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Departamento de Biologia

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Cátia Alexandra Ribeiro Venâncio

Salinization effects on coastal terrestrial and freshwater ecosystems

Efeitos de salinização em ecossistemas costeiros terrestres e de água doce

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Isabel Maria da Cunha Antunes Lopes (Investigadora Principal do CESAM e Departamento de Biologia da Universidade de Aveiro), do Professor Doutor Rui Godinho Lobo Girão Ribeiro (Professor Associado com Agregação do Departamento de Ciências da Vida da Universidade de Coimbra) e da Doutora Ruth Maria de Oliveira Pereira (Professora Auxiliar do Departamento de Biologia da Universidade do Porto).

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o júri

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palavras-chave

Salinização; NaCl; água do mar; ecossistemas dulçaquícolas; compartimento terrestre; efeito letal; efeito subletal; comunidade; resiliência

resumo

Os relatórios de avaliação do Painel Intergovernamentl para as Alterações Climáticas (IPCC) baseados nas últimas décadas preveem, até 2100, cenários de contínua subida do nível médio da água do mar e eventos climatéricos extremos. Como consequência destas alterações prevê-se a ocorrência de intrusões de água do mar e subsequente salinização destes ecossistemas costeiros (tanto terrestres como dulçaquícolas). A salinização destas regiões costeiras pode ocorrer diretamente por inundações de superfície pela água do mar, por exemplo, devido a tempestades costeiras violentas (provavelmente, causando sobretudo efeitos letais), mas também pode ocorrer por intrusão de água do mar nos sistemas subterrâneos de água doce (o que pode ocorrer de forma mais gradual, provavelmente causando efeitos subletais). Tendo em conta estes aspectos, o presente trabalho pretendeu avaliar os efeitos adversos que tal salinização pode causar nos ecossistemas costeiros. Para tal, foram delineados os seguintes objectivos específicos: (i) determinar se o cloreto de sódio (NaCl) pode ser utilizado como substituto da água do mar, em avaliações preliminares de risco ecológico. Esta alternativa seria vantajosa uma vez que existem muitos dados de toxicidade para NaCl e, portanto, reduziria o número de ensaios de toxicidade que seria necessário realizar; (ii) identificar quais os receptores ecológicos mais sensíveis à salinização, utilizando protocolos padronizados; (iii) estabelecer se a biota é capaz de aumentar a sua tolerância a baixos níveis de salinização através de mecanismos de plasticidade fenotípica; (iv) avaliar os efeitos do aumento da salinidade nas relações interespecíficas; e (v) identificar os efeitos de salinização nas comunidades dulçaquícolas e terrestres em cenários de exposição mais realistas. Estes objetivos foram abordados ao longo de sete capítulos, recorrendo a abordagens ecotoxicológicas padronizadas e não padronizadas desde o nível de organização biológica indivíduo (expondo organismos, de espécies pertencentes a diferentes níveis tróficos, a níveis de salinidade crescentes) até ao nível da comunidade (realizando exposições com várias espécies em cenários mais realistas de exposição). Os resultados obtidos revelaram que, de um modo geral, o NaCl exerceu uma toxicidade similar ou superior à provocada pela água do mar, quer nas espécies dulçaquícolas quer nas terrestres. Esses resultados sugerem que o NaCl pode ser usado como substituto da água do mar nos primeiros estágios de avaliação do risco ecológico de salinização causada pela intrusão de água do mar. No entanto, o seu uso deve ser cauteloso, uma vez que houve algumas espécies para as quais a água do mar apresentou maior toxicidade, e no caso de exposições multigeracionais de espécies de microalgas, estas mostraram um aumento na sensibilidade à agua do mar.

