3 – Average brood size (number of neonates per female), with the respective standard deviation (error bars), of clonal lineage N31 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.002).

Regarding body size, females of clonal lineage N31 exposed to concentrations higher than 0.86g/L of NaCl exhibit a significantly smaller body size comparatively to the control ($F_{5,53}$=6.79; p=5.9x10^{-5} followed by Dunnett's: p≤0.01) (Fig. 3.4). Thus, the NOEC and LOEC were, respectively, 0.86 g/l and 1.0g/l of NaCl.

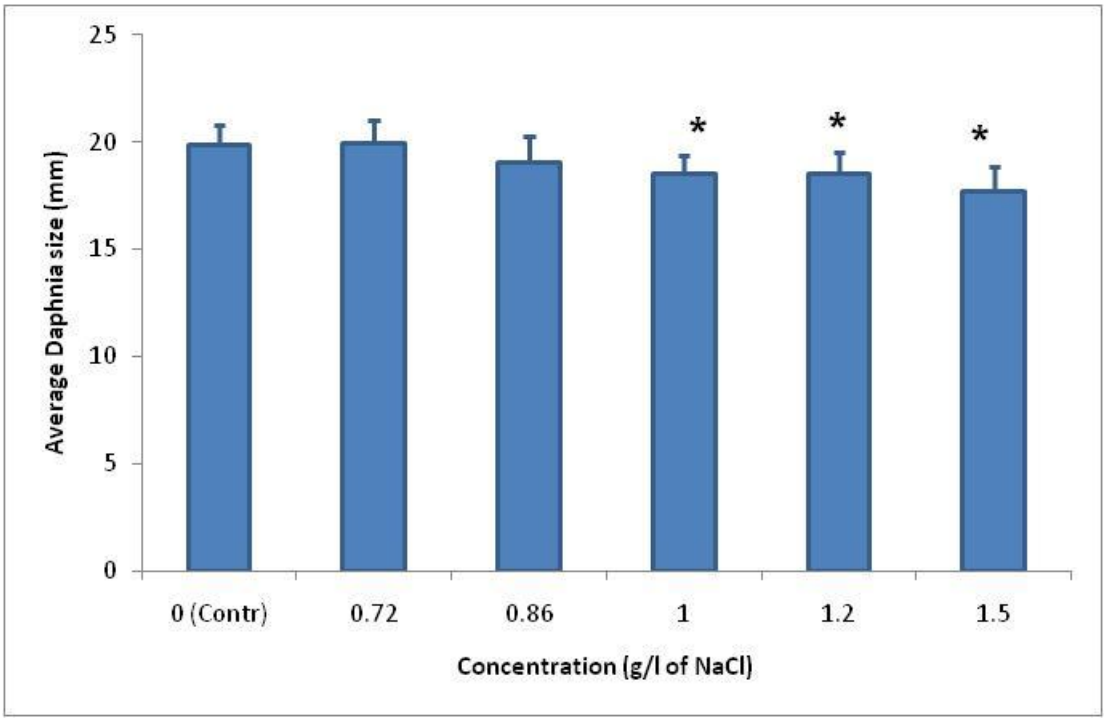

Figure 3.4 – Average body size (mm), with the respective standard deviation (error bars), of the clonal lineage N31 of *Daphnia logispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.01).

All tested concentrations of NaCl provoked a significant decrease in the intrinsic rate of natural increase, relatively to the control ($F_{5,54}$=971.3; p=0.00 followed by Dunnett's: p≤0.002) (Fig. 3.5). Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

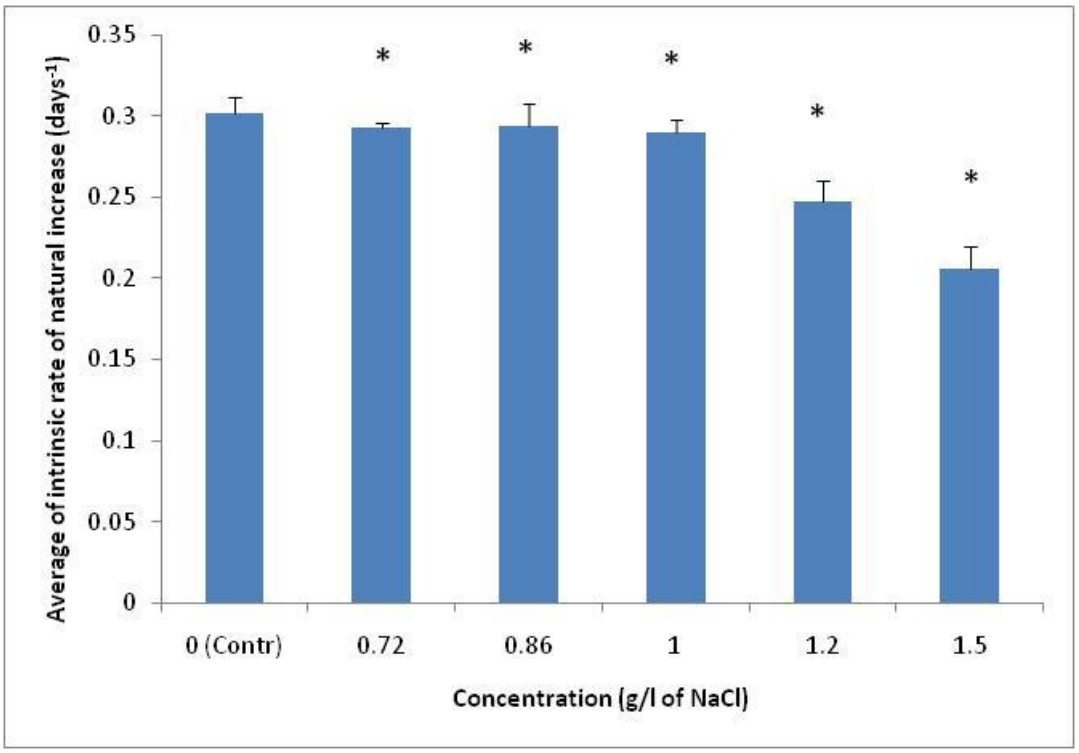

Figure 3.5 – Average of intrinsic rate of natural increase (r), with the respective standard deviation (error bars), of the clonal lineage N31 of *Daphnia logispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.002).

Clonal lineage N91

A significant increase in age at first brood, comparatively with the control, was only observed at the two highest concentration of NaCl ($F_{5,50}$=4.0; p = 0.0001; followed by Dunnett's: p≤0.009) (Fig. 3.6.). The determined NOEC was 1.0g/L and the LOEC was 1.2g/L of NaCl.

