



# DEPARTAMENTO DE ZOOLOGIA

FACULDADE DE CIÊNCIAS E TECNOLOGIA  
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## **Efeitos ecológicos em espécies de água doce sob *stress* salino**

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Mário Jorge Pereira (Universidade de Aveiro) e do Professor Doutor Miguel Ângelo Pardal (Universidade de Coimbra).

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## RESUMO

O estudo da intrusão salina tem-se tornado um problema cada vez mais preocupante, uma vez que afecta, em termos mundiais, vários sistemas de água doce. O incremento de salinidade nesses ecossistemas, resultante de alterações ambientais, faz com que as espécies sob *stress* salino desenvolvam mecanismos de adaptação a essas mesmas condições. Assim, no desenvolvimento deste trabalho foram usadas três espécies de microalgas verdes – *Pseudokirchneriella subcapitata* (Korshikov) Hindak, *Chlorella vulgaris* Beijerinck e *Pandorina morum* (Müller) Bory – e duas espécies de cladóceros – *Daphnia magna* Straus e *Daphnia longispina* O. F. Müller. Na primeira parte do trabalho foi desenvolvida uma metodologia aplicável em testes de inibição de crescimento algal tendo em conta a minimização dos gastos e rentabilização de todo o procedimento laboratorial, mantendo o rigor e a fiabilidade dos dados. Estatisticamente fez-se a análise de variância (ANOVA) de uma via e, no caso de haver diferenças significativas, aplicou-se o teste de Tukey. O coeficiente de correlação de Pearson foi aplicado para comparar os vários métodos de determinação da biomassa (densidade óptica, concentração da clorofila *a*, peso seco e número de células). Esta análise permitiu identificar diferenças significativas apenas no crescimento da espécie *Chlorella vulgaris*, quando submetida a condições nutritivas inferiores a 50% de MBL. A aplicação da correlação de Pearson permitiu verificar uma elevada correlação entre os parâmetros de biomassa medidos para as três espécies de microalgas possibilitando, assim, escolher o parâmetro mais prático e menos dispendioso. Na segunda parte do trabalho pretendeu-se avaliar o impacto do incremento de salinidade nas cinco espécies planctónicas representativas dos primeiros níveis tróficos da cadeia alimentar, tendo-se definido concentrações de salinidade para os vários grupos de organismos. Neste estudo foi aplicada a metodologia desenvolvida nos testes de microalgas, tendo-se utilizado,



neste caso, dois tipos de meio salino: meio sintético (NaCl) e meio marinho. A análise estatística permitiu verificar diferenças significativas com o incremento de salinidade, através da aplicação da análise de variância (ANOVA) de uma via e do teste Tukey, quando se verificavam diferenças significativas. Deste modo, a alga mais halotolerante foi *C. vulgaris* e a mais sensível *P. morum*, sendo ambas as espécies mais halotolerantes em meio marinho do que em meio sintético. Relativamente às espécies de cladóceros, realizaram-se testes agudos e crónicos com meio sintético (NaCl). Aplicou-se a análise de variância (ANOVA) unifactorial aos dados obtidos nos testes crónicos e, no caso de se verificarem diferenças significativas, aplicou-se o teste de Dunnett. Os valores de  $CE_{50}$ <sup>1</sup> para as diferentes espécies foram calculados através da análise Probit.

*Daphnia magna* revelou ser mais halotolerante do que *Daphnia longispina*, no entanto, ambas as espécies revelaram sensibilidades agudas e crónicas ao incremento salino. *D. magna* apresentou diferenças significativas nas concentrações mais elevadas, enquanto que *D. longispina* apresentou diferenças significativas nas primeiras concentrações de NaCl.

Neste trabalho estabeleceu-se uma metodologia aplicável em testes de inibição de crescimento algal para três espécies de algas de água doce, rentabilizando o tempo e o material necessário à sua execução. Este trabalho permitiu também avaliar a tolerância de cinco espécies planctónicas de ecossistemas de água doce, estimando-se o efeito do incremento de salinidade no crescimento de espécies algais e de cladóceros e também na reprodução destes.

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<sup>1</sup>  $CE_{50}$  – Concentração efectiva a 50%. Concentração que provoca 50% de efeito (mortalidade, fecundidade, crescimento) na população de organismos teste. No caso das microalgas, a  $CE_{50}$  representa a concentração de tóxico que provoca 50% de inibição do crescimento algal relativamente ao controlo. No caso dos cladóceros, a  $CE_{50}$  aguda representa a concentração de tóxico que provoca 50% de imobilização; a  $CE_{50}$  crónica representa a concentração de tóxico que provoca 50% de inibição reprodutiva relativamente ao controlo. É o equivalente ao  $EC_{50}$ , da língua inglesa.

## ABSTRACT

Salinity is one of the threats to freshwater ecosystems and is considered an environmental key factor changing aquatic community structure. Thus, some species develop mechanisms in order to adapt to salinity conditions, to survive and reproduce. Therefore, in this work was used three freshwater green algae – *Pseudokirchneriella subcapitata* (Korshikov) Hindak, *Chorella vulgaris* Beijerinck e *Pandorina morum* (Müller) Bory – and two cladocerans species – *Daphnia magna* Straus e *Daphnia longispina* O. F. Müller. The main aim of the first part of the work was to establish an easier and less expensive methodology with fewer requirements to perform algal inhibition tests. The results of the different treatments were compared using analysis of variance (ANOVA). If applicable, a Tukey multiple comparison test was applied with statistically significant differences in growth reported for  $p < 0.05$ . The coefficient of correlation of Pearson was performed to compare the different methods of biomass determination (optical density, chlorophyll *a*, dry weight and cell counting). The results showed no significant differences at MBL medium dilution of 50% to *P. subcapitata* and *P. morum*. The growth of *C. vulgaris* was not significant different under 60% MBL medium dilution. Thus, the biomass parameters measured for these algae showed high correlation among them and it was chosen the most adequate for each alga. At the second part of this work was studied the effect of the increase of salinity in the five planktonic species. It was applied the methodology established in tests of algae inhibition growth. The results obtained were compared by variance analysis (ANOVA). A Tukey multiple comparison test and a Dunnett test were applied to the data of algae tests and cladocerans chronic tests respectively, when statistically significant differences were reported ( $p < 0.05$ ). The  $EC_{50}$  values were calculated by Probit analysis. The results proved salinity affects the growth of these five species and also the reproduction of

*Daphnia* spp.. Thus, the sensitiveness differs between them, being some species more halotolerant than others.

In this work was established a methodology to perform inhibition tests for three freshwater green algae. It was also evaluated the effect of the raise of salinity in five freshwater species.

Capítulo 1

Introdução



## 1.1 – Intrusão salina em ecossistemas aquáticos

A intrusão salina em sistemas de água doce é um problema que se tem tornado cada vez mais preocupante devido às constantes alterações ambientais. Factores geológicos e climáticos como a meteorização das rochas, a subida do nível do mar, o transporte por acção das chuvas e do vento e a evaporação têm contribuído significativamente para a salinização em ecossistemas de água doce. Estes *habitats* caracterizam-se por águas com salinidade inferior a  $0,5 \text{ g.L}^{-1}$  (Hammer et al., 1983). Assim, as espécies destes ecossistemas toleram, geralmente, níveis muito baixos ou praticamente nulos de salinidade, sendo notórios efeitos adversos na comunidade biótica em gamas de salinidade superiores a  $1 \text{ g.L}^{-1}$  (Nielsen et al., 2003). Por outro lado, a sensibilidade das espécies é variável quando sujeitas a condições de *stress* salino, havendo espécies mais halotolerantes do que outras. Assim, à medida que a salinidade aumenta, a riqueza específica e abundância das espécies diminui, alterando a funcionalidade e a integridade dos ecossistemas (Schallenberg et al., 2003). A salinidade altera a capacidade osmorreguladora destes organismos, os quais têm de desenvolver mecanismos de adaptação ou evitarem o incremento salino de modo a sobreviverem em águas sujeitas a variações salinas. Alguns estudos realizados com espécies planctónicas revelaram adaptações genéticas e/ou fisiológicas a condições de *stress* salino (Avron, 1986; Fisher et al., 1994; Rokneddine & Chentoufi, 2004), permitindo a estes organismos tolerar elevadas concentrações de salinidade. Embora existam factores ambientais que alteram o comportamento de determinadas espécies aquáticas como a temperatura, o pH, a luminosidade e a concentração de oxigénio dissolvido, a salinidade é considerada o factor chave na determinação da estrutura das comunidades aquáticas de água doce. Nestes sistemas, a salinização pode provocar

alterações nos ciclos de nutrientes e de energia levando à formação de *blooms* algais (Nielsen et al., 2003).

## **1.2 – Importância dos sistemas de água doce e das comunidades planctónicas**

Os sistemas de água doce ocupam uma porção inferior à ocupada pelos sistemas marinhos e terrestres. No entanto, aqueles *habitats* são de crucial importância para o Homem dado fornecerem a água para consumo e serem importantes nas actividades domésticas, agrícolas e industriais, não necessitando de sofrer um processo de dessalinização como aconteceria no uso de águas marinhas (Odum, 1997). Por outro lado, os sistemas de água doce caracterizam-se por uma elevada biodiversidade e produtividade biológica, as quais podem sofrer flutuações devido a factores ambientais, principalmente salinos (Odum, 1997; Scallenberg et al., 2003; Rokneddine & Chentoufi, 2004).

O estudo das comunidades fitoplanctónicas revelou-se de extrema importância dado ocuparem a base da cadeia alimentar, sendo uma das principais fontes de energia e matéria dos sistemas aquáticos. Alterações ao nível dos produtores primários repercutir-se-ão nos níveis tróficos superiores, tornando-se indispensável o estudo deste grupo de organismos para inferir acerca das restantes unidades básicas da cadeia trófica e, posteriormente, estudar esses níveis superiores. Por outro lado, o zooplâncton ocupa o nível trófico imediatamente a seguir ao dos produtores (herbívoros), dependendo grandemente daqueles. Os organismos zooplanctónicos são frequentemente utilizado como indicadores da qualidade da água e a sua composição é fortemente afectada por gradientes de mineralização (Odum, 1997; Boronat et al., 2001). Deste modo, a

utilização de espécies fitoplanctónicas e zooplanctónicas é fundamental no estudo da alteração das condições ambientais (Odum, 1997; Boronat et al., 2001). Por outro lado, a cultura de espécies planctónicas em laboratório é relativamente fácil, sendo algumas espécies consideradas boas bioindicadoras e usadas regularmente em testes ecotoxicológicos (ASTM, 2002; OECD, 2002; USEPA, 2002).

### **1.3 – Objectivos**

As espécies de microalgas de água doce são muito usadas em testes ecotoxicológicos apresentando diferentes sensibilidades de acordo com o tóxico ou efluente utilizado (ASTM, 2002; OECD, 2002; USEPA, 2002; de Figueiredo et al., 2004). Assim, é fundamental a utilização de várias espécies algais em estudos de inibição do crescimento algal, sendo esta prática laboratorial extremamente dispendiosa e morosa. Deste modo, tornou-se importante o desenvolvimento de uma metodologia aplicável tendo em conta a minimização dos gastos e rentabilização de todo o procedimento laboratorial, mantendo o rigor e a fiabilidade dos dados.

O estudo da intrusão salina em sistemas de água doce é extremamente importante na avaliação da haloterância e na comparação da sensibilidade das espécies integrantes desses ecossistemas, permitindo inferir sobre o efeito do incremento salino em comunidades de água doce. Alguns trabalhos foram já desenvolvidos relativamente ao efeito do incremento de salinidade em espécies de água doce (Zeng & Vonshak, 1998; Barron et al., 2002; Moisander et al., 2002; Nielsen et al., 2003; Sbrilli et al., 2003; Schallenberg et al., 2003; Rokneddine & Chentoufi, 2004). No entanto, a informação disponível relativamente ao efeito do incremento salino na integridade dos ecossistemas é escassa. Por outro lado, os trabalhos realizados neste âmbito não



utilizam, normalmente, espécies fitoplanctónicas e zooplanctónicas em simultâneo, e poucos estudos têm sido feitos para as espécies usadas nesta investigação. Assim, pretendeu-se avaliar a sensibilidade de cinco espécies planctónicas, de sistemas de água doce, ao incremento da salinidade bem como analisar o seu efeito no crescimento de microalgas verdes e no crescimento e reprodução de cladóceros.

Neste trabalho serão abordados os seguintes objectivos específicos:

- ↻ Desenvolver uma metodologia aplicável em estudo da inibição do crescimento algal, de modo a rentabilizar os gastos e todo o procedimento laboratorial;
- ↻ Avaliar o crescimento algal em diferentes condições nutritivas;
- ↻ Avaliar a halotolerância de espécies planctónicas ao incremento da salinidade;
- ↻ Avaliar o efeito da salinidade no crescimento algal;
- ↻ Avaliar o efeito da salinidade no crescimento e reprodução de cladóceros;
- ↻ Calcular os valores de  $CE_{50}$  para três espécies de microalgas e duas espécies de cladóceros.

Capítulo 2

Material e métodos



## 2.1 – Espécies teste e condições de cultura

Os testes laboratoriais foram realizados para três espécies de microalgas pertencentes à Divisão Chlorophyta (classe Chlorophyceae) (van den Hoek et al., 1998) e para duas espécies de cladóceros (Phylum Arthropoda, classe Cladocera) (Alonso, 1996).

### 2.1.1 – Microalgas

Foram utilizadas três culturas de microalgas de água doce obtidas através de Carolina Biological Supply Company<sup>®</sup> ou isoladas por micromanipulação, após terem sido recolhidas no campo. As espécies algais pertencentes à família Chlorophyceae – *Pseudokirchneriella subcapitata* (Korshikov) Hindak, vulgarmente designada por *Selenastrum capricornutum*, *Chorella vulgaris* Beijerinck e *Pandorina morum* (Müller) Bory foram utilizadas com o intuito de testar o efeito de diferentes condições nutritivas, de diferentes capacidades volumétricas (utilização de Erlenmeyers com diferentes capacidades de volume que permitam obter resultados equivalentes, Anexo I) e também o efeito da salinidade (Anexo II) no crescimento das mesmas. A utilização destas três espécies algais deve-se ao facto de que *Pseudokirchneriella subcapitata* (Korshikov) Hindak ser a microalga mais recomendada na realização de testes de inibição do crescimento algal (ASTM, 2002; OECD, 2002; USEPA, 2002), enquanto que as outras duas espécies permitem comparar os resultados obtidos relativamente ao crescimento nas diferentes condições acima referidas. As microalgas foram cultivadas em meio MBL (Marine Biological Culture<sup>®</sup>) (Stein, 1973) e mantidas numa câmara com

fotoperíodo (16h<sup>L</sup>:8h<sup>E</sup>) e temperatura controlada (20±2°C), renovando-se o meio periodicamente.

### 2.1.2 – Cladóceros

Foram utilizadas duas espécies de cladóceros existentes em culturas, *Daphnia magna* Straus e *Daphnia longispina* O. F. Müller, com o intuito de testar o efeito da salinidade no crescimento e sua reprodução. A utilização destas espécies deve-se ao facto de *D. magna* ser uma espécie muito usada em testes toxicológicos, enquanto *Daphnia longispina* é uma espécie indígena em Portugal.

As espécies de *Daphnia* utilizadas nos testes de incremento de salinidade foram mantidas em laboratório, em culturas monoclonais, ao longo de várias gerações, de acordo com os procedimentos para *Daphnia magna* (Baird et al., 1989; Soares et al., 1992; ASTM, 1997). O meio sintético utilizado na cultura destes organismos foi ASTM (ASTM, 1980) ao qual se adicionou um aditivo orgânico (extracto de *Ascophyllum nodosum*; Baird et al., 1989) e como alimento utilizou-se a alga *Pseudokirchneriella subcapitata* (Korshikov) Hindak ( $3.0 \times 10^5$  cells.mL<sup>-1</sup> para *D. magna* e  $1.5 \times 10^5$  cells.mL<sup>-1</sup> para *D. longispina*); as culturas foram renovadas periodicamente e mantidas nas mesmas condições de temperatura e fotoperíodo, descritas anteriormente para as culturas de microalgas (Anexo III).