No compartimento dulçaquícola, os cladóceros e os rotíferos foram os dois grupos taxonómicos que apresentaram maior sensibilidade à salinização (tanto para NaCl como para água do mar), enquanto que os peixes e as macrófitas mostraram ser os grupos mais tolerantes. Os dados de ecotoxicidade obtidos para plantas terrestres e fungos (gerados no presente trabalho) foram integrados com dados recolhidos da literatura, permitindo identificar os microinvertebrados terrestres (*Folsomia candida* e *Enchytraeus crypticus*) como o grupo mais sensível à salinização, enquanto que os

fungos e as plantas demonstraram ser os mais tolerantes. Os dados de toxicidade gerados a partir de ensaios padronizados (ou em parte compilados a partir de literatura para espécies terrestres) possibilitaram calcular as concentrações de risco que permitem proteger 95% das espécies num ecossistema (HC_5), com base em curvas de distribuição de sensibilidade das espécies. Os valores de HC_5 foram calculados para espécies dulçaquícolas e terrestres e para NaCl e água do mar; todos esses valores revelaram-se muito baixos ($HC_5 \leq 2{,}26$ $mScm^{-1}$) quando comparados com a condutividade da água do mar natural (≈ 52 $mScm^{-1}$), o que faz prever que os ecossistemas costeiros estarão em alto risco devido a salinização. De um modo geral, a pré-exposição a curto e a longo (multigeracional) prazo a baixos níveis de salinidade, não causou uma alteração significativa na tolerância da biota à salinização. No entanto, algumas espécies revelaram uma maior tolerância (associada à aclimatação fisiológica ou outros mecanismos de plasticidade fenotípica) à salinidade após curta exposição (espécie de anfíbio *Pelophylax perezi*) e exposição multigeracional (a cianobactéria *Cylindrospermopsis raciborskii* e o cladócero *Daphnia longispina*) a baixos níveis de salinidade. Contrariamente, observou-se também que algumas espécies apresentavam uma maior sensibilidade à salinização após exposição multigeracional (a microalga *Raphidocelis subcapitata* e a macrófita *Lemna minor*). Estes resultados diversos podem ser devidos a diferenças na intensidade de salinidade e à duração dos períodos de exposição a baixos níveis de salinização. Para compreender com maior exactidão a influência da exposição prolongada a baixos níveis de salinidade na tolerância da biota à salinidade, devem ser realizados estudos adicionais que envolvam exposição multigeracional a níveis subletais de salinização.

Em relação ao objetivo específico (iv), os resultados obtidos revelaram que os níveis de salinização subletal influenciaram as relações interespecíficas. Para as microalgas dulçaquícolas, observou-se que, a baixos níveis de salinidade, ocorreu uma alteração na competição entre as duas espécies estudadas. Adicionando ao efeito direto que estas alterações irão provocar na estrutura da comunidade de microalgas, também se preveem efeitos indiretos em outras espécies (por exemplo, espécies de cladóceros que se alimentam delas). Para as plantas terrestres, na ausência de salinização, foi registada uma menor produtividade para algumas plantas testadas quando as mesmas se apresentavam num cenário de policultura comparativamente às condições de monocultura. A exposição ao limiar de salinidade de 4 $mScm^{-1}$ pareceu não alterar este padrão de respostas.

Finalmente, a exposição em cenários mais realistas (mesocosmos) sugeriu uma toxicidade inferior da salinização nos ecossistemas dulçaquícolas do que aquela prevista através de abordagens padronizadas, sugerindo uma maior resiliência das comunidades à salinização em cenários de exposição mais complexos e relevantes. Esses resultados sugerem que o risco de salinização para os ecossistemas dulçaquícolas pode ser sobrestimado quando se utilizam metodologias padrão e que cenários ecologicamente relevantes devem ser considerados em estágios avançados do processo de avaliação do risco ecológico para salinização.

keywords

Salinization; NaCl; natural seawater; freshwater ecosystem; terrestrial compartment; lethal effects; sublethal effects; community; resilience