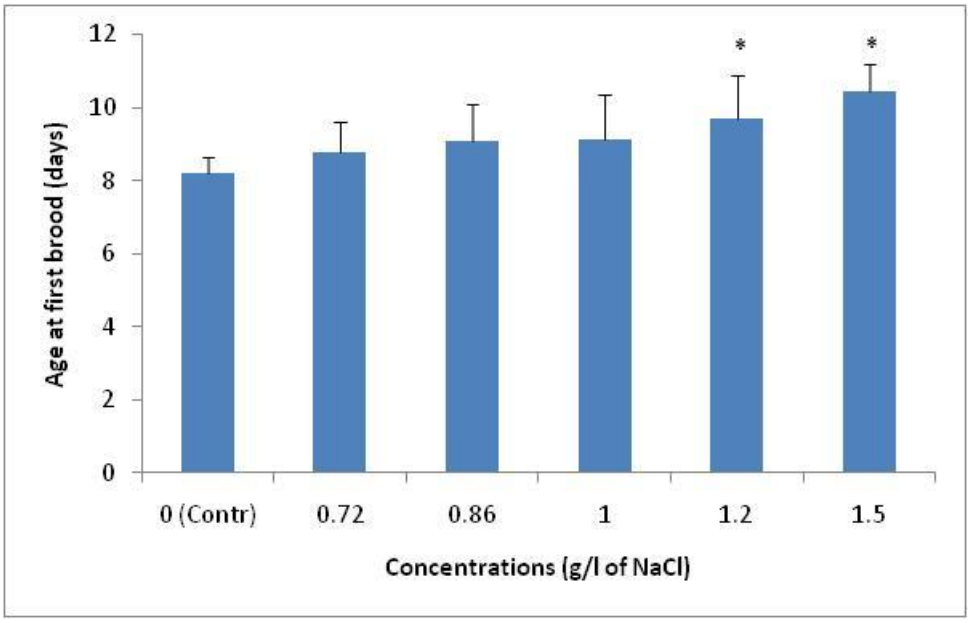

Figure 3.6 – Average of age at first brood (days), with the respective standard deviation (error bars), of clonal lineage N91 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.009).

For clonal lineage N91 a significant decrease in the total number of neonates produced per female was observed at the two highest concentrations of NaCl ($F_{5,50}$=6.96; P=$5.3x10^{-5}$ followed by Dunnett's: p≤0.00) (Fig. 3.7). Thus, for this parameter, the NOEC was 0.86g/l and LOEC was 1.0g/l of NaCl. The EC20 computed at the end of the assay for total reproduction was 0.84 (confidence limits: 0.51g/l to 1.19g/l). The EC50 could not be computed, as a reduction in total number of neonates equal or higher than 50% was not observed.

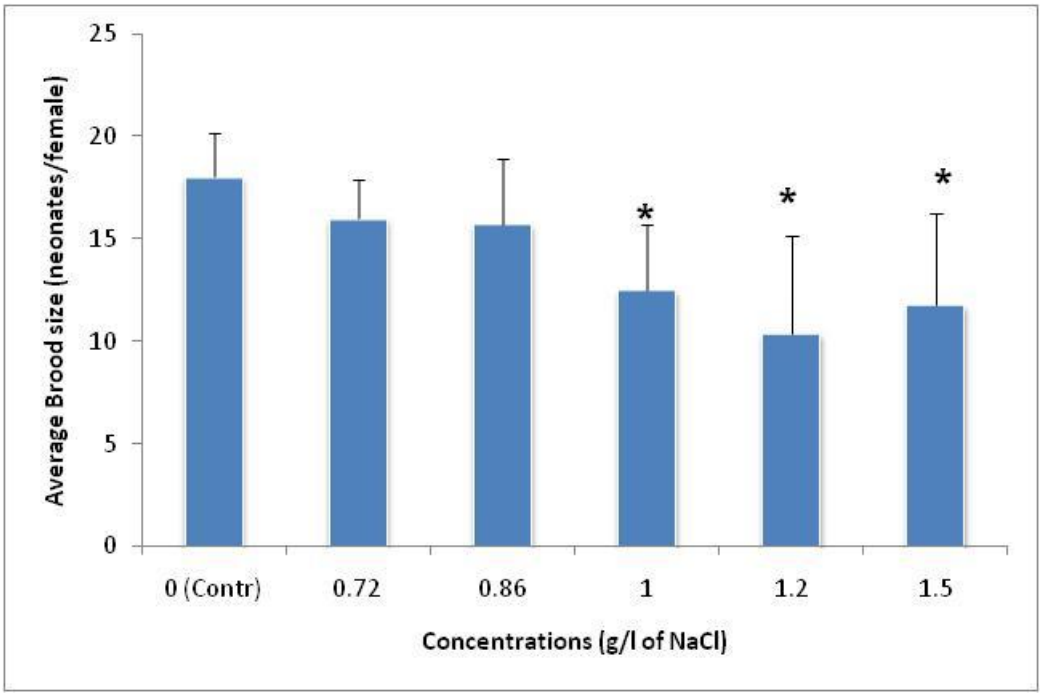

Figure 3.7 - Average brood size (number of neonates per female), with standard deviation (error bars), of clonal lineage N91 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.0035).

Regarding body size, females clonal lineage N91 exposed to concentrations higher than 1.0g/L of NaCl exhibited a significantly smaller body size comparatively to the control ($F_{5,50}$=4.20; P=0.003 followed by Dunnett's: p≤0.004) (Fig. 3.8). Thus, the NOEC and LOEC were, respectively, 1.0g/l and 1.2g/l of NaCl.