## 2.2 – Desenhos e procedimentos experimentais

### 2.2.1 – Optimização da metodologia para testes de crescimento algal

Dois a três dias antes do início de cada teste, procedeu-se à incubação de três inóculos pertencentes a cada cultura algal, colocando-os, em seguida, nas mesmas condições de temperatura, luminosidade e agitação do teste experimental para que as algas se adaptassem a essas mesmas condições e para obter o crescimento exponencial recomendado (OECD, 2002). Deste modo, tanto os inóculos como o teste experimental foram colocados numa câmara de cultura com agitação constante (100 r.p.m), sob condições de fotoperíodo (16h<sup>L</sup>:8h<sup>E</sup>) e temperatura controlada (20±2°C). Os testes experimentais tiveram uma duração de 96 h, e não de 72 h, uma vez que o material experimental esteve sujeito a condições de fotoperíodo e não a luz contínua, como recomendado pela OECD (2002), relativamente a testes de inibição de crescimento (Anexos I e II). A densidade inicial de células de cada microalga utilizada nos testes foi de  $5 \times 10^4$  células.mL<sup>-1</sup> (OECD, 2002). A actividade experimental seguiu os procedimentos estabelecidos para a preparação e manipulação de material. Em cada teste algal foram utilizadas três réplicas para cada tratamento (OECD, 2002). O efeito das condições nutritivas foi testado utilizando Erlenmeyers de 250 mL, cujo volume de teste final foi de 100 mL, e em que foram usados cinco tratamentos – meio MBL concentrado até diluições de 80, 70, 60 e 50 % de MBL em água destilada (Anexo I). As diferentes capacidades de volume do material de vidro foram testadas utilizando Erlenmeyers de 250 mL, 150 mL e de 100 mL aos quais correspondia um volume final de 100 mL, 60 mL e de 40 mL (Anexo I), respectivamente. Foram utilizados seis tratamentos para cada espécie de alga, tendo sido testada uma concentração saturada de

meio MBL e de 70 % de MBL diluído em água destilada, para cada volume final (Anexo I). No final do teste foram medidos quatro parâmetros de biomassa: densidade óptica a 440 nm, número de células, concentração de clorofila *a* e peso seco. A densidade algal foi determinada através da contagem directa em câmaras de Neubauer, para as espécies *C. vulgaris* e *P. subcapitata*, e de Sedgwick-Rafter, para a espécie *P. morum* (APHA, 1995). A determinação da concentração de clorofila *a* foi feita através da filtração de 40 mL, 20 mL e 18 mL (Erlenmeyers de 250 mL, 150 mL e 100 mL de capacidade, respectivamente) da cultura teste em filtros GF/C. Seguidamente, colocaram-se os filtros em tubos de centrifugação com 10 mL de acetona (90%) para, posteriormente, ser feita a leitura, num espetofotómetro, a 665nm e a 750 nm, antes e depois da acidificação com HCl (0,1M). O peso seco foi determinado através da filtração, em filtros GF/C, do restante volume da cultura teste. Os filtros foram previamente tarados a 103°C e, após a filtração, colocaram-se na estufa à mesma temperatura durante 24h (APHA, 1995).

### **2.2.2 – Tolerância à salinidade de microalgas**

O efeito de diferentes concentrações de salinidade no crescimento das três espécies de microalgas verdes foi testado através da metodologia descrita anteriormente. Foram utilizados dois meios distintos: meio sintético [meio MBL esterilizado (Stein, 1973)] com diferentes concentrações de cloreto de sódio (NaCl) (desde 0,0 g.L<sup>-1</sup> até 14,0 g.L<sup>-1</sup>) e meio marinho (EW) (36 psu) (Anexo II). Este último meio foi filtrado em filtros GF/C e adicionado às soluções de MBL, obtendo-se uma gama de salinidade desde 0,0 até 14,3 psu (Anexo II). Nesta experiência foram utilizados Erlenmeyers de 100 mL (40 mL de volume de teste final), com base nos dados obtidos na experiência

anterior (Anexo I). Ao fim de 96 h foi medido um dos quatro parâmetros de biomassa para cada espécie algal: densidade óptica a 440 nm para *C. vulgaris* e *P. subcapitata* e concentração de clorofila *a* para *P. morum*. O procedimento utilizado na determinação da clorofila *a* foi igual ao descrito anteriormente.

### 2.2.3 – Tolerância à salinidade de cladóceros

#### - Testes agudos

Os testes agudos foram realizados nas mesmas condições de fotoperíodo e temperatura descritas anteriormente e efectuados de acordo com os protocolos padrão (ISO, 1996; OECD, 2000; USEPA, 2002). Foram utilizados seis tratamentos, cada um com quatro réplicas, cuja gama de salinidade se situou entre 4,40 g.L<sup>-1</sup> e 7,09 g.L<sup>-1</sup>, para *D. magna*, e 2,50 g.L<sup>-1</sup> e 4,03 g.L<sup>-1</sup> para *D. longispina*. Estas concentrações foram obtidas através da diluição de uma solução mãe de NaCl em meio sintético ASTM, o qual foi utilizado como tratamento controlo (Anexo III). Em cada tratamento foram colocados, em 100 mL de solução teste, vinte neonatos (cinco organismos por réplica) de cada espécie, com idade inferior a 24 h e nascidos entre as 3<sup>a</sup> e 5<sup>a</sup> ninhadas. O teste teve a duração de 48h e decorreu na ausência de alimento e extracto orgânico. Ao fim de 24h e 48h foi registada a imobilidade dos organismos (Anexo III).

#### - Testes crónicos

O procedimento adoptado na realização dos testes crónicos foi semelhante ao dos testes agudos. No entanto, nos testes crónicos foram utilizados dez organismos de cada espécie (um organismo por réplica) para cada tratamento (incluindo o controlo). O



volume de solução final usado foi de 50 mL, ao qual se adicionou alimento (*P. subcapitata*) e extracto orgânico (Anexo III). A gama de salinidade foi determinada com base nos resultados dos testes agudos para cada espécie de cladóceros, tendo sido definidas as seguintes concentrações de salinidade: 3,42; 3,76; 4,14; 4,55; 5,00 e 5,50 g.L<sup>-1</sup> para *D. magna* e 1,55; 1,71; 1,88; 2,07; 2,27 e 2,50 g.L<sup>-1</sup> para *D. longispina* (Anexo III). O teste crónico teve a duração de vinte e um dias, com uma renovação periódica do meio de dois em dois dias (OECD, 1998; ISO, 2000). Procedeu-se à observação dos organismos teste para registo da mortalidade e reprodução (presença de ovos, número de neonatos), em cada um dos dias do teste.

No início de cada teste, foi medido (com o auxílio de uma lupa) o tamanho corporal de vinte neonatos retirados da mesma cultura dos organismos teste. Realizou-se idêntico procedimento a cada um dos organismos sobreviventes (mães) no final do teste (vinte e um dias). Os tamanhos inicial ( $l_i$ ) e final ( $l_f$ ) das mães (em mm) permitiram calcular a taxa de crescimento somático (TCS – dias<sup>-1</sup>), de acordo com a seguinte fórmula:

$$TCS = \frac{\ln(l_f) - \ln(l_i)}{\Delta t},$$

onde  $\Delta t$  é o intervalo de tempo (21 dias).

Adicionalmente, a taxa intrínseca de crescimento populacional ( $r$ ) foi obtida com base nos dados de sobrevivência e fecundidade, de acordo com a equação de Euler–Lotka:

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

onde  $r$  é expresso em dia<sup>-1</sup>,  $x$  corresponde à classe de idades (1...n dias),  $l_x$  é a probabilidade de sobreviver até à idade  $x$ , e  $m_x$  é a fecundidade à idade  $x$  (Anexo III).

## **2.3 – Análise estatística**

### **2.3.1 – Ensaio de crescimento de microalgas**

Os resultados dos testes de inibição de crescimento algal (Anexos I e II) foram comparados através da análise de variância (ANOVA) unifactorial. Foi aplicado o teste de Tukey quando se verificavam diferenças significativas entre os vários tratamentos, incluindo o controlo ( $p < 0,05$ ) (Zar, 1996). Nos trabalhos experimentais, relativos a diferentes condições nutritivas e a diferentes capacidades volumétricas, foi ainda calculado o coeficiente de correlação de Pearson de modo a comparar os diferentes métodos de determinação de biomassa (Anexo I). No teste experimental, relativo ao *stress* salino em algas de água doce (Anexo II), foi determinado o valor de  $CE_{50}$  (ver página VIII) através da análise Probit (Finney, 1971).

### **2.3.2 – Ensaio agudo e crónico com cladóceros**

Os valores de  $CE_{50}$  para a mortalidade (testes agudos) e reprodução (testes crónicos) em *Daphnia* spp. foram calculados através da análise Probit (Finney, 1971). O efeito da salinidade nos parâmetros de crescimento e reprodução de ambas as espécies (testes crónicos) foi avaliado através da aplicação de análises de variância (ANOVA) de uma via a cada um dos parâmetros. Quando se verificaram diferenças significativas entre tratamentos, aplicou-se o teste de Dunnett para determinar as diferenças significativas entre cada uma das concentrações e o controlo. Este procedimento

permitiu obter os valores de CENO<sup>2</sup> e CEO<sup>3</sup>. Todas as análises foram efectuadas com um nível de significância ( $\alpha$ ) de 0,05.

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<sup>2</sup> CENO – concentração de efeito não observado. É a concentração mais alta onde não se registaram efeitos tóxicos significativos, relativamente ao controlo. É o equivalente ao NOEC (no observed effect concentration), da língua inglesa.

<sup>3</sup> CEO – concentração de efeito observado. É a concentração mais baixa onde se registaram efeitos tóxicos significativos, relativamente ao controlo. É o equivalente ao LOEC (lowest observed effect concentration), da língua inglesa.

Capítulo 3

Resultados



### 3.1 Análise do crescimento de microalgas

Os resultados obtidos com espécies algais submetidas a diferentes condições nutritivas mostraram não haver diferenças significativas entre os vários tratamentos para as espécies *P. subcapitata* e *P. morum*, apresentando as mesmas um crescimento elevado mesmo no último tratamento (50% de MBL diluído) (figura 1.A e B, Anexo I). O crescimento de *C. vulgaris* foi significativamente diferente entre 50% de MBL e os restantes tratamentos (incluindo o controlo) (figura 1.C, Anexo I). Os resultados obtidos aquando do uso de Erlenmeyers com diferentes capacidades volumétricas e diferentes quantidades de meio sintético [MBL concentrado (CTL) e 70% de MBL diluído em água destilada], mostraram que *P. subcapitata* e *C. vulgaris* apresentavam diferenças significativas entre MBL<sup>4</sup> e 70 MBL<sup>5</sup> e também entre ambos os tratamentos em Erlenmeyers de 250 mL (MBL e 70 MBL) e 70 MBL<sup>5</sup> (figura 2.A e B, Anexo I). *P. morum* não apresentou diferenças significativas entre os vários tratamentos (figura 2.C, Anexo I). No final do teste (após 96 h) foram medidos quatro parâmetros de biomassa, para *P. subcapitata* e *C. vulgaris*: densidade óptica (440 nm), número de células (contagem), concentração de clorofila *a* e peso seco. A densidade óptica mostrou ser o parâmetro de biomassa mais rápido e fácil de medir, existindo uma elevada correlação entre este e o número de células (*C. vulgaris*:  $r = 0,932$ ;  $p < 0,05$ ;  $n = 48$ ; *P. subcapitata*:  $r = 0,891$ ;  $p < 0,05$ ;  $n = 22$ ), a clorofila *a* (*C. vulgaris*:  $r = 0,704$ ;  $p < 0,05$ ;  $n = 31$ ; *P. subcapitata*:  $r = 0,812$ ;  $p < 0,05$ ;  $n = 26$ ) e o peso seco (*C. vulgaris*:  $r = 0,976$ ;  $p < 0,05$ ;  $n = 32$ ; *P. subcapitata*:  $r = 0,819$ ;  $p < 0,05$ ;  $n = 31$ ), em ambas as espécies (tabelas I e II). Para *P. morum* mediram-se três parâmetros de biomassa, não tendo sido medida a densidade óptica dado tratar-se de uma alga colonial. Assim, a determinação da concentração de clorofila *a* mostrou ser o parâmetro de biomassa mais eficaz e

adequado para esta espécie colonial, sendo também de salientar a correlação ( $r = 0,670$ ;  $p < 0,05$ ;  $n = 26$ ;  $r = 0,853$ ;  $p < 0,05$ ;  $n = 35$ ) entre este parâmetro e o número de células e o peso seco, respectivamente (tabela III).

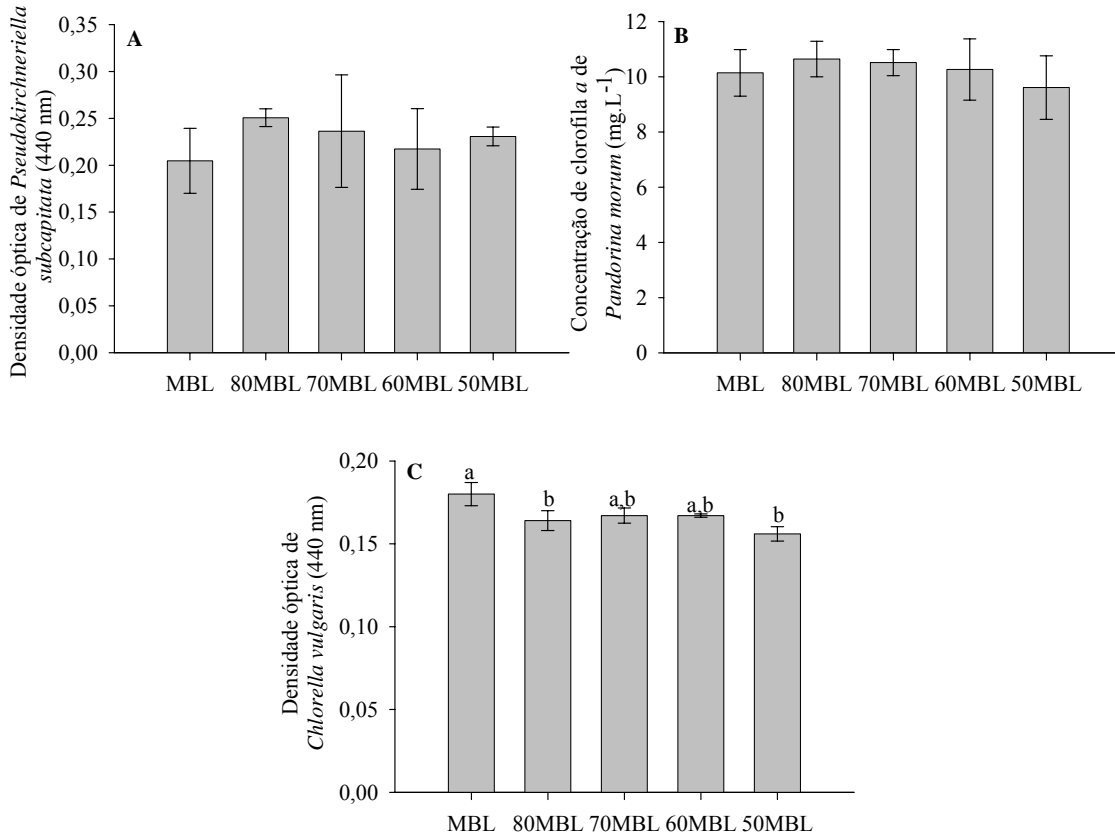


Figura 1 – Crescimento algal após 96h de incubação em meio saturado de MBL e em 80, 70, 60 e 50% de meio sintético diluído em água destilada para (A) *P. subcapitata*, (B) *P. morum* e para (C) *C. vulgaris*. Os dados correspondem a três réplicas por tratamento e as barras de erro representam o desvio padrão. As letras referem-se a diferenças significativas entre os tratamentos ( $p < 0,05$ ); a inexistência de letras corresponde a casos onde não se verificaram diferenças significativas entre os tratamentos.