abstract

The projections made by the International Panel on Climate Changes (IPCC) until the year 2100 foresee scenarios of increased sea level rise and extreme weather events. As a consequence of these changes it is anticipated that coastal ecosystems (both terrestrial and freshwater) will suffer from seawater (SW) intrusions and, consequently, become impacted with salinization. Such salinization, caused by SW intrusion, may occur through surface flooding (for example due to violent storms, which may lead to pulses of SW intrusion with high peaks of salinity that, most probably, will cause lethal effects on biota) and/or groundwater intrusion (which may occur more gradually, and, most likely starts by inducing sublethal effects in the ecological receptors). In this context, the present work intended to evaluate the adverse effects that salinization, due to SW intrusion, may cause to coastal ecosystems. For this, several specific goals were identified: (i) to determine if sodium chloride (NaCl) may be used as a surrogate of SW at early stages of ecological risk assessment frameworks. This possibility would be advantageous since many toxicity data exist for NaCl and, therefore, it would reduce the number of toxicity assays needed to be carried out; (ii) to identify the ecological receptors most sensitive to salinization, by using standard approaches; (iii) to establish if biota is capable of acquire an increased tolerance to low levels of salinization through mechanisms of phenotypic plasticity; (iv) to assess the effects of increased salinity on interspecies relationships; and (v) to identify the effects of salinization for freshwater and soil communities under realistic exposure scenarios. These objectives were addressed along seven chapters by using standard and non-standard ecotoxicological approaches from the individual (by exposing organisms, from species belonging to different trophic levels, to increased salinity levels) to the community level (by performing multispecies exposures under more realistic scenarios of exposure). Obtained results revealed that, in general, NaCl exerted a similar or higher toxicity than SW, both to freshwater and terrestrial species. These results, suggest that NaCl could be used as a surrogate of SW at early stages of ecological risk assessment of salinization caused by SW intrusion. However, caution must be taken since there were a few species for which SW revealed higher toxicity, and multigenerational exposures showed an increase in the sensitivity to SW for microalgae species. Within the freshwater compartment, cladocerans and rotifers were the two taxonomic groups exhibiting the highest sensitive to salinization (both for NaCl and SW) while fish and macrophytes showed to be the most tolerant groups. Ecotoxicity data obtained for terrestrial plants and fungi (generated in the present work) was integrated with data obtained from literature, allowing to identify microinvertebrates (*Folsomia candida* and *Enchytraeus crypticus*) as the most sensitive group to salinization while fungi and plants were the most tolerant ones. The toxicity data generated from standard assays (or in part compiled from literature for terrestrial species) was used to compute the hazard concentrations that allowed protecting 95% of species (HC_5), on the basis of species sensitive distribution curves. The values of HC_5 were computed for freshwater and terrestrial species and for NaCl and SW; all of these values were very low ($HC_5 \leq 2.26$ $mScm^{-1}$) when compared to the conductivity of natural seawater (≈ 52 $mScm^{-1}$), foreseeing that coastal ecosystems will be at a high risk due to salinization. Experiments

involving a pre-short and long-term (multigerational) exposure to low levels of salinity, overall, did not caused a change in the tolerance of biota to this stressor. However, some species revealed an increased tolerance (either associated with physiological acclimation or other mechanisms of phenotypic plasticity) to salinity after short (the amphibian species *Pelophylax perezi*) and multigenerational exposure (the cyanobacteria *Cylindrospermopsis raciborskii* and the cladoceran *Daphnia longispina*) to low levels of salinity. Contrarily to this, it was also observed that a few species revealed a higher sensitivity to salinization after multigenerational exposure (the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*). These diverse results may be due to differences in the intensity of salinity stress and to the duration of the periods of exposure to low levels of salinization. To more accurately understand the influence of prolonged exposure to low levels of salinity in biota tolerance to this stressor, further studies should be carried out involving multigenerational exposure to sublethal levels of salinization.

Regarding the specific objective (iv), the obtained results revealed that sublethal salinization levels influenced interspecific relationships. For freshwater microalgae, it was observed that, at low levels of salinity, a shift in the competition between the two microalgae species occurred. Adding to the direct effect that these changes will caused in the structure of microalgae community, indirect effects on other species (e.g. cladoceran species that feed on them) are also foresee. For terrestrial plants in the absence of salinization, a lower productivity was registered for some tested plants when exposure occurred under polyculture conditions comparatively to monoculture conditions. However, exposure to the salinity threshold of 4 $mScm^{-1}$ under such conditions seemed not to alter the pattern of responses.