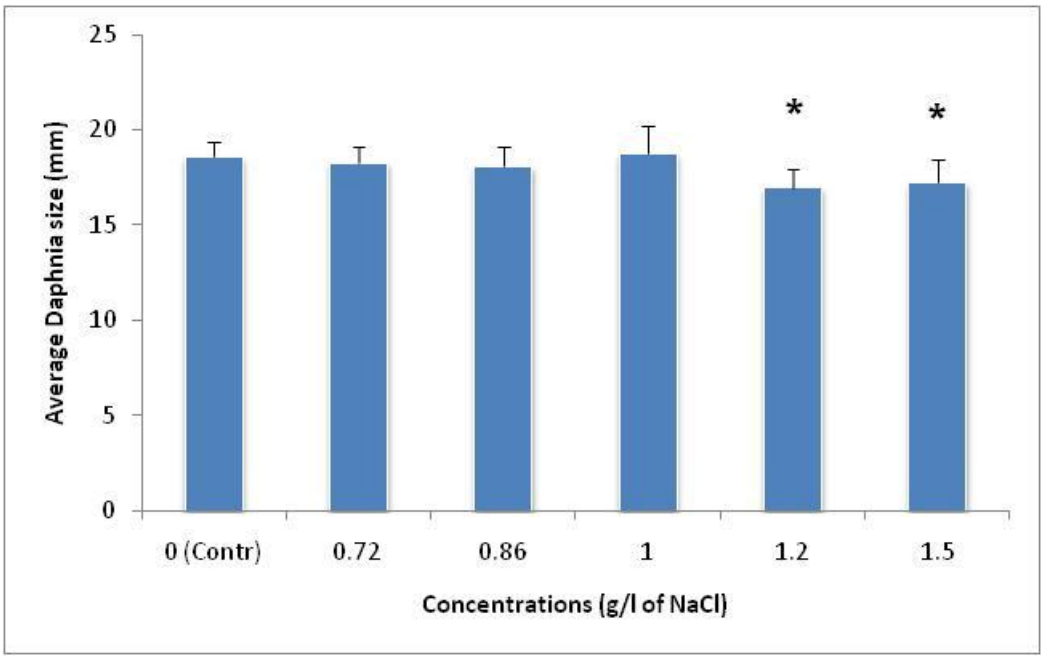

Figure 3.8 - Average body size (mm), with the respective standard deviation (error bars), of the clonal lineage N91 of *Daphnia logispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.004).

All tested concentrations of NaCl provoked a significant decrease in the intrinsic rate of natural increase, relatively to the control ($F_{5,54}$=591; p=0.00 followed by Dunnett's: p≤0.002) (Fig. 3.9). Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

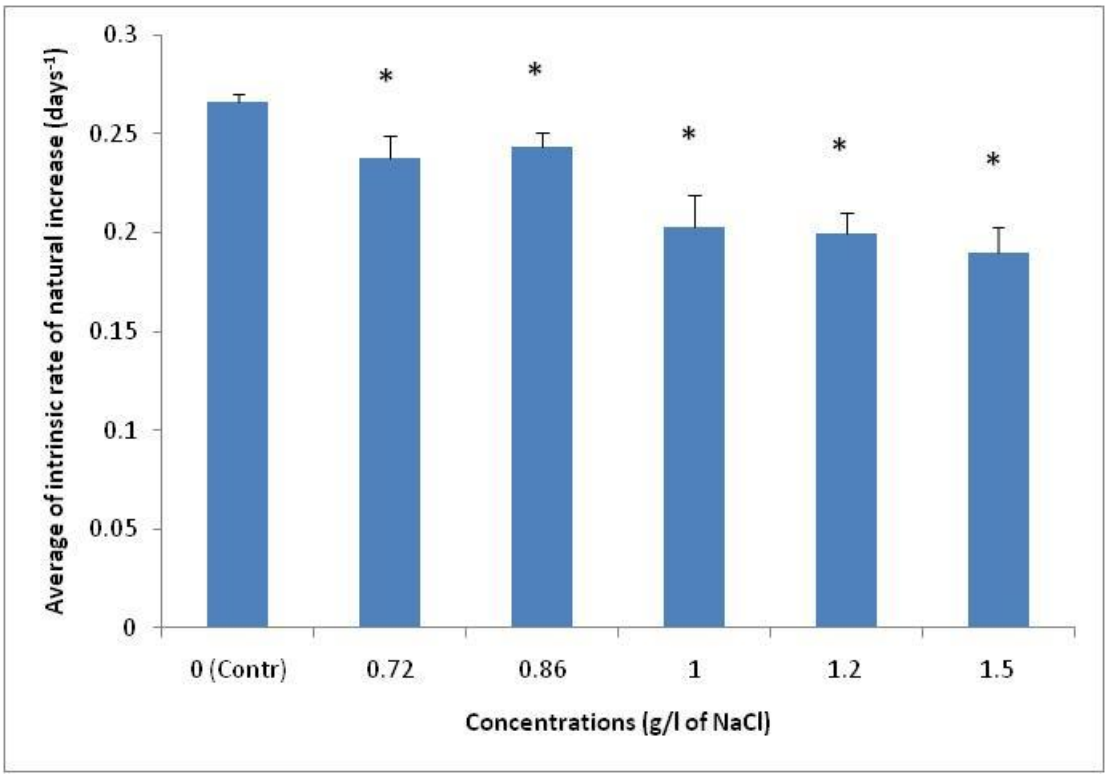

Figure 3.9 – Average of intrinsic rate of natural increase (r), with the respective standard deviation (error bars), of the clonal lineage N91 of *Daphnia logispina* exposed to a gradient of NaCl * symbolizes significant differences relatively to the Control (p≤0,002).

Clonal lineage N116

A significant increase in age at first brood, comparatively with the control, was only observed at the two highest concentration of NaCl ($F_{5,42}$=11.3; p=10^{-6}; followed by Dunnett's: p≤10^{-4}) (Fig. 3.10). The determined NOEC was 1.0g/L and the LOEC was 1.2g/L of NaCl.