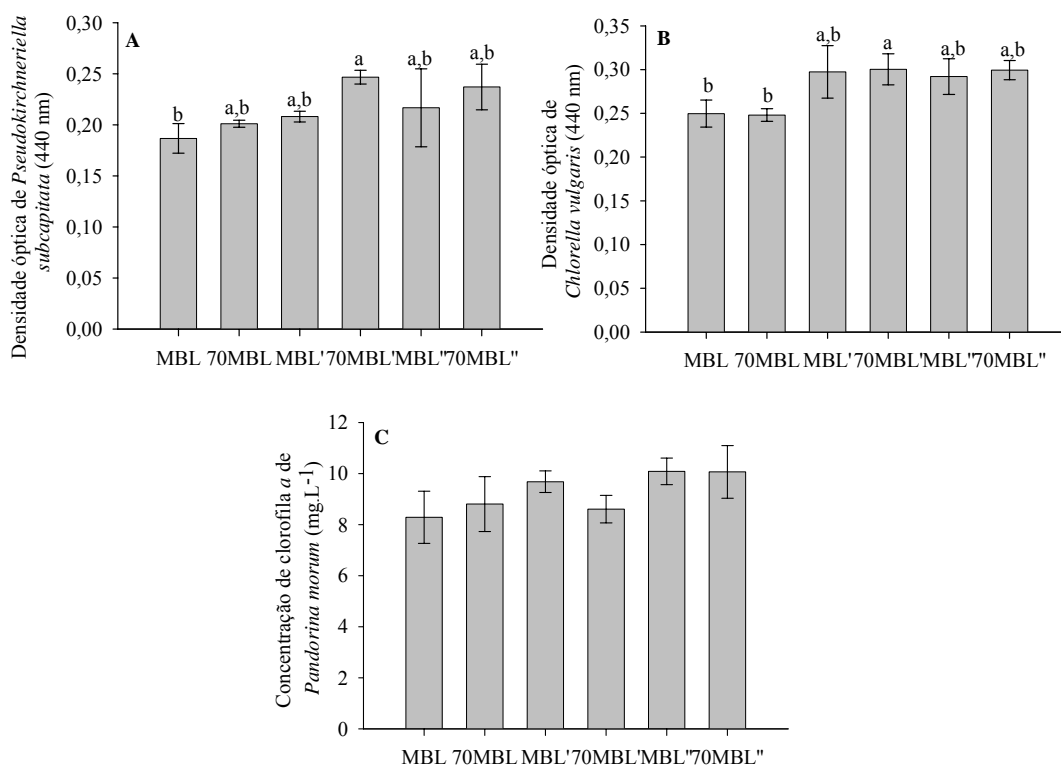


Figura 2 – Crescimento algal em Erlenmeyers com diferentes capacidades de volume, após 96h de incubação, para (A) *P. subcapitata*, (B) *C. vulgaris* e (C) *P. morum*. MBL e 70MBL correspondem a Erlenmeyers de 250mL, MBL' e 70MBL' correspondem a Erlenmeyers de 150mL e MBL'' e 70MBL'' correspondem a Erlenmeyers de 100mL. Os dados correspondem a três réplicas por tratamento e as barras de erro representam o desvio padrão. As letras referem-se a diferenças significativas entre os tratamentos ( $p < 0,05$ ); a inexistência de letras corresponde a casos onde não se verificaram diferenças significativas entre os tratamentos.

Tabela I – Correlação entre os parâmetros de biomassa para *C. vulgaris*

Parâmetro de biomassa	Densidade óptica
Nº de células (contagem)	$r=0,932$ ; $p<0,05$ ; $n=48$
Chl <i>a</i>	$r=0,704$ ; $p<0,05$ ; $n=31$
Peso seco	$r=0,976$ ; $p<0,05$ ; $n=32$

Tabela II – Correlação entre os parâmetros de biomassa para *P. subcapitata*

Parâmetro de biomassa	Densidade óptica
Nº de células (contagem)	$r=0,891$ ; $p<0,05$ ; $n=22$
Chl <i>a</i>	$r=0,812$ ; $p<0,05$ ; $n=26$
Peso seco	$r=0,819$ ; $p<0,05$ ; $n=31$



Tabela III – Correlação entre os parâmetros de biomassa para *P. morum*

Parâmetro de biomassa	Clorofila <i>a</i>
Nº de células (contagem)	$r=0,670$ ; $p<0,05$ ; $n=26$
Peso seco	$r=0,853$ ; $p<0,05$ ; $n=35$

### 3.2 Análise do incremento salino para microalgas

O incremento da salinidade em microalgas de água doce inibiu significativamente o crescimento de *C. vulgaris*, *P. subcapitata* e *P. morum* a concentrações superiores a 5 g.L<sup>-1</sup>, 4 g.L<sup>-1</sup> e 2 g.L<sup>-1</sup>, respectivamente (Figura 3, Anexo II). Os valores de CE<sub>50</sub> obtidos para *C. vulgaris*, *P. subcapitata* e *P. morum* foram: 5,1 g.L<sup>-1</sup> (4,3-6,1); 4,1 g.L<sup>-1</sup> (3,6-4,7); 0,6 g.L<sup>-1</sup> (0,002-3,6), respectivamente.

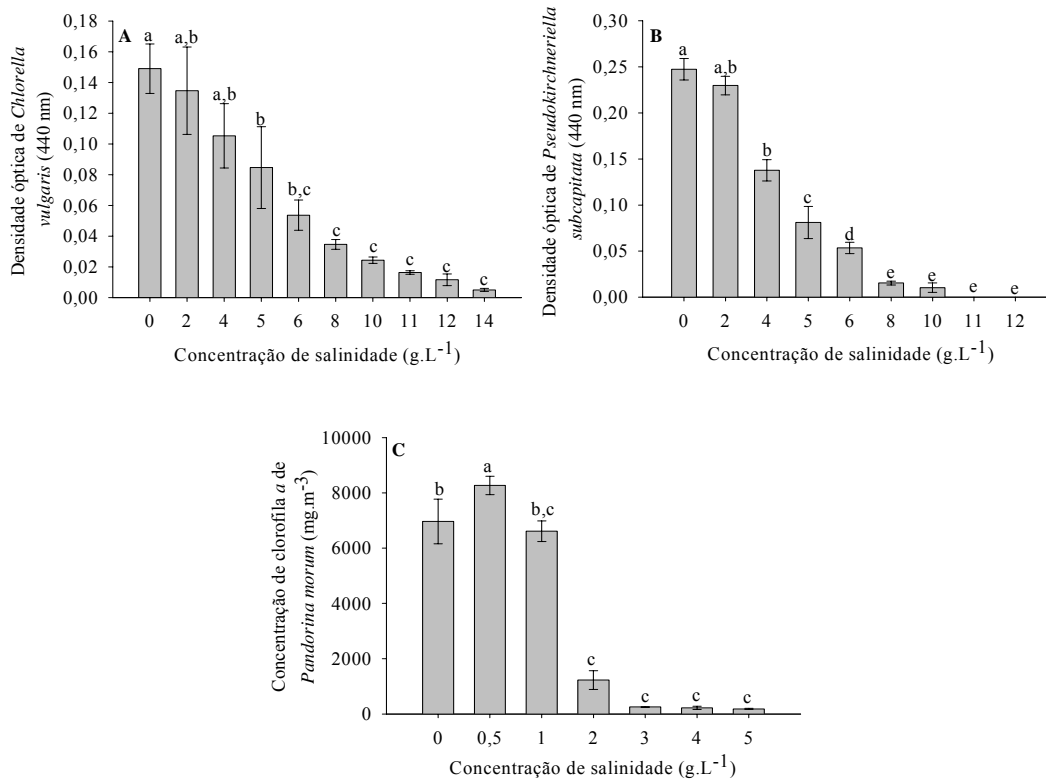


Figura 3 – Crescimento algal em diferentes concentrações de NaCl (desde 0,0 até 14,0 g.L<sup>-1</sup>), após 96h de incubação, para (A) *C. vulgaris*, (B) *P. subcapitata* e (C) *P. morum* [a, b, c, d, e representam diferenças significativas ( $p<0,05$ ) entre os vários tratamentos].

Os resultados obtidos com água do mar (EW) mostraram que o crescimento de *C. vulgaris*, *P. morum* e *P. subcapitata* foi significativamente inibido acima de 10,7 psu; 0,9 psu e 3,6 psu, respectivamente (figura 4, Anexo II). Os valores de CE<sub>50</sub> calculados para as três espécies algais foram 7,0 psu (4,6-10,7) para *C. vulgaris*, 3,7 psu (2,7-5,1) para *P. subcapitata* e 1,4 psu (0,8-2,1) para *P. morum*.

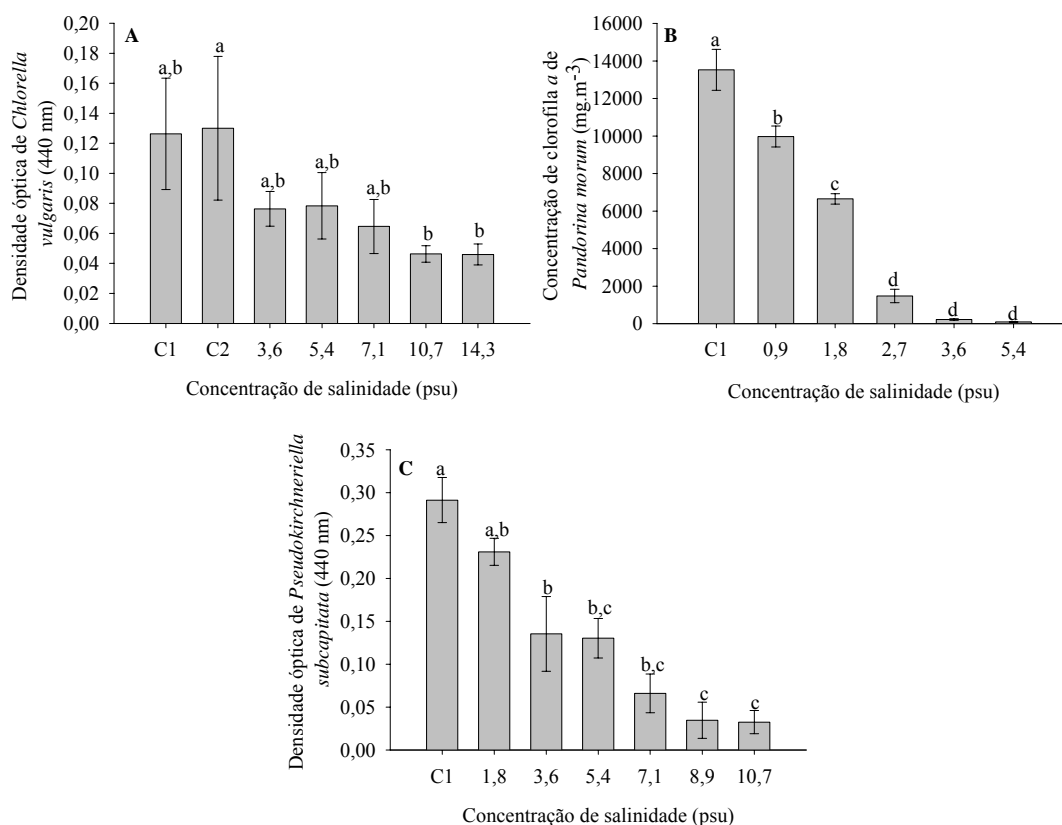


Figura 4 – Crescimento algal em meio marinho [gama de salinidade entre 0,0 (C1 e C2) e 14,3 psu], após 96 h de incubação, para (A) *C. vulgaris*, (B) *P. morum* e (C) *P. subcapitata* [a, b, c, d correspondem a diferenças significativas ( $p < 0,05$ ) entre os tratamentos].

### 3.3 Análise do incremento salino para cladóceros

Os valores de CE<sub>50</sub> agudos e crônicos determinados para *D. magna* são aproximadamente o dobro dos valores obtidos para *D. longispina* (tabela IV). Contudo, registou-se uma grande proximidade entre os valores agudos e os crônicos, dentro de

cada espécie. Com base nestes valores, verificou-se que *D. longispina* é menos halotolerante do que *D. magna* (Anexo III).

Os resultados obtidos nos testes crônicos mostraram que ambas as espécies de *Daphnia* são afectadas pela salinidade, observando-se diferenças significativas entre as concentrações e o controlo, à excepção do número de ninhadas de *D. magna* (tabela V, Anexo III). Nas figuras 5 e 6 está representado o efeito da salinidade nos parâmetros populacionais medidos para *D. magna* e *D. longispina*, sendo o número de neonatos o parâmetro mais drasticamente afectado (ver também Anexo III). A mortalidade manteve-se inferior a 20% nas primeiras concentrações, atingindo o valor máximo (90%) na última concentração em ambas as espécies (figuras 5 e 6). Os resultados obtidos mostraram uma diminuição do número de neonatos e um atraso no desenvolvimento (aumento da idade à primeira reprodução) com o incremento de salinidade, para ambas as espécies (figuras 5 e 6, Anexo III). Consequentemente, houve uma diminuição da taxa intrínseca de crescimento populacional ( $r$ ) com o incremento da salinidade, em *D. magna* e *D. longispina*, registando-se, apenas nesta última espécie, uma diminuição significativa do número de ninhadas (Anexo III). A taxa de crescimento somático também foi significativamente afectada pelo incremento salino em ambas as espécies, sendo observado um efeito estimulador inicial para *D. longispina* (figuras 5 e 6, Anexo III).

Na tabela 6 estão registados os valores de CEO e CENO para ambas as espécies de cladóceros. Assim, 5 g.L<sup>-1</sup> foi a concentração mais baixa de salinidade para a qual se registaram efeitos significativos nos parâmetros populacionais medidos para *D. magna*, sendo os valores de CEO e CENO comuns aos parâmetros populacionais desta espécie de *Daphnia*. Para *D. longispina*, os valores de CEO encontraram-se entre 1,71 e 2,07 g.L<sup>-1</sup> (tabela VI, Anexo III).