Finally, exposure under more realistic scenarios (outdoor mesocosms) suggested a much lower toxicity of salinization to freshwater ecosystems than that predicted from standard approaches, suggesting a higher resilience of communities to salinization under more complex and relevant scenarios of exposure. These results suggest that risk of salinization to freshwater ecosystems may be overestimated when using standard methodologies and that ecologically relevant scenarios at higher stages of ecological risk assessment for this stressor should be considered.

Contents

Chapter I: General Introduction

1. Introduction 3
   1.1 Climate Change: human and natural drivers 3
   1.2 Sea level rise 5
   1.3 Salinization of Low Elevation Coastal Zones (LECZ) 6
   1.4 Assessment of Salinization effects 10
2. Goals and Thesis Structure 13

References 17

Chapter II: Sea level rise as a threat to coastal freshwater ecosytems: How sensitive are ecological receptors?

Abstract 25
1. Introduction 26
2. Material and Methods 29
   2.1 Test solutions 29
   2.2 Culturing conditions of tested species 29
   2.3 Toxicity Assays 30
      2.3.1 Toxicity assays with microalgae/phytoplankton 31
      2.3.2 Toxicity assays with macrophytes 32
      2.3.3 Toxicity assays with consumers 32
      2.3.4 Toxicity assays with detritivores 34
   2.4 Multigenerational exposure to low levels of salinity 35
3. Data analysis 39
4. Results 39
   4.1 NaCl vs SW toxicity 39
   4.2 Multigenerational exposure to increased salinity 41
5. Discussion 43
   5.1 Sodium chloride as a surrogate of natural seawater 43
   5.2 Most sensitive freshwater ecological receptors 45
   5.3 Multigenerational exposure to low levels of salinity 47

6. Conclusions 49
7. Acknowledgements 50
References 51

Chapter III: May amphibians and fish acclimate to low levels of salinization?

Abstract 59
1. Introduction 60
2. Material and Methods 63
2.1 Test solutions 63
2.2 Test species 63
2.3 Toxicity Assays 63
2.4 Short-term exposure to low levels of salinity 65
3. Data analysis 66
4. Results 66
4.1 NaCl *versus* SW 66
4.2 Short-term exposure to low levels of salinity 68
5. Discussion 69
5.1 NaCl *versus* Seawater 69
5.2 Short-term exposure to low levels of salinity 72
6.Conclusions 73
7. Acknowledgements 74
References 75

Chapter IV: Multigenerational effects of salinity in six clonal lineages of *Daphnia longispina*

Graphical Abstract 79
Abstract 79
1. Introduction 80
2. Material and Methods 83
2.1 Test solutions 83
2.2 Test organisms 84
2.3 Toxicity Assays 85

2.4 Lethal toxicity assays 86
2.5 Sub-lethal assays 86
2.5.1 Feeding inhibition assays 86
2.5.2 Juvenile growth rate 87
2.5.3 Reproduction assays 87
3. Data analysis 88
4. Results 89
4.1 Toxicity of NaCl and SW 89
4.2 Multigenerational effects 90
4.2.1 Lethal assays 90
4.2.2 Sub-lethal assays 91
4.3 Association between tolerance to metals and to NaCl 92
5. Discussion 93
5.1Toxicity of NaCl and Seawater 93
5.2 Multigenerational effects 95
5.3 Association between tolerance to metals and to NaCl 98
6.Conclusions 99
7. Acknowledgements 100
References 101
Supplementary Information 105

Chapter V: Does increased salinity influence the competitive outcome of two producers?