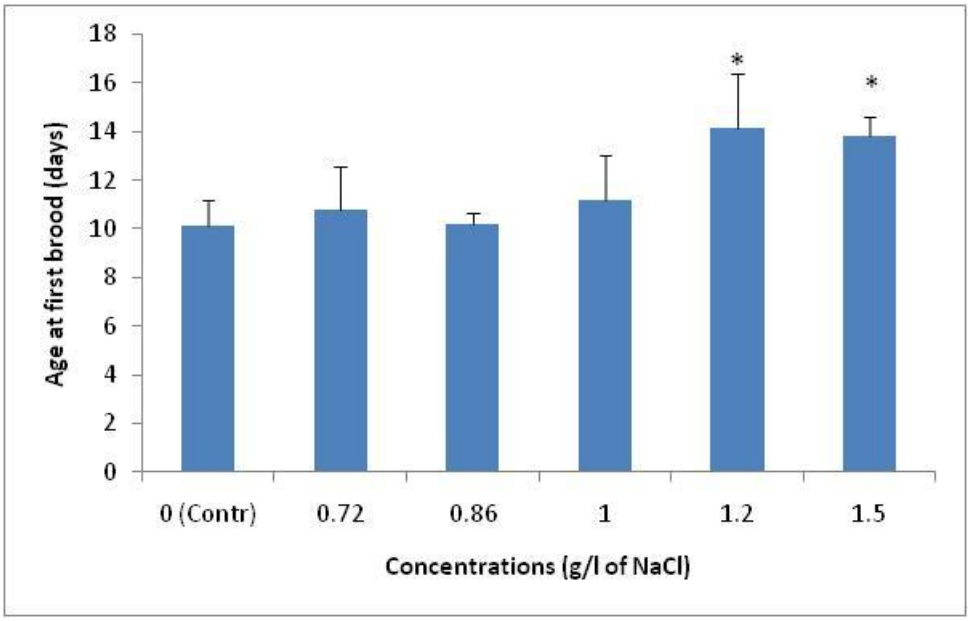

Figure 3.10 – Average of age at first brood (days), with the respective standard deviation (error bars), of clonal lineage N116 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤10^{-4}).

For clonal lineage N116 a significant decrease in the total number of neonates produced per female was observed at the three highest concentrations of NaCl ($F_{5,50}$=14.5; P=0.00 followed by Dunnett's: p≤0.0058) (Fig. 3.11). Thus, for this parameter, the NOEC was 0.86g/l and LOEC was 1.0g/l of NaCl. The EC50 computed at the end of the assay for total reproduction was 1.13 (confidence limits: 1.01g/l to 1.26g/l) and the EC20 was 0.86g/l (confidence limits: 0.70g/l to 1.03g/l).

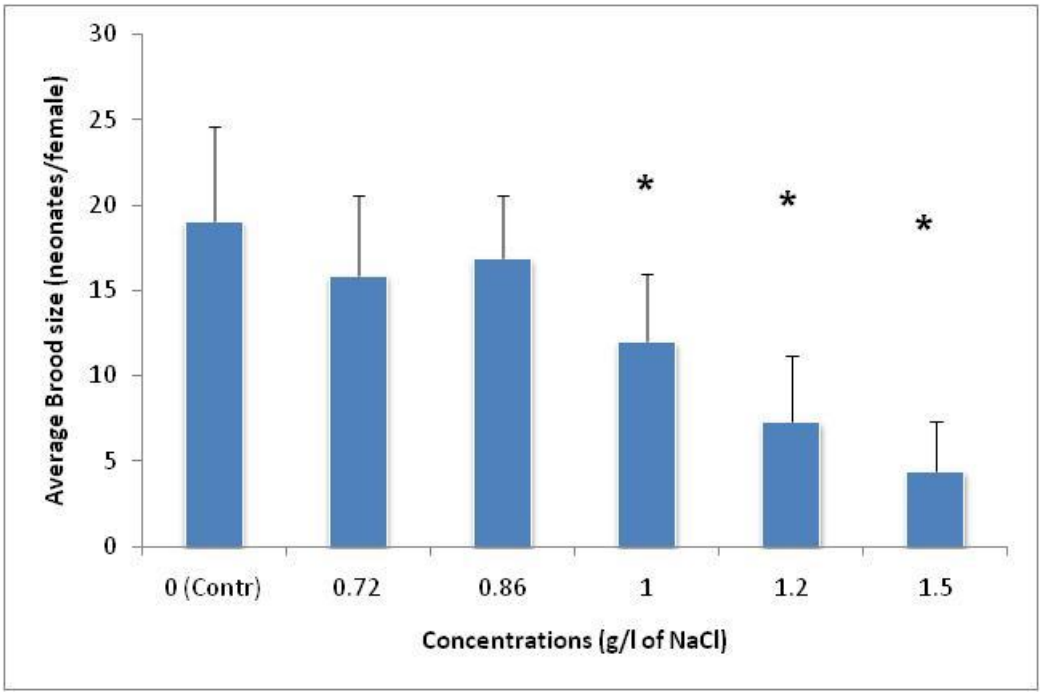

Figure 3.11 - Average brood size (number of neonates per female), with standard deviation (error bars), of clonal lineage N116 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.0058).

Regarding body size, females clonal lineage N116 exposed to all concentrations of NaCl exhibited a significantly smaller body size comparatively to the control ($F_{5,50}$=25.2; p=0.00 followed by Dunnett's: p≤0.0028) (Fig. 3.12). Thus, the NOEC and LOEC were, respectively, 1.0g/l and 1.2g/l of NaCl. Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

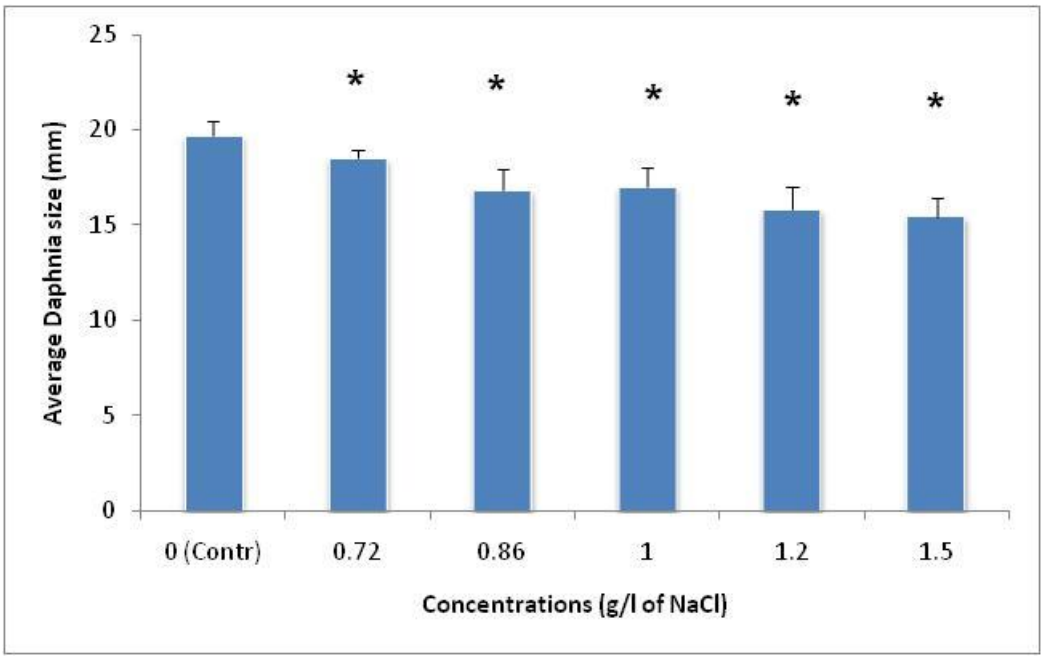

Figure 3.12 - Average body size (mm), with the respective standard deviation (error bars), of the clonal lineage N116 of *Daphnia logispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.0028).