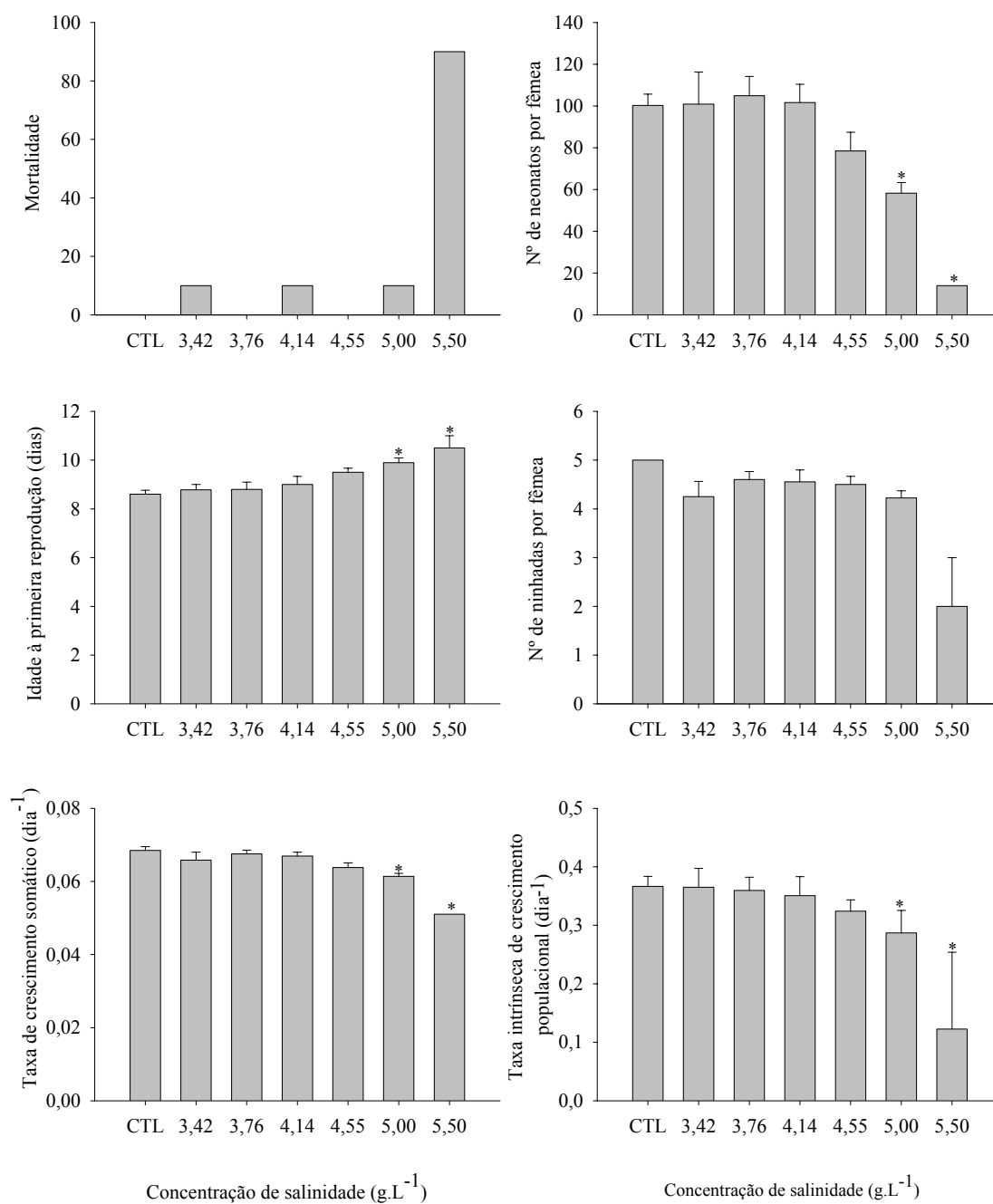


Figura 5 – Parâmetros populacionais de *Daphnia magna* quando exposta a diferentes concentrações de salinidade (NaCl), durante 21 dias. As barras de erro representam o erro padrão e \* corresponde a diferenças significativas (teste de Dunnett,  $p \leq 0,05$ ) entre as concentrações e o controlo (CTL).

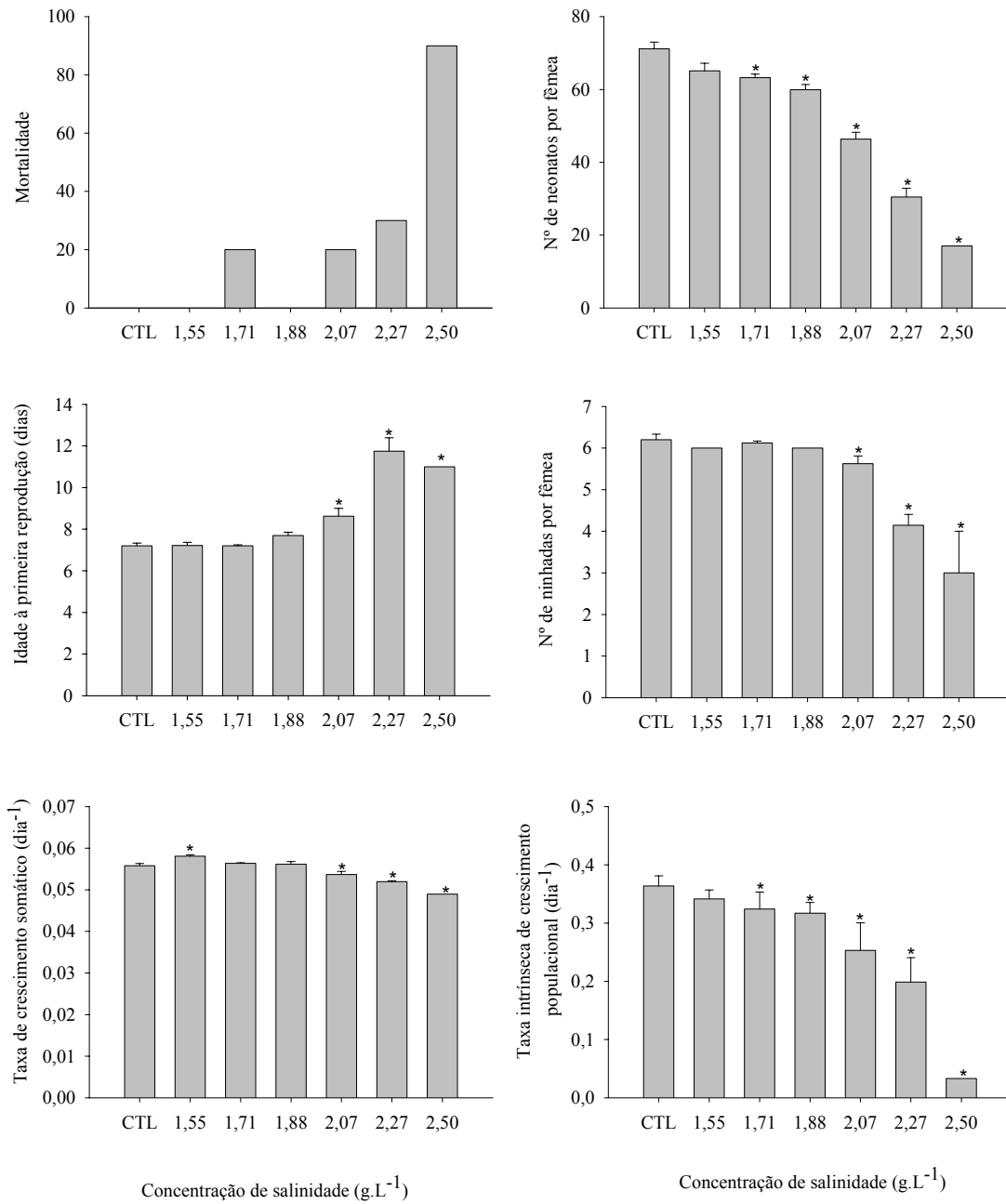


Figure 6 – Parâmetros populacionais de *Daphnia longispina* quando exposta a diferentes concentrações de salinidade (NaCl), durante 21 dias. As barras de erro representam o erro padrão e \* corresponde a diferenças significativas (teste de Dunnett,  $p \leq 0,05$ ) entre as concentrações e o controle (CTL).

Tabela IV – Valores de  $CE_{50}$  agudos e crônicos de salinidade (NaCl) para *D. magna* e *D. longispina*, com intervalos de confiança de 95% (entre parêntesis).

Espécies	$CE_{50}$ Aguda	$CE_{50}$ Crônica
<i>D. magna</i>	<b>5,9 g.L<sup>-1</sup></b> (5,5 – 6,5)	<b>5,0 g.L<sup>-1</sup></b> (4,9 – 5,1)
<i>D. longispina</i>	<b>2,9 g.L<sup>-1</sup></b> (2,8 – 3,0)	<b>2,2 g.L<sup>-1</sup></b> (2,2 – 2,3)

Tabela V – Resumo da análise de variância (ANOVA) de uma via, aplicada aos parâmetros populacionais de *D. magna* e de *D. longispina* (g.l. – graus de liberdade; p – probabilidade)

Parâmetros	<i>D. magna</i>			<i>D. longispina</i>		
	F	g.l.	p	F	g.l.	p
Número de neonatos	4,1	5, 48	0,004	49	5, 46	<0,001
Idade à primeira reprodução	5,5	6, 55	<0,001	34	5, 49	<0,001
Número de ninhadas	2,3	5, 50	0,057	29	5, 46	<0,001
Taxa de crescimento somático	4,3	5, 48	0,003	15	5, 46	<0,001
Taxa intrínseca de crescimento populacional	24	6, 62	<0,001	39	5, 53	<0,001

Tabela VI – Valores de CENO e de CEO (g.L<sup>-1</sup>) para os parâmetros populacionais de *D. magna* e de *D. longispina*, após exposição a diferentes concentrações de salinidade, durante 21 dias.

Parâmetros	<i>D. magna</i>		<i>D. longispina</i>	
	CENO	CEO	CENO	CEO
Número de neonatos	4,55	5,00	1,55	1,71
Idade à primeira reprodução	4,55	5,00	1,88	2,07
Número de ninhadas	-	-	1,88	2,07
Taxa de crescimento somático	4,55	5,00	1,88	2,07
Taxa intrínseca de crescimento populacional	4,55	5,00	1,55	1,71



Capítulo 4

Discussão





#### **4.1 – Crescimento de microalgas em diferentes condições nutritivas**

Os resultados obtidos nos testes de inibição de crescimento algal, sob diferentes condições nutritivas, permitiram concluir que são asseguradas as condições nutritivas básicas utilizando meio MBL como meio base para o crescimento algal, quando o volume de efluente se situou entre 0 e 50 % para *P. subcapitata* e *P. morum*, e entre 0 e 60% para *C. vulgaris* (Anexo I).

Alguns autores (e.g., Chen et al., 1997; De Figueiredo et al., 2004) obtiveram resultados muito semelhantes aos que se obtiveram no presente trabalho relativamente à densidade celular de *P. subcapitata* e *C. vulgaris*, salientando que a temperatura e a presença de luz contínua são factores importantes no desenvolvimento destas espécies algais (Anexo I). A agitação a 100 r.p.m. mostrou ser um procedimento bastante adequado de modo a evitar a sedimentação algal, promovendo melhores condições de arejamento (Anexo I). Por outro lado, dos parâmetros de biomassa medidos para cada alga, a densidade óptica pareceu ser o mais adequado para *C. vulgaris* e *P. subcapitata*, enquanto que para *P. morum* foi a medição da concentração de clorofila *a* (Anexo I). A utilização de Erlenmeyers de 150 mL e de 100 mL é apropriado nos testes de inibição de crescimento algal permitindo, assim, reduzir a área ocupada pelo material de vidro nos agitadores, possibilitando o aumento do número de réplicas usadas (Anexo I).

#### **4.2 Efeito da salinidade no crescimento de microalgas**

O crescimento das três espécies de microalgas foi afectado significativamente pelo aumento da salinidade (Anexo II), considerando-se, de acordo com os resultados obtidos, que *C. vulgaris* é a mais halotolerante e *P. morum* é a mais sensível (Anexo II).

As espécies *C. vulgaris* e *P. morum* mostraram ser mais sensíveis em meio com NaCl do que em meio marinho, provavelmente devida à toxicidade de NaCl e à presença de estimuladores de crescimento algal presentes em meio marinho. Por outro lado, *P. subcapitata* mostrou ser mais sensível em meio marinho do que em meio com NaCl (Anexo II). Trabalhos realizados com cianobactérias (Moisander et al., 2002) evidenciaram diferenças significativas no seu crescimento, tendo sido referido mecanismos de adaptação a condições de salinidade por algumas espécies fitoplanctónicas (Barron et al., 2002; Anexo II). Assim, o crescimento algal mostrou estar dependente dos mecanismos de adaptação às condições salinas, os quais definem a sensibilidade ou tolerância da alga em meios salinos (Anexo II).

#### **4.3 Efeito da salinidade em parâmetros populacionais de cladóceros**

O incremento salino inibiu o crescimento e reprodução de cladóceros (Anexo III). Assim, com base no estudo realizado, concluiu-se que *D. magna* é mais halotolerante do que *D. longispina*, sendo esta diferença de sensibilidade notória entre outras espécies de *Daphnia* (tabela VII, Anexo III). Tal como no presente trabalho, outros estudos (e.g. Boronat et al., 2001; Rokneddine & Chentoufi, 2004; Hairston et al., 2005) concluíram que há espécies de cladóceros mais halotolerantes do que outras, podendo este grupo de organismos habitar desde águas doces até águas salinas, classificando-se *D. longispina* de estenohalina e *D. magna* de eurihalina. Os resultados obtidos no presente trabalho mostraram que, de um modo geral, todos parâmetros populacionais medidos foram afectados pelo incremento salino, tendo sido o número de neonatos o parâmetro mais drasticamente afectado (Anexo III).

Tabela VII – Valores agudos e crônicos de CE<sub>50</sub> calculados para a salinidade por outros autores para a família Daphniidae.

<b>Espécies</b>	<b>CE<sub>50</sub> aguda</b>	<b>CE<sub>50</sub> crónica</b>	<b>Referências bibliográficas</b>
<i>D. longispina</i>	2,9 g.L <sup>-1</sup>	2,2 g.L <sup>-1</sup>	Presente estudo
<i>D. magna</i>	8,3g.L <sup>-1</sup>	-	Cowgill & Milazzo (1990)
<i>D. magna</i>	11,3 g.L <sup>-1</sup>	8,57 g.L <sup>-1</sup>	Schuytema et al. (1997)
<i>D. magna</i>	0,49 g.L <sup>-1</sup>	-	Semsari & Haït-Amar (2001)
<i>D. magna</i>	7,3 g.L <sup>-1</sup>	-	Do Hong et al. (2004)
<i>D. magna</i>	5,9 g.L <sup>-1</sup>	5,0 g.L <sup>-1</sup>	Presente estudo
<i>D. carinata</i>	7,7 g.L <sup>-1</sup> [água do mar artificial (25°C )]	-	Kefford (2000)
<i>D. carinata</i>	5,3 g.L <sup>-1</sup> [água salgada de lago (25°C)]	-	Kefford (2000)
<i>D. carinata</i>	0,9 g.L <sup>-1</sup> (20°C)	-	Hall & Burns (2002)
<i>D. carinata</i> (adultos)	2,2 g.L <sup>-1</sup> (10°C) 1,7 g.L <sup>-1</sup> (20°C)	-	Hall & Burns (2002)
<i>D. thomsoni</i> (7 clones)	7,5 – 8,8 g.L <sup>-1</sup>	-	Barry et al. (2005)
<i>C. cornuta</i>	2,6 g.L <sup>-1</sup>	-	Do Hong et al. (2004)

Por outro lado, os resultados mostraram que *D. magna* reproduz-se e vive satisfatoriamente em concentrações de NaCl inferiores a 4,55 g.L<sup>-1</sup> (Anexo III). Estes resultados são comparáveis aos obtidos por Teschner (1995) relativamente à diminuição do crescimento e retardamento da idade à maturação de clones de *D. magna* em águas salobras. O trabalho realizado por Schuytema et al. (1997) também apresentou resultados idênticos a este, referindo que *D. magna* é capaz de viver sem perturbações de reprodução nem crescimento em concentrações inferiores a 4 g.L<sup>-1</sup> (Anexo III). Tal como algumas espécies fitoplanctónicas, as espécies de cladóceros também desenvolvem mecanismos de adaptação a alterações das condições ambientais, nomeadamente ao *stress* salino (Teschner, 1995; Schallenberg et al., 2003; Rokneddine & Chentoufi, 2004).

Os resultados obtidos neste trabalho sugerem que o aumento da salinidade em sistemas de água doce pode alterar significativamente a biodiversidade, dado que afecta a sobrevivência, o crescimento e a reprodução de cladóceros, eliminando-as em casos de irreversibilidade das concentrações salinas, quando estas estiverem acima do limite tolerado por esses organismos.