All tested concentrations of NaCl provoked a significant decrease in the intrinsic rate of natural increase, relatively to the control ($F_{5,54}$=1055; p=0.00 followed by Dunnett's: p≤0.002) (Fig. 3.13). Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

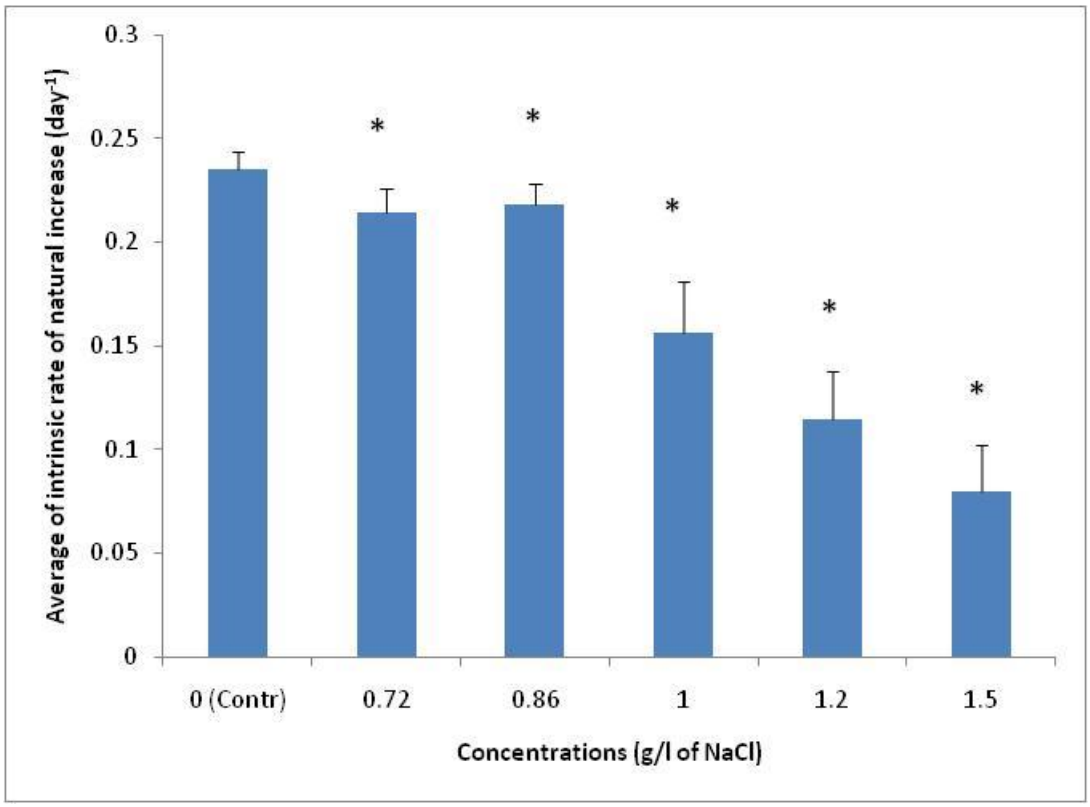

Figure 3.13 – Average of intrinsic rate of natural increase (r), with the respective standard deviation (error bars), of the clonal lineage N116 of *Daphnia logispina* exposed to a gradient of NaCl * symbolizes significant differences relatively to the Control (p≤0.002).

Clonal lineage E89

A significant decrease in age at first brood, comparatively with the control, was observed at NaCl concentrations of 0.86, 1.2, and 1.5g/L ($F_{5,50}$=5.14; p=0.0007; followed by Dunnett's: p≤0.018) (Fig. 3.14). The determined NOEC was 0.72g/L and the LOEC was 0.86g/L of NaCl.

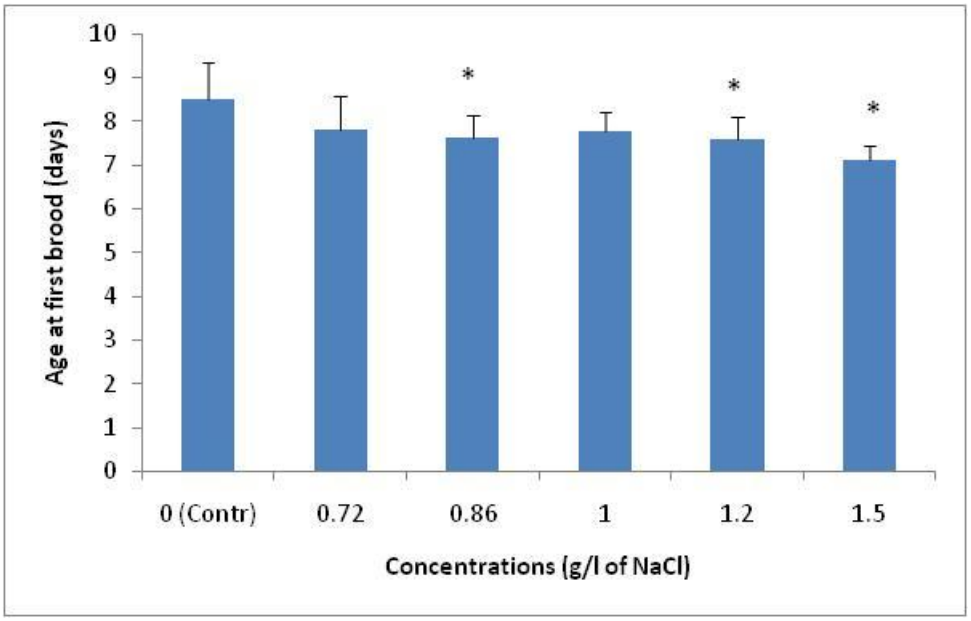

Figure 3.14 – Average of age at first brood (days), with the respective standard deviation (error bars), of clonal lineage E89 of *Daphnia longispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.018).

For clonal lineage E89 a significant decrease in the total number of neonates produced per female was observed at any concentrations of NaCl ($F_{5,50}$=0.70; P=0.62) (Fig. 3.15). Thus, for this parameter, the NOEC was 1.5g/l and LOEC was higher than the highest tested concentration of NaCl.

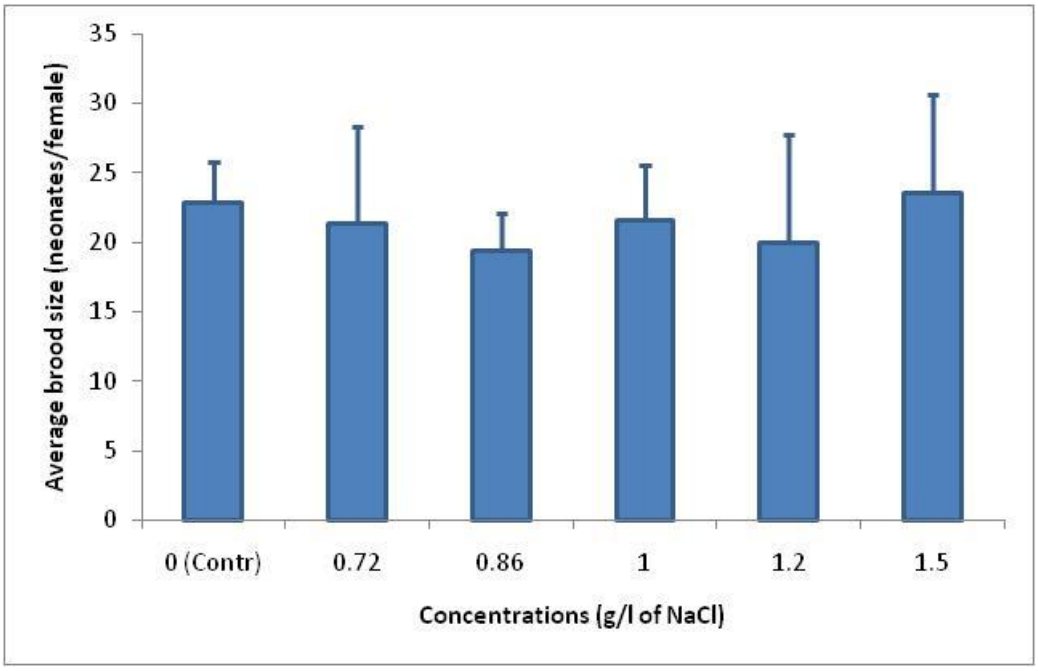

Figure 3.15 - Average brood size (number of neonates per female), with standard deviation (error bars), of clonal lineage E89 of *Daphnia longispina* exposed to a gradient of NaCl.

Regarding body size, females of clonal lineage E89 exposed to all concentrations of NaCl exhibited a significantly smaller body size comparatively to the control ($F_{5,50}$=24.7; p=0.00 followed by Dunnett's: p≤0.00003) (Fig. 3.16). Thus, the NOEC and LOEC were, respectively, 1.0g/l and 1.2g/l of NaCl. Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

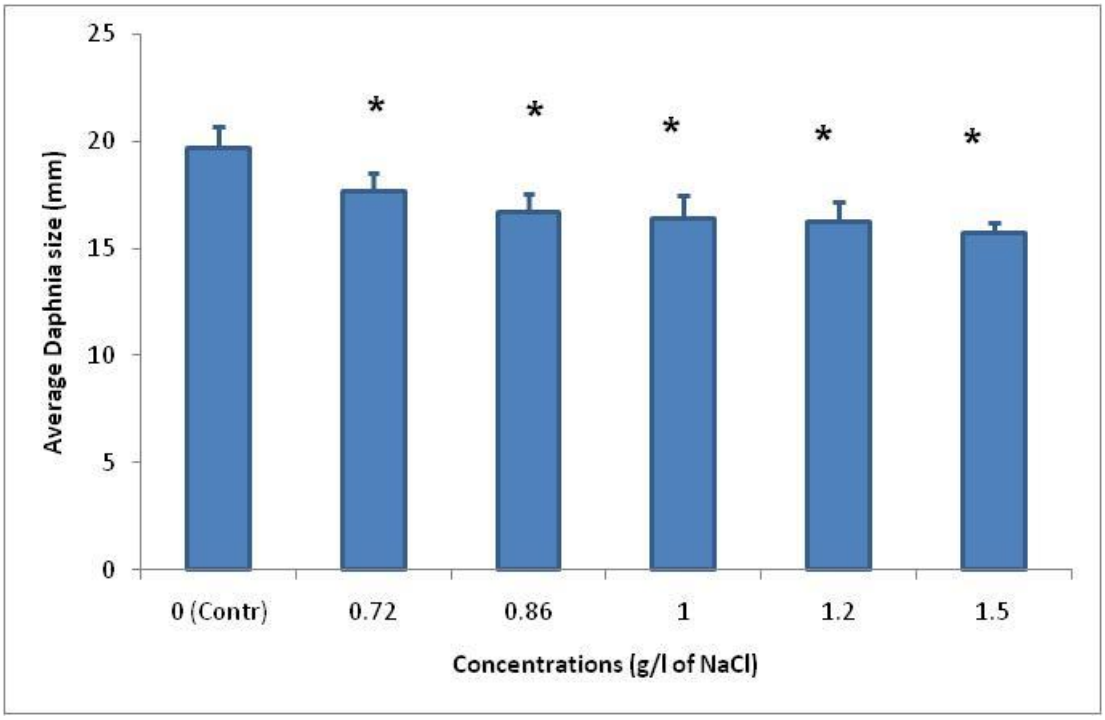

Figure 3.16 - Average body size (mm), with the respective standard deviation (error bars), of the clonal lineage E89 of *Daphnia logispina* exposed to a gradient of NaCl. * symbolizes significant differences relatively to the Control (p≤0.00003).

All tested concentrations of NaCl provoked a significant increase in the intrinsic rate of natural increase, relatively to the control ($F_{5,54}$=68.9; p=0.00 followed by Dunnett's: p≤0.00002) (Fig. 3.17). Thus, the NOEC was lower than the lowest tested concentration and the LOEC was 0.72g/l of NaCl.