Capítulo 5

Considerações finais



Com base nos objectivos propostos, a presente dissertação contribuiu para definir uma metodologia rápida, simples e aplicável em testes de inibição de crescimento algal para três espécies de microalgas (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* e *Pandorina morum*). Por outro lado, este estudo também serviu para um melhor conhecimento do efeito da intrusão salina em espécies de algas verdes e de cladóceros, mostrando que o aumento da salinidade afecta significativamente o crescimento de microalgas e de cladóceros e também tem efeitos inibidores ao nível da reprodução e crescimento populacional destes últimos. Assim, sendo a salinidade um factor importante e fundamental na determinação da presença e sucessão das espécies, pode-se inferir que o aumento de salinidade poderá levar à alteração da estrutura da comunidade de um ecossistema de água doce, caso esses organismos não possuam mecanismos de adaptação a esse incremento salino ou este se mantenha em níveis superiores aos tolerados por estas espécies aquáticas.

Numa perspectiva futura, esta investigação deverá levar à realização de um maior número de estudos com espécies de água doce de modo a compreender porque há espécies mais halotolerantes do que outras e quais os mecanismos adaptativos desses organismos a ambientes sob condições de *stress* salino. Esta abordagem poderá fornecer informação mais relevante em termos ecológicos ao englobar no seu estudo outros organismos (e.g. peixes) de níveis tróficos diferentes





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Anexos



## **Anexo I**

### **A low-cost methodology for algal growth inhibition tests using three freshwater green algae**

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## **Anexo II**

### **The effects of different salinity concentrations on growth of three freshwater green algae**

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### **Anexo III**

#### **Acute (immobilisation) and chronic (life history) responses of two freshwater cladocerans (*Daphnia magna* and *Daphnia longispina*) to salinity**

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## Anexo I



**A LOW-COST METHODOLOGY FOR ALGAL GROWTH  
INHIBITION TESTS USING THREE FRESHWATER GREEN  
ALGAE**

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## SUMMARY

Freshwater microalgae are considered good bioindicators in ecotoxicology being used as test organisms in several standard ecotoxicological testing procedures. The sensitivity to a determined toxicant may differ among species and thus it is important to use of a set of different species and to cross the final sensitivity of those microalgae to assess the toxicity of a compound or effluent. It is important to know the nutritive conditions that are enough to guaranty basic nutritive needs, allowing the performance of tests using effluents. It is also important to know the final test volume which is proper for algal growth tests, according to the surface apparatus.

An adapted methodology for three freshwater green algae (*Chlorella vulgaris* Beijerinck, *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory) growth tests establishing an easier, with fewer requirements and less expensive procedure to perform algal inhibition tests is proposed. It concerns different nutritive and apparatus conditions and most adequate biomass parameter chosen for each species. Several nutritive conditions were tested (ranging from MBL nutrient saturated medium to MBL dilutions). Different test vials conditions (250, 150 and 100 mL Erlenmeyer flasks) were also tested for these green algae.

**KEYWORDS:** Growth tests conditions, biomass parameters, *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Pandorina morum*.

## INTRODUCTION

Factors such as nutrient availability, light intensity and temperature influence the microalgal development [1]. Besides, each species may have different optimal values to growth and the response to a toxic may also vary under different test conditions. Standard procedures for algal growth inhibition tests describe the method to adopt and make several indications about the apparatus (vessels and culturing apparatus), test medium (nutritive conditions), incubation conditions (temperature, light and agitation) and other parameters that are important for algal growth and, consequently, for validation of the test. But sometimes the conditions in laboratories are quite different than one expects and alterations must be done [2]. For instance, the incubator chamber may be used for other tests or culture maintenance with specific parameters

(temperature and photoperiod, for example) that can not be modified; other items like the minimum final test volume on the flasks, the flasks capacity and the recommended medium for algal growth tests may also not be the same as indicated in standard guidelines [3, 4, 5]. Therefore, the conditions used may not be specifically the same of the standard procedures and the growth algal inhibition results may be different, depending on those alterations. Nevertheless, the results may follow the validation rules of the standard procedures such as the minimum 16-fold biomass increase in control flasks during the test period [4].

The aim of this study was to establish the most accurate, easiest and least time-consuming procedure to algal inhibition tests concerning different nutritive and apparatus conditions and algal biomass assessment (such as cell counting, dry weight, optical density or Chlorophyll *a*) in order to use only the parameter which combines better all those characteristics.

## **MATERIALS AND METHODS**

The green algae *Chlorella vulgaris* Beijerinck, *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory were used to test the effects of different conditions over their growth. The freshwater green alga most recommended for algal growth inhibition testing procedures is *Pseudokirchneriella subcapitata* [3, 4, 5]. Nevertheless, the performance of *C. vulgaris* and *P. morum* as test organisms was assessed by comparison with the *P. subcapitata* growth behaviour under the same conditions.

The nutritive culture medium used was the Marine Biological Laboratory medium (MBL – Sterilized Hoods Hole Culture) [6] but several nutritive conditions were tested (ranging from MBL nutrient saturated medium to 80, 70, 60 and 50 % dilutions of MBL in distilled water). In the preparation of MBL medium it is used sterile distilled water in which is added the correct quantity of stock solutions:  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (36.76  $\text{g} \cdot \text{L}^{-1}$ );  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (36.97  $\text{g} \cdot \text{L}^{-1}$ );  $\text{NaHCO}_3$  (12.60  $\text{g} \cdot \text{L}^{-1}$ );  $\text{K}_2\text{HPO}_4$  (8.71  $\text{g} \cdot \text{L}^{-1}$ );  $\text{NaNO}_3$  (85.01  $\text{g} \cdot \text{L}^{-1}$ );  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  (28.42  $\text{g} \cdot \text{L}^{-1}$ );  $\text{Na}_2\text{EDTA}$  (4.36  $\text{g} \cdot \text{L}^{-1}$ );  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (3.15  $\text{g} \cdot \text{L}^{-1}$ );  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.01  $\text{g} \cdot \text{L}^{-1}$ );  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.022  $\text{g} \cdot \text{L}^{-1}$ );  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.01  $\text{g} \cdot \text{L}^{-1}$ );  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.18  $\text{g} \cdot \text{L}^{-1}$ );  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.006  $\text{g} \cdot \text{L}^{-1}$ ) and Tris(hydroxymethyl) – aminomethane (50g.200mL<sup>-1</sup>) – 1mL of each stock solution per litre of medium and 2mL of Tris(hydroxymethyl) – aminomethane per litre of medium.

The medium is sterilised by autoclaving. The vitamins (sterilised by filtration) are only added after medium sterilisation and in cold conditions.

For each freshwater green alga the samples were placed in 250 borosilicate Erlenmeyer flasks with 100mL of final test volume. To each treatment corresponds a set of three replicates. An inoculum culture was incubated under the same conditions as the test cultures 3 days before starting the experiment to adapt each alga to the test conditions and achieve exponential growth such as usually recommended [4]. The initial cell density used for all green algae corresponded to  $5 \times 10^4$  cells.mL<sup>-1</sup>. The preparation of the MBL medium and flasks handling followed aseptic procedures. The tests were located in an incubation chamber (F10 000 EDTU model) with continuous agitation (GFL 3015) at 100 rpm, a 16h<sup>L</sup>:8h<sup>D</sup> photoperiod (43 – 46  $\mu$ mol quantum<sup>-2</sup>s<sup>-1</sup> provided by cool white fluorescent lights) and controlled temperature at 20±2°C. Standard growth inhibition procedures recommend that algal growth tests should be terminated after 72h of incubation under continuous light. In this experiment the test was finished after 4 days of incubation (after 96h) due to the photoperiod. At 96h there were performed four biomass parameters for all algae: optical density at 440 nm, dry weight, cell counting and chlorophyll *a* (chl *a*) concentration. Dry weight determination was made by filtering 40mL of the test volume from Erlenmeyer flasks through whatman GF/C filters, previously tarred at 103°C, and drying those filters at 103°C for 24h [7] (in an incubation chamber venticell 111 type). Algal density was achieved by direct counting using a Neubauer chamber for *C. vulgaris* and *P. subcapitata* and a Sedgwick-Rafter chamber for *P. morum* [7].

Chl *a* concentration was determined by filtering the remaining culture volume through GF/C filters, which were then treated with acetone (90%) to extract the chl *a* that was measured at 665nm and 750nm, before and after acidification with HCl (0.1M), in a 6505 UV/VIS spectrophotometer.

For the three algae, different test vials capacities (250, 150 and 100mL Erlenmeyer flasks) were also tested for MBL nutrient saturated medium and 70% of MBL in distilled water. For each treatment there were set up three replicates. The final test volume was 100, 60 and 40mL for the 250, 150 and 100mL Erlenmeyer flasks, correspondingly. The apparatus followed the same conditions described above. The parameters for algal biomass determination were the same as above but with different filtering volumes for dry weight (40mL, 20mL and 18mL from the cultures of 250, 150 and 100mL Erlenmeyer flasks, respectively) and, inherently, for chl *a*.

The results (expressed as optical density for *C. vulgaris* and *P. subcapitata* and chl *a* for *P. morum*) among the different treatments were compared using analysis of variance (ANOVA). If applicable, a Tukey multiple comparison test was applied with statistically significant differences in growth reported for  $p < 0.05$  [8]. The coefficient of correlation of Pearson was performed to compare the different methods of biomass determination: cell counting, dry weight, optical density and chlorophyll *a*.

## RESULTS

After 96h of incubation the results obtained for the algal growth, under different nutritive conditions (from MBL nutrient saturated medium to 80, 70, 60 and 50% MBL dilutions in distilled water), showed no significant differences (ANOVA,  $p > 0.05$ ), allowing the green algae *P. subcapitata* and *P. morum* to achieve maximal growth even at the MBL medium dilution of 50% (Figure 1.A and B). On the other hand, the growth of *C. vulgaris* with nutrient saturation was not significant different from the growth under 60% MBL medium dilution although it differed significantly from the growth at 50% MBL (Figure 1.C).

Figure 2 shows the three algae growth responses under different test vials and final test volume conditions (250, 150 and 100mL Erlenmeyer flasks with 100, 60 and 40mL of final test volume, respectively) using MBL medium and a dilution corresponding to 70% of MBL (the dilution of 70% of MBL medium was chosen due to the better results obtained for the different tested MBL dilutions). The results show that *P. morum* grew with no significant differences between the test vials conditions but also between the MBL samples and the 70MBL samples. Nevertheless, *C. vulgaris* and *P. subcapitata* presented significantly higher cell densities in 150 and 100mL Erlenmeyer flasks (ANOVA,  $p < 0.05$ ).

In all the tests, for *C. vulgaris* and *P. subcapitata*, there were performed four biomass parameters: cell counting, optical density (440nm), dry weight and Chlorophyll *a* concentration. Due to its easier and accurate procedure, optical density proved to be the most adequate parameter for current use in algal biomass determination for *C. vulgaris*, showing high correlation with cell counting ( $r = 0.932$ ,  $p < 0.05$ ,  $n = 48$ ), chl *a* ( $r = 0.704$ ,  $p < 0.05$ ,  $n = 31$ ) and dry weight ( $r = 0.976$ ,  $p < 0.05$ ,  $n = 32$ ). The same was observed for *P. subcapitata* which shows also a high correlation between optical density and cell counting ( $r = 0.891$ ,  $p < 0.05$ ,  $n = 22$ ), chl *a* ( $r = 0.812$ ,  $p < 0.05$ ,  $n = 26$ ) and dry



weight ( $r=0.819$ ,  $p<0.05$ ,  $n= 31$ ). In contrast, for *P. morum* optical density was not possible to measure due to the variation caused by colonies movement. Chl *a* showed an important correlation with dry weight ( $r=0.853$ ,  $p<0.05$ ,  $n= 35$ ) and cell counting determinations ( $r=0.670$ ,  $p<0.05$ ,  $n= 26$ ) and therefore was chosen as the most adequate biomass parameter for this alga.

## DISCUSSION AND CONCLUSION

From the results obtained we may infer that the nutritive conditions in 50% MBL medium are enough to guaranty *P. subcapitata* and *P. morum* basic nutritive needs, allowing the performance of tests using effluents which volume can range from 0 to 50% of the MBL volume. The same can be established for *C. vulgaris* using 60% MBL medium as control reference. This may be useful for the experimental design of toxicity tests using effluents. On the other hand, the reduction of final test volume in smaller flasks enables the reduction of the orbital shaker surface needed for the test apparatus, comparing to the standard procedures that indicate the use of 250mL Erlenmeyer flasks with 100mL of final test volume [4].

*P. subcapitata* achieved high cell densities in nutrient saturated medium ( $1.52 \times 10^7$  cells.mL<sup>-1</sup>) by comparison with other studies using this species under continuous light after a 96h test period at 24°C, but with air agitation in transparent chemostat reactors [9]. Under optimized conditions (temperature of 24°C, continuous light and agitation at 100 rpm conditions) and after 72h, this alga showed final test cell densities higher [10] than in the present work at 96h, possibly indicating the importance of temperature and continuous light to this species performance. De Figueiredo et al [10] also showed that, under the same conditions and after 72h, *C. vulgaris* presented cell densities similar to the ones achieved in this study for the 96h, indicating that the conditions described in this work are valid in terms of this species growth. Other studies using another *Chlorella* species [11] report growth values much lower than the ones achieved in the present study ( $2.25 \times 10^7$  cells.mL<sup>-1</sup>) after the same 96h test period but using continuous illumination and daily manual shaking of samples. Actually, *C. vulgaris* presented higher cell densities than *P. subcapitata* after the test period indicating the high potential of *C. vulgaris* as test organism in ecotoxicological studies and with the procedure presented in this study. *P. morum* achieved the lower cell density values of the three green algae, although it may be more sensitive to alterations

in the media or to a toxicant [12]. Agitation at 100 rpm proved to be an important procedure feature by preventing the algal sedimentation, particularly of *P. subcapitata* and *C. vulgaris*, allowing better aeration conditions for their growth.

In conclusion, our results established an adapted methodology to perform algal inhibition tests with *P. subcapitata*, but also with other freshwater green algae, such as *C. vulgaris* and *P. morum* which may prove to be more sensitive to toxicants than the former one [10, 12]. Also, our study supports the use of MBL as the media basis for algal growth tests when effluent volumes range from 0 to 50% for *P. subcapitata* and *P. morum* and from 0 to 60% of the total test volume for *C. vulgaris* without compromising the basic nutritive needs of these three green algae. For *C. vulgaris* and *P. subcapitata* the optical density showed to be the most adequate algal biomass determination parameter and for *P. morum* the chlorophyll *a* concentration. Algal growth inhibition tests support the use of 150 or 100 mL Erlenmeyer flasks which allow the reduction of apparatus area on an orbital shaker and thus the increase of test samples/replicates at the same time. Thus, the reduction of final test volume, experimental flasks capacity and surface apparatus showed to be proper for current algal growth tests.