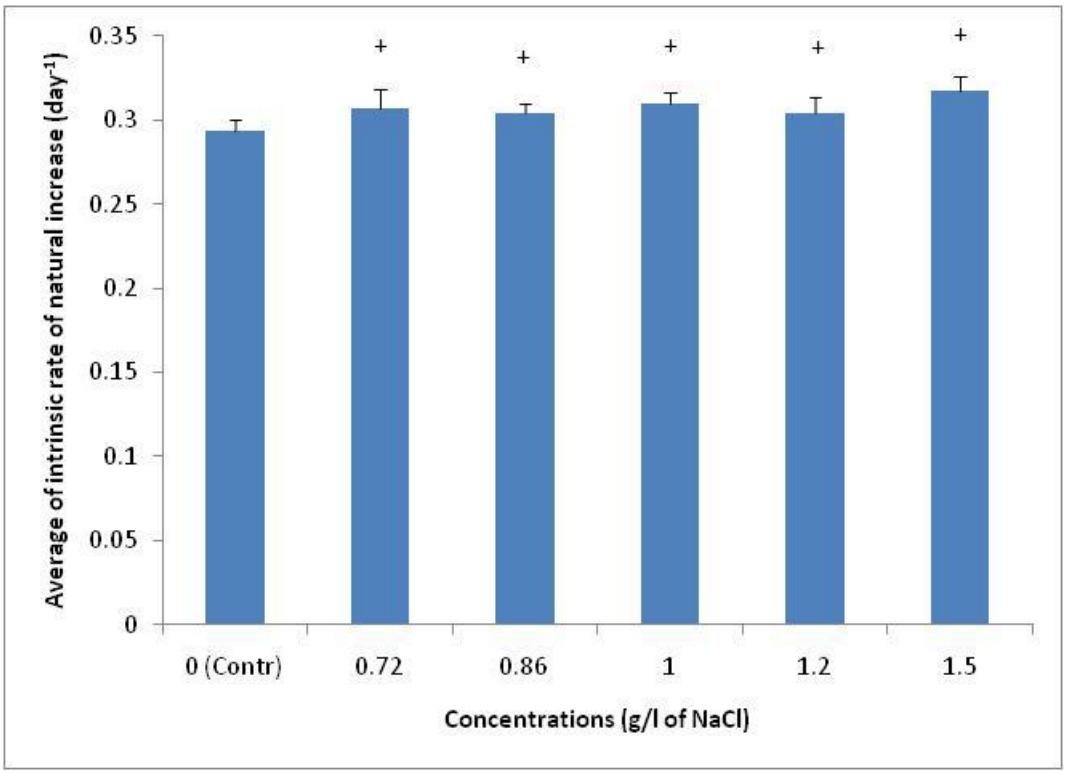

Figure 3.17 – Average of intrinsic rate of natural increase (r), with the respective standard deviation (error bars), of the clonal lineage E89 of *Daphnia logispina* exposed to a gradient of NaCl + symbolizes significant differences relatively to the Control (p≤0.00002).

In general, three of the tested clonal lineages of *D. longispina* (N91, N116, and N31) showed an increase in age at first reproduction, and a significant decrease in the total number of neonates produced per female, in body size and in intrinsic rate of increase when exposed to NaCl (Table 2). Clonal lineage E89, exhibited a different response to this salt. Though a significant decrease was also observed in the body size, no significant effects in the total number of neonates per female, a significant decrease in age at first reproduction, and a significant increase in the intrinsic rate of increase were observed (Table 2).

Table 2 – No observable effect concentration (NOEC) and lowest observed effect concentration (LOEC) values (g/L) determined for the life-history parameters of the four clonal lineages of *Daphnia longispina* exposed to NaCl.

		N91	N116	N31	E89
Age at first brood	NOEC	1.00*	1.00*	1.20*	0.86
	LOEC	1.20*	1.20*	1.50*	0.72
Total number of neonates	NOEC	0.86	0.86	1.00	a
	LOEC	1.00	1.00	1.20	a
Body size	NOEC	1.00	-	0.86	-
	LOEC	1.20	0.72	1.00	0.72
Intrinsic rate of increase	NOEC	-	-	-	-
	LOEC	0.72	0.72	0.72	0.72*

* - A significant increase relatively to the control were registered in these parameter.
a – No significant differences relatively to the control were observed ($p>0.05$).

Association between tolerance to copper and NaCl

No significant correlations were observed between resistance to lethal levels of copper and to lethal levels of NaCl, neither between lethal levels of copper and sublethal levels (EC20 for total reproduction) of NaCl (Table 3).

Though no significant associations were computed between resistance to copper and NaCl, it must be highlighted that the two clonal lineages most resistant to lethal levels of copper (N91 and N116), were the ones exhibiting the highest sensitivity to NaCl for total reproduction (Figs. 3.7 and 3.11).

Table 3. Values of correlation coefficients calculated for the four *Daphnia longispina* clonal lineages exposed to copper and NaCl.

	$LC_{50,48h}$ Cu
$LC_{50,48h}$ NaCl	$r = 0.55$; $p = 0.45$
EC_{20} (total reproduction) NaCl	$r = -0.92$; $p = 0.08$

Discussion:

The tested clonal lineages of *Daphnia longispina* revealed a high sensitivity to both lethal and sublethal levels of NaCl. The range of lethal sensitivities varied between 2.48 (N31) and 2.85g/L (N116), while the concentrations causing 20% of reduction in total reproduction varied between 0.84 (N91) and values higher than 1.5g/L (E89). But, lower sublethal concentrations of NaCl (0.72g/L) were able to induced significant effects in life-history parameters, namely body size and age at first reproduction. The results obtained in the present study, for lethal resistance to NaCl, were similar to those reported by Gonçalves et al. (2007) for another clone of *D. longispina* (an $LC_{50,48h}$ of 2.9g/L NaCl). However, the sublethal sensitivity of the clonal lineages here tested was slightly higher than that reported by Gonçalves et al. (2007) (LOEC values for the life history parameters always ≥1.71 g/L of NaCl). This is not a surprising conclusion, since a high intraspecific variability in responses to contaminants may exist (e.g. for cladocerans: Baird et al., 1990; Soares et al., 1992; Barata et al., 2000). Furthermore, the sensitivity of *D. longispina* to lethal and sublethal levels of NaCl is within the range found for other cladocerans. *Daphnia longispina* being more tolerant than, for example, *Ceriodaphnia dubia* ($LC_{50,48h}$ = 1.59g/L and EC50 for reproduction =1.35g/L) and *Daphnia ambigua* ($LC_{50,48h}$ = 2.00g/L and EC50 for reproduction =0.65g/L), and being more sensitive to NaCl than *D. magna* (Harmon et al., 2003; Gonçalves et al., 2007; Martinez-Jerónimo and Martinez-Jerónimo, 2007; please see also data from chapter 2), a standard species commonly used for ecotoxicological assessment.