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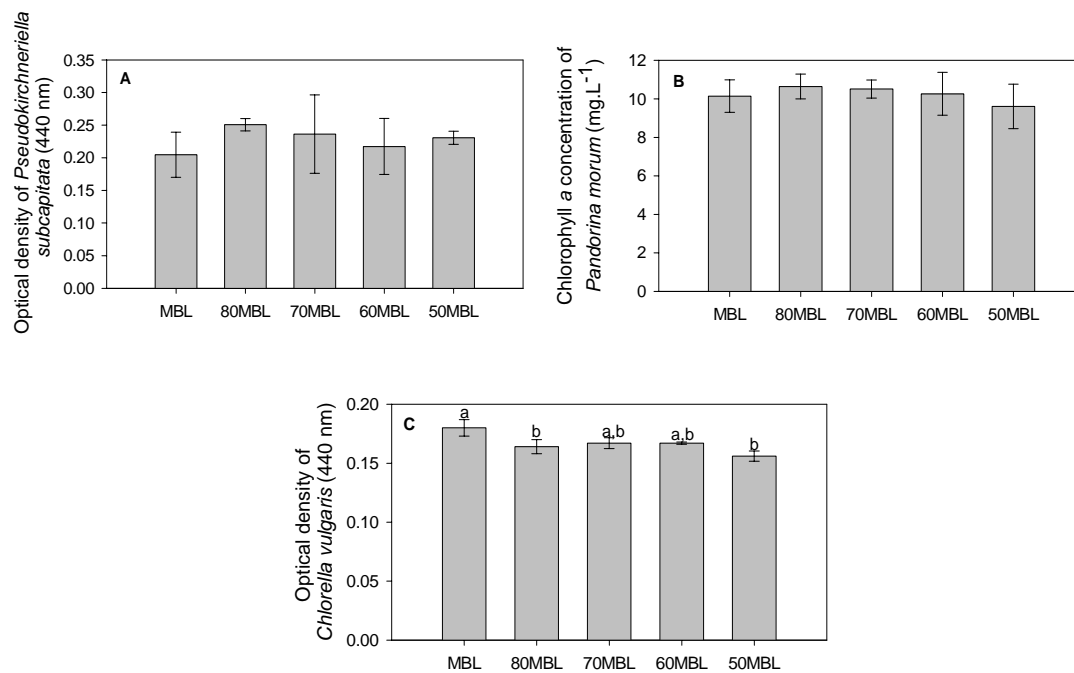


Figure 1 – Algal growth (in saturated nutrient medium and in 80, 70, 60 and 50% of MBL) after 96h of incubation using (A) *P. subcapitata*, (B) *P. morum* and (C) *C. vulgaris*. Data are the mean of at least 3 replicates and error bars represent the standard deviation. The different letters correspond to significant differences between the treatments ( $p < 0.05$ ). The absence of letters on the graphics means there are no significant differences among all the treatments.

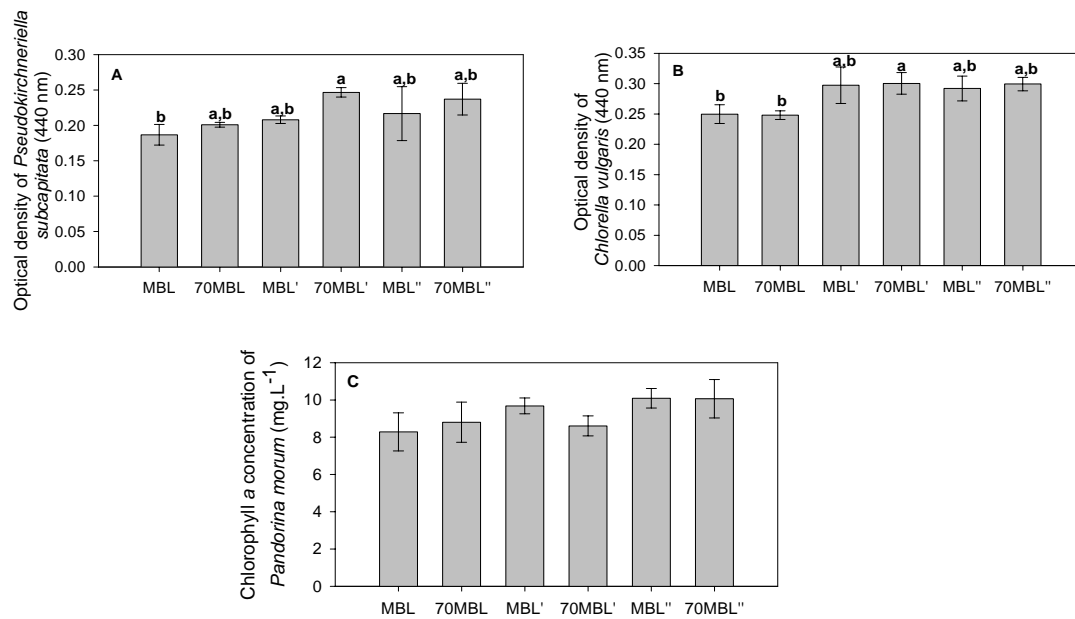


Figure 2 – Algal growth (on different vials and final test volume) after 96h of incubation using (A) *P. subcapitata*, (B) *C. vulgaris* and (C) *P. morum*. The MBL and 70MBL correspond to 250mL flasks, the MBL' and 70MBL' correspond to 150mL flasks, and the MBL'' and 70MBL'' correspond to 100mL. Data are the mean of at least 3 replicates and error bars represent the standard deviation. The different letters correspond to significant differences between the treatments ( $p < 0.05$ ). The absence of letters on the graphics means there are no significant differences among all the treatments.

## Anexo II



**THE EFFECTS OF DIFFERENT SALINITY  
CONCENTRATIONS ON GROWTH OF THREE  
FRESHWATER GREEN ALGAE**

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## SUMMARY

In low-salinity systems the phytoplankton is commonly composed by freshwater species. The native species grow under specific salinity concentrations which must not suffer major changes to avoid the inhibition of their growth. Organisms living in systems with high NaCl concentrations had to adapt themselves in order to maintain intracellular concentration of Na<sup>+</sup> lower than toxic values. This is a problem that becomes more and more serious due to the salt intrusion in freshwater aquiferous. As freshwater is being used salt water replaced it and the freshwater organisms must adapt to survive. In that order, it is important understand the tolerance of freshwater organisms to salinity.

The potential effect of different salinity concentrations were tested in three freshwater green algae: *Chlorella vulgaris* Beijerinck, *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory. Salinity experiments were based in synthetic medium (with a NaCl concentration ranging from 0.00 to 14.00 gL<sup>-1</sup>) and in environmental-derived water medium with salinity ranging between 0.00 and 14.28 psu (psu – practical salinity units for ocean seawater).

**KEYWORDS:** Salinity, growth inhibition, EC<sub>50</sub>, *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Pandorina morum*.

## INTRODUCTION

Phytoplankton growth may be affected by several environmental parameters such as light, nutrients, temperature and salinity [1, 2, 3]. Freshwater microalgae are often used in standard growth inhibition tests [4, 5, 6]. The response of each species towards those factors varies under different test conditions because sensitivity differs among species. Thus, a set of different species instead of a single species should be used to determine the influence of a particular environmental factor.

High salinity concentrations become a serious problem due to environmental changes. Freshwater species must adapt themselves to stress salinity conditions. Thus, it is important to understand how these organisms adapt to high NaCl concentrations.

Several works have been performed in order to understand the effects of salinity on these organisms [2, 7, 8, 9, 10, 11], being rare the use of several microalgae at the same time and the comparison of their growth using synthetic and environmental – derived media. On the other hand, there are few works using *Pseudokirchneriella subcapitata*, *Chlorella vulgaris* or *Pandorina morum*. In halotolerance tests microalgae are often used [1, 11, 12]. Some algae (such as the ones belonging to the genus *Dunaliella*) are haloterant, growing at high saline media due to a certain mechanisms; glycerol intracellular accumulation [13], and a salt-induced protein (p60) present in plasma membrane [7].

This study aims to estimate the halotolerance of *Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Pandorina morum* under different salinity concentrations using a synthetic and an environmental-derived water media. Also, this work pretends to evaluate if there are different sensitivities among them.

## **MATERIALS AND METHODS**

The potential growth inhibition effects of different salinity concentrations on three freshwater green algae (*Chlorella vulgaris* Beijerinck, *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory) were tested. *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* were obtained by Bacteria – Free Protease Agar, Carolina Biological Supply Company – Burlington, North Carolina 27215 and Alga – Gro<sup>®</sup> Freshwater, Carolina Biological Supply Company – Burlington, North Carolina 27215, respectively. *Pandorina morum* was obtained in the environment and isolated by micromanipulation in laboratory. Then, the identification of *P. morum* followed Huber-Pestalozzi [14]. The experiments were based in synthetic medium (sterilised MBL medium [15] with the addition of NaCl concentrations ranging from 0.0 to 14.0 gL<sup>-1</sup>) and environmental-derived water medium with salinity ranging between 0.0 to 14.3 (psu – practical salinity units for ocean seawater).

Different environmental-derived water medium concentrations were obtained by using seawater (with a salinity value of 36 psu) filtered through Whatman GF/C filters and to which the adequate MBL quantities were added in order to have a final volume of 40 mL in 100 Erlenmeyer flasks. The controls (salinity of 0.0 psu) used for the following

tests were 70 % MBL + 30 % distilled water (C1) and 60 % MBL + 40 % distilled water (C2) for *C. vulgaris*, 70 % MBL + 30 % distilled water (C1) for *P. subcapitata* and 85 % MBL + 15 % distilled water (C1) for *P. morum* where growth differences proved to be not significant in relation to the growth of these species in MBL saturated medium [16]. The psu values for the MBL and the environmental water (EW) mixtures were obtained as follows: 0.9; 1.8; 2.7; 3.6; 5.4; 7.1; 10.7 and 14.3 corresponding to 97.5 % MBL + 2.5 % EW for *P. morum*; 95 % MBL + 5 % EW for *P. subcapitata* and *P. morum*; 92.5 % MBL + 7.5 % EW for *P. morum*; 90 % MBL + 10 % EW for *C. vulgaris*, *P. subcapitata* and *P. morum*; 85 % MBL + 15 % EW for *C. vulgaris*, *P. subcapitata* and *P. morum*; 80 % MBL + 20 % EW for *C. vulgaris* and *P. subcapitata*; 70 % MBL + 30 % EW for *C. vulgaris* and *P. subcapitata* and 60 % MBL + 40 % EW for *C. vulgaris*, respectively.

By using these proportions of MBL/EW, the basic nutritive requirements were assured as proved by the growth tests using the same proportions of distilled water already described above for these microalgae. Thus, if a significant negative effect is detected, the nutritive deficiency would not be the main responsible.

A preliminary test was carried out in which 100 mL borosilicate Erlenmeyer flasks were used with a final test volume of 40 mL for the three freshwater green algae. The biomass results obtained for *P. morum* by dry weight proved to be less accurate than chlorophyll *a* concentration due to the crystallisation process occurring at higher salinities and which masked the real biomass value (even after several washes with distilled water).

To compare both methods, 250 mL Erlenmeyer flasks were used to guaranty an enough quantity of final test volume (100 mL) to perform dry weight and chlorophyll *a* determinations. Therefore, for *P. morum* chlorophyll *a* (chl *a*) was the chosen biomass parameter for this colonial alga once it has proven to be highly correlated with algal biomass in previous studies [16]. For *C. vulgaris* and *P. subcapitata*, optical density was chosen, according to the same study previously referred [16] reporting high correlations between optical density and cell counts for both species. For each treatment and control there were set up three replicates. OECD [5] recommends preparing an inoculum culture in the test medium 2-3 days before starting the test. Therefore, inoculum cultures were incubated under the same conditions as the test cultures 3 days before starting the

experience to adapt the test alga to the test conditions although the growth was made in synthetic medium without NaCl. The initial cellular concentration used for the three algae, in the growth inhibition tests, was  $5 \times 10^4$  cells·mL<sup>-1</sup> as recommended by OECD [5], in order to assure a minimum cell density that allows a higher sensitivity of the toxicity test. There were followed aseptic conditions. The Erlenmeyer flasks with the different salinity concentrations were placed in an incubation chamber (F10 000 EDTU model), during 96h with photoperiod (16 hours light – 8 hours dark), continuous agitation (GFL 3015) at 100 rpm and controlled temperature at  $20 \pm 2^\circ\text{C}$ . At 96h optical density at 440 nm was determined for *C. vulgaris* and *P. subcapitata* and chlorophyll *a* for *P. morum*. Chl *a* determination was made by filtering 40-50 mL from the culture Erlenmeyer flasks volume through GF/C filters, by treatment with acetone (90%) and measuring at 665nm and 750nm, with and without hydrochloric acid (HCl) 0.1M, in a 6505 UV/VIS spectrophotometer. OECD [5] recommends that algal growth tests should be finished after 72h of incubation. In this experiment it was determined after 4 days of incubation (after 96h) because the test flasks were incubated under a photoperiod.

The results obtained after 96h of incubation (as optical density for *C. vulgaris* and *P. subcapitata* and chl *a* for *P. morum*) among different salinity concentrations were compared by variance analysis (ANOVA) in both media (synthetic medium and environmental-derived water medium). A Tukey multiple comparison test was used [17], when statistically significant differences in inhibition were reported  $p < 0.05$ . For each species, it was also calculated by probit analysis the EC<sub>50</sub> based on inhibition concentrations.

## RESULTS

At the end of the 96h test period, the results using the saline artificial MBL media showed that the growth of *C. vulgaris* was significantly reduced at  $5 \text{ gL}^{-1}$  and it was greatly inhibited at  $8 \text{ gL}^{-1}$  or more (Figure 1.A). *P. subcapitata* growth was significantly reduced at  $4 \text{ gL}^{-1}$  (Figure 1.B). Finally, for *P. morum* the growth of this colonial alga was highly decreased with salinities of  $2 \text{ gL}^{-1}$  or more, with statistical differences (Figure 1.C). The EC<sub>50</sub> values obtained were  $5.1 \text{ gL}^{-1}$  (4.3-6.1),  $4.1 \text{ gL}^{-1}$  (3.6-4.7),  $0.6 \text{ gL}^{-1}$  (0.002-3.6) for *C. vulgaris*, *P. subcapitata* and *P. morum*, respectively.

The results obtained with the environmental-derived water medium for *C. vulgaris* and *P. morum* showed significant inhibition for 10.7 psu (almost twice than the result indicated for the artificial medium) and 0.9 psu (lower than the obtained for synthetic medium), correspondingly (Figure 2.A and B). *P. subcapitata* showed significant inhibition at 3.6 psu, a result not very different from that obtained using synthetic medium (Figure 2.C). The EC<sub>50</sub> values obtained were 7.0 psu (4.6-10.7), 3.7 psu (2.7-5.1), 1.4 psu (0.8-2.1) for *C. vulgaris*, *P. subcapitata* and *P. morum*, respectively. For *C. vulgaris* and *P. morum* the differences between the results obtained with artificial medium and environmental-derived water medium might be explained by the toxicity of NaCl and the presence of a stimulation algal growth in the environmental-derived water that is not in the compounds of MBL.

## **DISCUSSION AND CONCLUSION**

From the results obtained we may infer that salinity affects the growth of these three freshwater green algae as NaCl concentrations become higher. We may state that *C. vulgaris* is the most halotolerant alga and *P. morum* the most sensitive. The tests also proved that *C. vulgaris* and *P. morum* were more sensitive to saline synthetic medium than to seawater derived medium. On the other hand, *P. subcapitata* was more sensitive to environmental-derived water medium than to saline synthetic medium.

Moisander et al [9] also showed differences in growth rate of estuarine cyanobacteria in different concentrations of NaCl (0, 2, 6, 10, 15 and 20 gL<sup>-1</sup>). *Nodularia* strain FL2f showed no significant difference in the experiments, although *Anabaenopsis* was the only tested alga growing less in the treatments without salt [9]. The other tested cyanobacteria showed significant differences in higher NaCl concentrations. Zeng et al [8] and Neale et al [2] shown that under salt stress *Spirulina platensis* and *Chlamydomonas reinhardtii* decrease the photosynthetic activity, although Moisander et al [9] stated that estuarine cyanobacteria photosynthesis rate increases under salt concentrations. Other studies [10] also proved that phytoplankton can adapt to salinity conditions although this process becomes slower as NaCl concentration increases. The variations in sensitivity between the algae may be due to infrastructural differences such as specific wall features. In that way algae show different growths according to their adaptive mechanisms which define their tolerance or sensitivity in high saline media.

From the present study, it can be concluded that there is an important effect of salinity over the growth of the tested freshwater green algae and that some algae are more

halotolerant (such as *Chlorella vulgaris*) and adapt faster to salt stress conditions than others, which are more sensitive (such as *Pandorina morum*). However, more tests should be conducted to understand how it happens and which mechanisms are behind the halotolerance differences observed.

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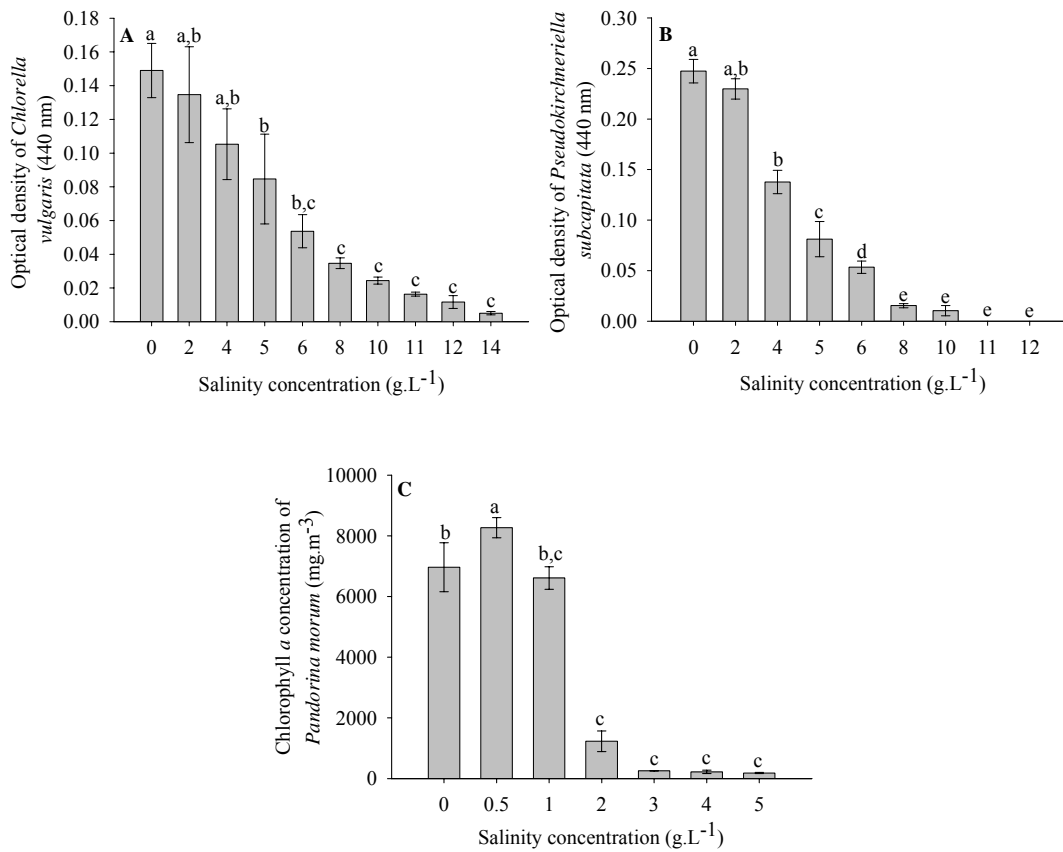


Figure 1 – Algal growth in synthetic medium, with NaCl concentrations ranging from 0.0 to 14.0 g.L<sup>-1</sup>, after 96 h of incubation using (A) *C. vulgaris*, (B) *P. subcapitata* and (C) *P. morum* [a, b, c, d, e represent statistical differences (p<0.05) in the algal growth between treatments].

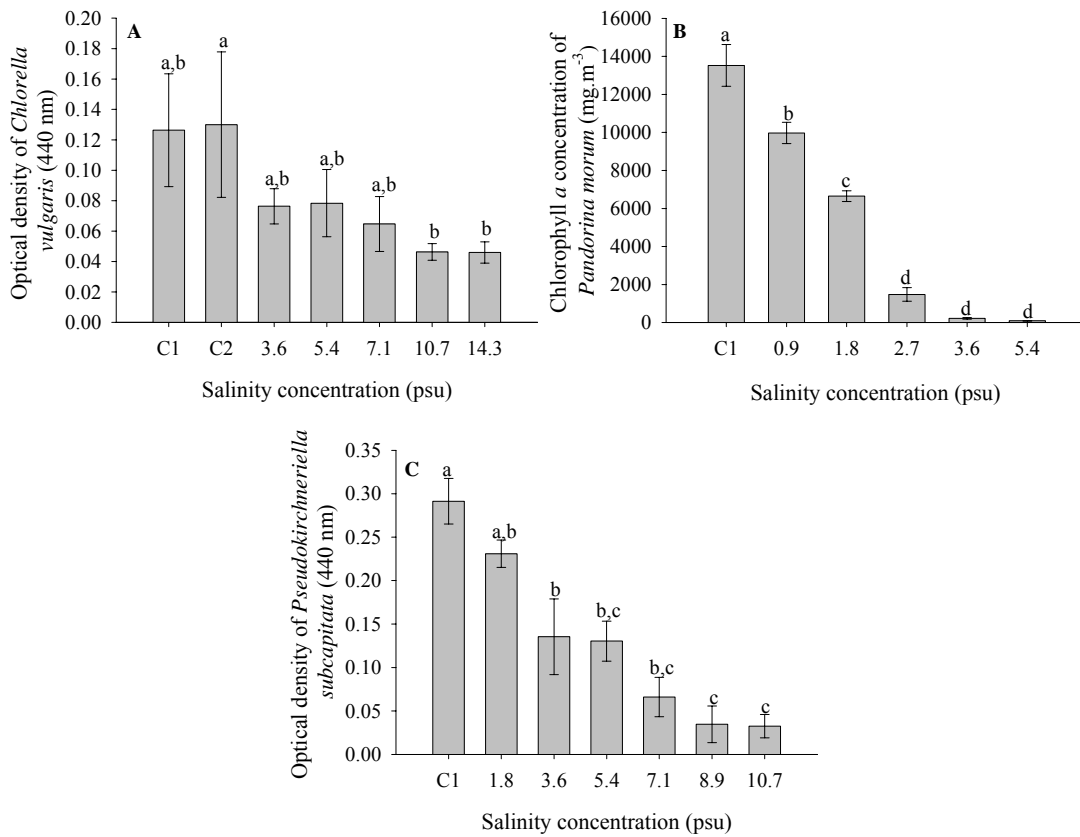


Figure 2 – Algal growth in environmental-derived water medium with salinity ranging between 0.0 (C1 and C2) to 14.3 psu by MBL dilutions, after 96 h of incubation using (A) *C. vulgaris*, (B) *P. morum* and (C) *P. subcapitata* [a, b, c, d represent statistical differences ( $p < 0.05$ ) in the algal growth between treatments].



## Anexo III



**Acute (immobilisation) and chronic (life history) responses of two  
freshwater cladocerans (*Daphnia magna* and *Daphnia longispina*) to  
salinity**

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**Abstract**

Salinity is one of the threats to freshwater ecosystems and is considered an environmental key factor changing aquatic community structure, whose effects can be irreversible. Thus, freshwater species must adapt to stress salinity conditions to survive and reproduce. In this work the effects of different salinity concentrations were tested in two species of cladocerans: *Daphnia magna* (standard test organism) and *Daphnia longispina* (autochthonous species). Salinity experiments were based in successive dilutions of a stock solution of NaCl in a synthetic medium. The results showed that *D. magna* is more tolerant than *D. longispina*, both in acute (EC<sub>50</sub> 5.9 and 2.9 g/L, respectively) and chronic (EC<sub>50</sub> 5.0 and 2.2 g/L, correspondingly) exposures. In the chronic exposure, salinity caused a significant reduction in fecundity and a developmental delay (increase in age at first reproduction), as well as a decrease in the growth rate of daphnids. However, these effects were mainly observed at salinity concentrations where mortality occurred.

**Additional keywords:** saline stress, sodium chloride, toxicity tests, zooplankton.

## **Introduction**

Salinity intrusion in freshwater ecosystems is a problem that is becoming more and more serious due to environmental change. These systems can suffer important alterations with the increase of salinity due to global climate change, such as sea level rise (Schallenberg et al., 2003). Several authors (e.g. Hall and Burns, 2003; Schallenberg et al., 2003) have reported a reduction in diversity and abundance of zooplankton communities exposed to salinity increase. Freshwater species must either adapt or avoid the saline stress in order to survive and reproduce. The ability of the organisms to survive and reproduce in such unstable environments is as higher as their amount of tolerance (Rokneddine and Chentoufi, 2004). Studies have been carried out in freshwater ecosystems, which are becoming threatened by salinity, in order to evaluate how the raise of salt affects the zooplankton species, altering ecosystems structure and function (Hall and Burns, 2003; Nielsen et al., 2003; Schallenberg et al., 2003; Rokneddine and Chentoufi, 2004). Still, there is little information on how the increase of salinity affects ecosystem integrity in order to predict and rehabilitate aquatic systems under salinity conditions (Nielsen et al., 2003). Therefore, it is important to study the effect of salinity stress in aquatic organisms. Among them, zooplankton has long been used as a suitable group to assess the impact of environmental change, in part due to its key intermediate position in the trophic food web. Zooplankton organisms are frequently used as indicators of water quality due to the narrow relationship between environmental factors and species composition, which are mainly affected by mineralization gradients (Sánchez-Colomer, 1996; Boronat et al., 2001). Salinity changes in freshwater systems may greatly affect the survival and the

life history (growth and reproduction) of zooplankters and, consequently, have an impact in higher trophic levels. Cladocerans, one of the most abundant primary consumer groups in lentic ecosystems, show different physiological tolerances to several ionic components (Bos et al., 1996) and are good indicators of environmental changes (water quality and/or historical differences), especially to salinity changes (Sánchez-Colomer, 1996; Boronat et al., 2001; Amsinck et al., 2003). The genus *Daphnia*, in particular, has a wide range of tolerance to salinity and these differences among species can have distinct effects in the system dynamics such as in the abundance of primary producers, the rate of primary production and rates of nutrient cycling (Boronat et al., 2001; Hairston et al., 2005). As an example, euryhaline species *Daphnia magna* lives in fresh, brackish and athalassohaline waters while daphnids from the *longispina* group (*D. longispina*, *D. galeata*, *D. hyalina*, *D. cucullata*) prefer waters with low mineralization and are very sensitive to magnesium (concentrations above 0.6 g.L<sup>-1</sup> become lethal for them) (Alonso, 1996).

In order to assess the tolerance of *Daphnia* to salinity (as NaCl), we tested its effects on the immobilisation (acute toxicity) and life history (chronic toxicity) responses of two *Daphnia* species: *Daphnia magna* Straus and *Daphnia longispina* O. F. Müller. The former (*D. magna*) is a common test organism, widely used in standard ecotoxicological procedures, while *D. longispina* is an ubiquitous European species. The main aims of this work were: 1) to determine salinity thresholds which cause adverse effects to *Daphnia* spp., both in acute and chronic exposures; 2) to compare these thresholds between species (i.e. to compare their tolerance).



## Materials and methods

### *Daphnid cultures*

Monoclonal cultures of *Daphnia magna* (clone A, *sensu* Baird et al., 1989a) and *Daphnia longispina* (clone EM7, *sensu* Antunes et al., 2003) have been reared in our laboratory for several generations using widespread procedures for *D. magna* (see Baird et al., 1989b; Soares et al., 1992; ASTM, 1997). Daphnids were cultured in ASTM hard water synthetic medium (ASTM, 1980), to which an organic additive (*Ascophyllum nodosum* extract; Baird et al., 1989b) was added. Cultures were reared under a 16h<sup>L</sup>:8h<sup>D</sup> photoperiod (provided by cool white fluorescent lights) and a temperature of 20±2°C. The medium was renewed every other day, to a total of three times per week. Animals were fed with *Pseudokirchneriella subcapitata* (Korshikov) Hindak every other day, at a concentration of 3.0×10<sup>5</sup> cells.mL<sup>-1</sup> for *D. magna* and 1.5×10<sup>5</sup> cells.mL<sup>-1</sup> for *D. longispina*. Algal (food) ration was determined spectrophotometrically, as described for *Chlorella vulgaris* by Carvalho et al. (1995).

### *Acute tests*

Tests were performed according to standard protocols (ISO, 1996; OECD, 2000; USEPA, 2002) under the same temperature and photoperiod regimes as described for rearing procedures. For both *Daphnia* species, experiments were initiated with neonates (< 24-h-old) obtained from the same bulk culture, born between the 3<sup>rd</sup> and 5<sup>th</sup> broods. The experimental saline concentrations were obtained by successive dilutions of a stock solution of sodium chloride (NaCl) in the synthetic hard water medium, with salinity concentrations ranging from 4.40 g.L<sup>-1</sup> to 7.09 g.L<sup>-1</sup> and from 2.50 g.L<sup>-1</sup> to 4.03 g.L<sup>-1</sup> for *D. magna* and *D. longispina*, correspondingly. The culture medium was used as the control treatment. Tests were carried out in glass beakers (four per treatment) containing

100 mL of test solutions. A static design was employed, using 20 animals (randomly divided into four groups of five animals) per control and per NaCl concentration. Daphnids were exposed to the different salinity concentrations during 48h, without food or organic extract. Vessels were checked for immobilised individuals, at 24 and 48 h, for posterior determination of EC<sub>50</sub> values (see Statistical analyses).

### *Chronic tests*

Tests were conducted for 21 days according to standard protocols (ASTM, 1997; OECD, 1998; ISO, 2000), under the same temperature and photoperiod regimes as described for rearing procedures. Tests were initiated with neonates (< 24-h-old) obtained from the same bulk culture, born between the 3<sup>rd</sup> and 5<sup>th</sup> broods. The body lengths (from the base of the spine to the top of the head) of a subsample (n=20) of neonates from the same batch were measured under stereoscope magnification in order to determine their size at the beginning of the test ( $l_i$  – initial body length). The saline concentrations tested ranged from 3.42 g.L<sup>-1</sup> to 5.50 g.L<sup>-1</sup> for *D. magna* and from 1.55 g.L<sup>-1</sup> to 2.50 g.L<sup>-1</sup> for *D. longispina*. The culture medium was used as the control treatment. Experiments were carried out using glass beakers (10 per treatment) containing 50 mL of test solutions (including organic extract, unlike in the acute tests). A semi-static design was employed, using 10 individualised organisms (i.e. one per replicate) randomly assigned to the control and to each NaCl concentration. Daphnids were fed daily (with *P. subcapitata*) and transferred to freshly-prepared test solutions every other day. Animals were checked every single day at the same approximate hour of the day for mortality and reproductive state (presence of eggs or offspring). Every time neonates were released, they were counted and discarded. A life history table was built with the mortality and fecundity data. At the end of the test, all surviving

organisms (mothers) were measured (from the base of the spine to the top of the head) under stereoscope magnification ( $l_f$  – final body length). This allowed the calculation of the somatic growth rate, which was estimated from the initial ( $l_i$ , in mm) and final ( $l_f$ , in mm) body size of the daphnids, according to the following expression:

$$growth\ rate = \frac{\ln(l_f) - \ln(l_i)}{\Delta t},$$

where growth rate is expressed in  $\text{day}^{-1}$  and  $\Delta t$  is the time interval (21 days).

Survival and fecundity estimates were also used to compute the intrinsic rate of population increase ( $r$ ). This demographic parameter was iterated from the Euler–Lotka equation:

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

where  $r$  is expressed in  $\text{day}^{-1}$ ,  $x$  is the age class (1...n days),  $l_x$  is the probability of surviving to age  $x$ , and  $m_x$  is the fecundity at age  $x$ . Pseudovalues and standard errors for  $r$  were estimated using the jack-knifing technique described by Meyer et al. (1986).

### *Statistical analysis*

For each *Daphnia* species, the  $EC_{50}$  values for immobilisation (acute tests) and fecundity (chronic tests) were calculated by probit analysis (Finney, 1971). To test the significance of the effect of salinity on the life history parameters of the daphnids, a one-way ANOVA was employed on the chronic test data. When the ANOVAs were statistically significant, a Dunnett test was then applied to the data in order to determine which concentrations were significantly different from the control group. A significance level ( $\alpha$ ) of 0.05 was used in all the analyses.