Regarding an association between resistance to copper and to NaCl, after a literature revision (using the following combinations: Cu AND NaCl co-tolerance or resistance; copper AND NaCl multiple tolerance or resistance; NaCl tolerance or resistance; copper tolerance or resistance; chemcical co- or multiple tolerance or resistance) any work was found addressing a possible association in resistance between these two chemical. However, some works reporting the existence of co-tolerance between NaCl and other cations, or copper and other metals have been published (e.g. Tilstone and Macnair, 1997; Shah et al., 2002; Kobayashi et al., 2004; Lopes et al., 2005; Guo et al., 2008). As some examples, Langdon et

al.(1999) showed that the earthworm *Lumbricus rubellus* inhabiting a arsenic–contaminated soils developed resistance to this metal. In follow-up studies, Langdon et al. (2001) showed that this population of earthworms also exhibited increased resistance to copper. Furthermore, Hodson et al. (1981) compared the sensitivity of clones of the grass *Agrostis stolonifera*, from a salt marsh and an inland ecosystem to several ions (e.g. lithium, potassium, rubidium, caesium, magnesium, and calcium), and observed that the former was always more tolerant than the inland one.

In the present study, a significant association between lethal resistance to copper and lethal or sublethal resistance to NaCl was not observed among the studied clonal lineages of *D. longispina*, thus, suggesting the presence of different mechanisms responsible for the resistance to the two chemicals. Nevertheless, though not being statistically significant, the correlation coefficient obtained between lethal resistance to copper and sublethal resistance to NaCl (EC20 for total reproduction) was high. Probably, the lack of a significant correlation between these two responses was related with the small number of clonal lineages that were tested. These results agree with other works, which hypothesized that resistance to high ion concentrations may involve specific mechanisms, such as metallothionein production and changes in enzymatic activities, while sublethal responses, namely feeding behavior, involve more generalist mechanisms, including filtering rates (dependent onfilter screens, mesh sizes, and appendages beat rates), ingestion, and the physiology of the gut (Roesijadi, 1992; Hoffmann and Parsons, 1994; Macnair, 1997; Barata et al., 2000).

Though no association between copper and NaCl was observed in this study, the two lineages more resistant to lethal levels of copper revealed the lowest EC20 for total reproduction when exposed to NaCl. These results may evoke implications for ecological risk assessment, as it may suggest that a population genetically eroded by copper contamination may be at a higher risk if exposed for long periods of NaCl contamination. However, aiming for a better comprehension of a possible association between resistance to Cu and NaCl, further studies should be carried out with a higher number of clonal lineages of *D. longispina*. Despite this fact, the results obtained within this study highlight the

possibility of scenarios of great risk of extinction in genetically eroded populations due to inverse resistance between chemicals.

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Chapter 4

General conclusions:

Salinisation of coastal freshwater ecosystems due to sea level rise is currently a worldwide major concern. It is predicted that global sea level rise will lead to surface flooding and salt intrusions through groundwater in coastal freshwater lagoons, causing adverse effects in biota inhabiting these ecosystems, namely contributing to a decrease in the biodiversity (Hall & Burns 2003, Schallenberg et al. 2003). Actually, such adverse effects have already been reported by several case studies (Lyons et al., 2007; Santangelo et al., 2007; Anthony et al., 2009). For example, Heine-Fuster et al. (2010) reported that in a coastal freshwater ecosystem in Chile, that is experiencing an increase in salinity, the cladoceran *Daphnia exilis*, which has a high tolerance to salinity, is outcompeting the native species of cladocerans (exhibiting a lower sensitivity to salinity). As these coastal freshwater lagoons commonly constitute protected habitats holding a large biodiversity, it is imperative to understand how secondary salinisation will affect them. Several works have already been carried out in order to understand the tolerance of aquatic biota inhabiting these ecosystems to salinity (e.g. James et al, 2003; Gonçalves et al., 2007; Nielsen et al., 2008). Most of these studies were carried out using NaCl to establish the increased salinity. The results obtained in the present work (Chapter 2) showed that the use of NaCl as a surrogate to assess the effects of increased salinity in freshwater biota constitutes a worst case scenario, as the two tested species (*Pseudokirchenriella subcapitata* and *Daphnia magna*) exhibited a higher sensitivity to NaCl than to natural seawater. Other authors reported similar results when comparing the tolerance of freshwater biota to NaCl and to artificial seawater (Kefford et al., 2004); tested species being more tolerant to the artificial seawater. The use of artificial seawater would be advantageous over NaCl, when evaluating the effects of salinity in freshwater biota, as its chemical composition is similar to that of natural seawater. In addition, it would present an advantage over natural sweater, since its chemical composition can be controlled and therefore would enable the comparison of

results between different works. However, Kefford et al. (2004) reported that the lethal sensitivity of *D. carinata* to artificial seawater lead to an under-estimation of the toxicity of three saline lakes, though their ionic proportion being similar to that of the artificial seawater. Therefore, it is suggested that the use of NaCl (which is a major constituent of natural seawater) would be a more protective surrogate to evaluate the effects of secondary salinisation in coastal freshwater ecosystems.

Another issue to consider when assessing the effects of increased salinity in coastal freshwater lagoons is that some of these aquatic systems have already been historically exposed to chemical contamination. Depending on the intensity of the contamination, natural population historically exposed to such chemical contamination may have undergone genetic erosion through the elimination of the most sensitive genotypes to that particular contamination (e.g. van Straalen and Timmermans; Lopes et al., 2009). Whether the resistant genotypes remaining in the population are also resistant to other types of contamination, namely increased salinity, will determine the survival and persistence of the population under future exposure to secondary salinisation. The results obtained in Chapter 3 with clonal lineages of *Daphnia longispina* exhibiting different lethal sensitivities to copper, showed any association between resistance to copper and NaCl. Thus, suggesting the inexistence of multiple or co-resistance in responses to these two chemicals. However, it was observed that the two most resistant clones to lethal levels of copper exhibited the highest sensitive responses, for total reproduction, when exposed to NaCl. Thus, suggesting, that though no association was found between resistance to copper and NaCl, genetically eroded populations due to copper exposure may be at a higher risk under future long period exposure to NaCl contamination.

Finally, though it was not an objective of this work, during this work it was observed that the cladoceran *Daphina longispina* was more sensitive to salinity than the surrogate species *Daphnia magna*. These results are in line with those obtained by Gonçalves et al. (2007), and suggests that care should be taken when using surrogate species to predict effects in natural populations as an under-estimation of risk may occur.

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