## Results

Acute immobilisation tests showed that *D. magna* is more tolerant to salinity than *D. longispina*. The acute EC<sub>50</sub> value determined for *D. magna* (5.9 g.L<sup>-1</sup>) was about two-fold the value obtained for *D. longispina* (2.9 g.L<sup>-1</sup>) (table 1). In absolute terms, the NaCl concentrations used in the acute test represent low levels of salinity.

Salinity caused a significant effect on the life history of both daphnids, affecting all parameters, with the exception of number of broods in *D. magna* (table 2). Figures 1 and 2 show the life history responses of *D. magna* and *D. longispina* to salinity, respectively. Mortality was not higher than 10% in *D. magna*, except in the last concentration where it achieved the highest value (90%). In *D. longispina*, mortality increased in the last two concentrations (30 and 90%, correspondingly). A concentration-dependent decrease in the number of offspring was observed for both species, accompanied by a developmental delay, i.e. an increase in age at first reproduction (AFR). The number of broods produced per female was significantly reduced only in *D. longispina*. As a consequence of the lower fecundity and of the developmental delay, the intrinsic rate of increase (*r*) was also significantly reduced in both species, in a concentration-dependent manner. A decrease in the somatic growth rate of both species was also observed; however, in *D. longispina*, this reduction was preceded by a stimulatory effect at low levels (1.55 g.L<sup>-1</sup>). The number of offspring was the most affected parameter, being drastically reduced with the increase of salinity in both species.

The EC<sub>50</sub> values determined from the fecundity data showed once again that *D. magna* tolerate salinity concentrations two-fold higher than the salinity concentrations tolerated by *D. longispina* (table 1). Indeed, the LOEC value for all parameters in *D. magna* was 5 g.L<sup>-1</sup>, while for *D. longispina* it ranged between 1.71 and 2.07 g.L<sup>-1</sup> (table

3). Acute and chronic EC<sub>50</sub> values were very close within each species, for both *Daphnia* species (table 1). In fact, the concentrations used in the chronic test were very close to the acute EC<sub>50</sub> value for each species, with life history impairment occurring immediately prior to high mortality (see figs 1 and 2). For both *Daphnia* species, reproductive impairment and mortality occurred within a narrow range of salinities (5.00-5.50 g.L<sup>-1</sup> in *D. magna* and 1.71-2.50 g.L<sup>-1</sup> in *D. longispina*).

## Discussion

This study demonstrated that relatively low levels of salinity are deleterious to both freshwater cladoceran species: *D. magna* and *D. longispina*. The acute and chronic EC<sub>50</sub> values for *D. magna* (5.9 g.L<sup>-1</sup> and 5.0 g.L<sup>-1</sup>, respectively) were about two-fold the values obtained for *D. longispina* (2.9 g.L<sup>-1</sup> and 2.2 g.L<sup>-1</sup>, correspondingly), which confirmed that *D. longispina* is more sensitive to salinity stress than the standard species, *D. magna*. The results obtained in this work are comparable to other studies with *Daphniidae* (see table 4). A high variability of protocols was used when testing the effects of salinity, so comparison must be made with caution. Kefford et al. (2004), for example, stated that artificial sea salt is less toxic than NaCl. Differences in tolerance to artificial sea salt and NaCl may be due to pH, which tends to be lower in NaCl treatments (Kefford et al., 2004). The juvenile *D. carinata* acute EC<sub>50</sub> value obtained by Hall and Burns (2002) was much lower than that obtained by Kefford (2000) (table 4). Barry et al. (2005) established that salinities of about 4 g.L<sup>-1</sup> were tolerated by some species within the genus *Daphnia*, showing 48h EC<sub>50</sub>s for seven *D. thomsoni* clones that were between 7.49 and 8.79 g.L<sup>-1</sup> (table 4). The lowest 48h EC<sub>50</sub> value in the literature was obtained by Semsari and Haït-Amar (2001) for *D. magna*, which is an unusual

value when compared to those obtained in this work and in the works described above (table 4).

Boronat et al. (2001) demonstrated that cladoceran species have different halotolerances, ranging from fresh to saline water bodies, with *D. longispina* being estenohaline (range 0.5-1.2 g.L<sup>-1</sup>) and *D. magna* euryhaline (range 3.8-38.0 g.L<sup>-1</sup>). In fact, *D. magna* is one of the most halotolerant *Daphnia* species (Cowgill and Milazzo, 1990; Rokneddine and Chentoufi, 2004; Do Hong et al., 2004; Hairston et al., 2005). Tackx et al. (2004) observed that cladocerans were mostly present in the freshwater part (<0.7 p.s.u. – practical salinity units for ocean seawater: 35 p.s.u. = 35 g.Kg<sup>-1</sup>) of the Schelde estuary where a horizontal salinity gradient (0-10 p.s.u.) is present. On the other hand, other small zooplankters (e.g. rotifers and copepods) were abundant in the brackish water zone (0.7-10 p.s.u.) of the estuary, in this same study. Schallenberg et al. (2003) stated that, at salinities above 2.7 p.s.u., the zooplankton community changes from one consisting of copepods, rotifers, cladocerans, amphipods and amoebae, at low salinities, to one represented only by rotifers.

Life history parameters can also be affected, in general, by the increase of salinity, as shown in our study. In this work, the most drastic reduction caused by NaCl in life history traits was that observed in the number of neonates. Additionally, growth rate of both daphnids was also negatively affected and maturation was delayed at the highest concentrations. Similarly, Teschner (1995) concluded clones of *D. magna* under brackish conditions grew more slowly and matured on average later than under freshwater conditions. Nevertheless, our results demonstrated that *D. magna* reproduces and lives well at salinity concentrations below 4.55 g.L<sup>-1</sup>. These results are comparable to those obtained by Schuytema et al. (1997), who stated that *D. magna* reproduce and survive normally at salinity concentrations below 4 g.L<sup>-1</sup>. *D. magna* and *D. longispina*

showed the highest rate of mortality in the last concentration, being noticeable a drastic reduction in the number of survivors in this concentration.

Nielsen et al. (2003) stated that microinvertebrates, freshwater algae and aquatic plants appear to be the biological communities less tolerant to increase of salt, tolerating salinity concentrations below 3 g.L<sup>-1</sup>. Other studies (Barron et al., 2002) also proved phytoplankton can adapt to salinity conditions, although this process becomes slower as NaCl concentration increases. Some algae (such as the ones belonging to the genus *Dunaliella*) are haloterant, growing at high saline media due to physiological adaptations, such as glycerol intracellular accumulation (Avron, 1986) and a salt-induced protein (p60) present in the plasma membrane (Fisher et al., 1994). Correspondingly, other authors stated that some cladocerans develop mechanisms to adapt to environmental changes, particularly salinity (Teschner, 1995; Schallenberg et al., 2003; Rokneddine and Chentoufi, 2004). Genetic and physiological regulations to tolerate fluctuating environmental conditions determine the tolerance limit of these organisms to those factors (Rokneddine and Chentoufi, 2004).

Salinity is an important and crucial factor in determining the presence and dominance succession of organisms. This work has proved how the increase of NaCl affects significantly survival and life history traits, which can cause changes in the zooplankton community structure. Other authors (e. g. Nielsen et al., 2003; Bailey et al., 2004) stated that, if salinity changes occur very rapidly, organisms can not adapt and freshwater *taxa* can become locally extinct, transferring dominance to salt tolerant *taxa*. However, Bailey et al. (2004) demonstrated that resistance eggs of *Daphnia* sp. hatched when returned from high levels of salt to freshwater conditions, suggesting that fast recolonization is possible from local ephippial egg banks.

The results obtained in this work suggest that the increase of salinity in freshwater systems can have profound implications on biodiversity, affecting the survival, growth and reproduction of the species and eliminating them if salinity concentrations remain above their tolerance. Further studies with freshwater species under brackish and saline conditions are required for understanding how freshwater species adapt to such a stressful environment, in terms of their physiology, and to explain the inter-species differences in halotolerance. Studies in more realistic and complex scenarios (e.g. mesocosms) may be useful in order to understand the effects of salinity, as well as its interactions with other factors (nutrients, fish), at the guild/community level, thus providing more ecologically relevant information.

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## Figure legends

Figure 1 – Life history parameters of *Daphnia magna* exposed to different salinities (as NaCl concentrations) for 21 days. Error bars represent standard error and \* indicates statistically significant differences (Dunnett test,  $p \leq 0.05$ ) between the test concentrations and the control (CTL).

Figure 2 – Life history parameters of *Daphnia longispina* exposed to different salinities (as NaCl concentrations) for 21 days. Error bars represent standard error and \* indicates statistically significant differences (Dunnett test,  $p \leq 0.05$ ) between the test concentrations and the control (CTL).

Tables

**Table 1** – Acute (immobilisation) and chronic (fecundity) EC<sub>50</sub> values of salinity (as NaCl) for *D. magna* and *D. longispina*, with respective 95% confidence limits (in brackets).

Species	Acute EC <sub>50</sub>	Chronic EC <sub>50</sub>
<i>D. magna</i>	<b>5.9 g.L<sup>-1</sup></b> (5.5 – 6.5)	<b>5.0 g.L<sup>-1</sup></b> (4.9 – 5.1)
<i>D. longispina</i>	<b>2.9 g.L<sup>-1</sup></b> (2.8 – 3.0)	<b>2.2 g.L<sup>-1</sup></b> (2.2 – 2.3)

**Table 2** – Summary of one-way ANOVA analyses of variance applied to the life history parameters with *D. magna* and *D. longispina* (d.f. – degrees of freedom; p – probability; NS – non significant).

Parameters	<i>D. magna</i>			<i>D. longispina</i>		
	F	d.f.	p	F	d.f.	p
Number of offspring	4.1	5, 48	0.004	49	5, 46	<0.001
Age at first reproduction	5.5	6, 55	<0.001	34	5, 49	<0.001
Number of broods	2.3	5, 50	NS	29	5, 46	<0.001
Somatic growth rate	4.3	5, 48	0.003	15	5, 46	<0.001
Intrinsic rate of increase	24	6, 62	<0.001	39	5, 53	<0.001



**Table 3** – NOEC and LOEC values (in g.L<sup>-1</sup>) for each life history parameter of *D. magna* and *D. longispina* exposed to different salinity concentrations, during 21 days.

<b>Parameters</b>	<b><i>D. magna</i></b>		<b><i>D. longispina</i></b>	
	<b>NOEC</b>	<b>LOEC</b>	<b>NOEC</b>	<b>LOEC</b>
Number of offspring	4.55	5.00	1.55	1.71
Age at first reproduction	4.55	5.00	1.88	2.07
Number of broods	-	-	1.88	2.07
Somatic growth rate	4.55	5.00	1.88	2.07
Intrinsic rate of increase	4.55	5.00	1.55	1.71

**Table 4** – Acute and chronic EC<sub>50</sub> values of salinity calculated by other authors for Daphniidae.

Species	Acute EC <sub>50</sub>	Chronic EC <sub>50</sub>	Reference
<i>D. longispina</i>	2.9 g.L <sup>-1</sup>	2.2 g.L <sup>-1</sup>	This study
<i>D. magna</i>	8.3g.L <sup>-1</sup>	-	Cowgill and Milazzo (1996)
<i>D. magna</i>	11.3 g.L <sup>-1</sup>	8.57 g.L <sup>-1</sup>	Schuytema et al. (1997)
<i>D. magna</i>	0.49 g.L <sup>-1</sup>	-	Semsari and Hait-Amar (2001)
<i>D. magna</i>	7.3 g.L <sup>-1</sup>	-	Do Hong et al. (2004)
<i>D. magna</i>	5.9 g.L <sup>-1</sup>	5.0 g.L <sup>-1</sup>	This study
<i>D. carinata</i>	7.7 g.L <sup>-1</sup> [artificial sea water (25°C)]	-	Kefford (2000)
<i>D. carinata</i>	5.3 g.L <sup>-1</sup> [saline lake water (25°C)]	-	Kefford (2000)
<i>D. carinata</i>	0.9 g.L <sup>-1</sup> (20°C)	-	Hall and Burns (2002)
<i>D. carinata</i>	2.2 g.L <sup>-1</sup> (10°C)	-	Hall and Burns (2002)
(adults)	1.7 g.L <sup>-1</sup> (20°C)	-	Hall and Burns (2002)
<i>D. thomsoni</i> (7 clones)	7.5 – 8.8 g.L <sup>-1</sup>	-	Barry et al. (2005)
<i>C. cornuta</i>	2.6 g.L <sup>-1</sup>	-	Do Hong et al. (2004)

Figure 1

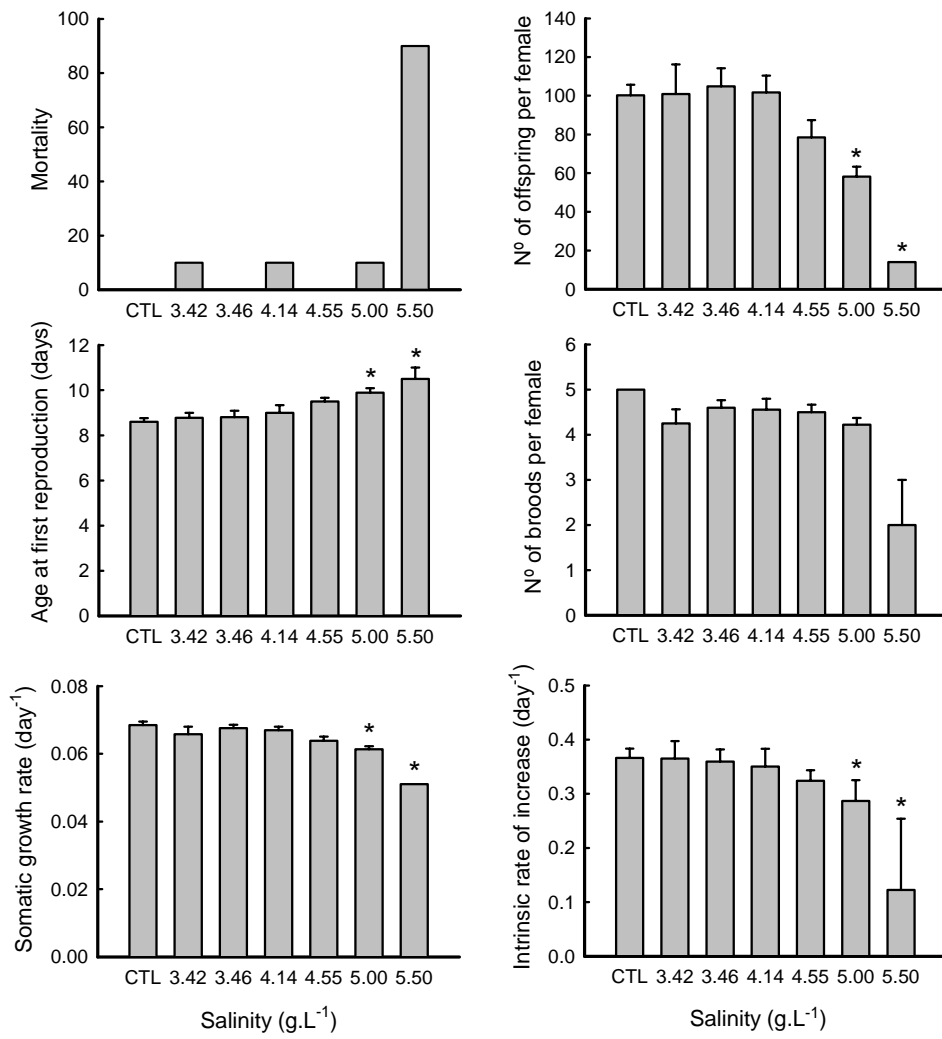


Figure 2