Graphical Abstract 113
Abstract 113
1. Introduction 114
2. Materials and Methods 116
2.1 Test solutions 116
2.2 Tested species 117
2.3 Growth inhibition assays 117
2.4 Acclimation to low levels of salt stress 118
2.5 Competition assays 118

3. Data analysis 119
4. Results 120
4.1 Monospecific growth inhibition assays 120
4.2 Exposure to low levels of sodium chloride 120
4.3 Exposure to low levels of natural seawater 122
5. Discussion 124
5.1 Monospecific growth inhibition assays 124
5.2 Exposure to low levels of salinity 127
5.3 Competition 128
6. Conclusions 130
7. Acknowedgements 131
References 132

Chapter VI: Mesocosm simulation of seawater intrusioninto warm temperate coastal freshwater lagoons and its aftermath dilution

Abstract 139
1. Introduction 140
2. Materials and Methods 146
2.1 Mesocosms 146
2.2 Experimental design 147
2.3 Biological measurements: macroinvertebrate, zooplankton and chlorophyll sampling 148
2.4 Physical and chemical measurements 149
3. Statistics and Calculations 151
4. Results 152
4.1 Physical, chemical and biological parameters 152
4.2 Effects of a pulse of seawater intrusion on the invertebrate's community 153
4.2.1 Macroinvertebrates 153
4.2.2 Zooplankton 156
5. Discussion 159
6. Conclusions 162
7. Acknowledgement 162

References 163

Chapter VII: The influence of salinization on seed germination and growth under mono and polyculture conditions

Abstract 169
1. Introduction 170
2. Materials and Methods 172
   2.1 Tested solutions 172
   2.2 Tested species 172
   2.3 Standard monospecific assays on emergence and growth of higher plants 173
   2.4 Polyculture assays 174
3. Data analysis 175
4. Results 176
   4.1 Standard monospecific assays on seed emergence and growth of higher plants 176
   4.2 Polyculture assays 178
5. Discussion 181
   5.1 Monospecific assays on seed emergence and growth of plants 181
   5.2 Polyculture assays 183
6. Conclusions 185
7. Acknowledgements 186

References 187

Chapter VIII: Salinity induced effects on the growth rates and mycelia composition of basidiomycete and zygomycete fungi

Graphical Abstract 191
Abstract 191
1. Introduction 192
2. Materials and Methods 194
   2.1 Test solutions 194
   2.2 Test species 195
   2.3 Growth inhibition assays 195
   2.4 Period of pre-exposure to low levels of NaCl (PE-NaCl) 197

2.5 Fourier Transform Spectoscopy (FTIR-ATR) 198

3. Data analysis 199

4. Results 200

4.1 Effects of NaCl *versus* Seawater on the growth of fungi 200

4.2 Pre-exposure to low levels of NaCl (PE-NaCl) 202

4.3 FTIR-ATR analysis 203

5. Discussion 205

5.1 NaCl *versus* SW 205

5.2 Effect of pre-exposure to low levels of NaCl in fungi 208

6. Conclusions 210

7. Acknowledgements 211

References 213

Supplementary Information 216

Chapter IX: General Discussion and Conclusions 221

References 242

Supplementary Information 246

List of Figures

Figure II.1: a) Lethal (LC_{50}) and b) Median effective conductivities (EC_{50}) of sodium chloride (NaCl, dark grey bars) and natural seawater (SW, light grey bars), for all the studied species. Vertical bars correspond to the 95% confidence limits. Different letters (a,b) indicate significant differences (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$). 41

Figure II.2: a) Lethal (LC_{50}) and b) Median effective conductivities (EC_{50}) of NaCl before (dark grey bars) and after multigenerational exposure to low levels of NaCl (light grey bars) for the studied species. Vertical bars correspond to the 95% confidence limits. Different letters (a,b) indicate significant differences (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$). 42

Figure III.1: Lethal conductivities ($mScm^{-1}$) causing X% of effect (LC_x) after exposure to natural seawater (SW; light gray lines) and to sodium chloride (NaCl; dashed black lines) for a) *Pelophylax perezi* embryos, b) *Pelophylax perezi* tadpoles and c) *Lepomis gibbosus* juveniles. Vertical bars correspond to confidence limits at 95% (CL at 95%). * represent statistical differences between LC_x computed for NaCl and LC_x computed for SW after analysis with a generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$). 68

Figure III.2: Lethal conductivities ($mScm^{-1}$) causing X% of effect (LC_x) computed for sodium chloride (NaCl) before (light gray lines) and after short-term exposure to low levels of salinity (dark gray bars) for a) *Pelophylax perezi* tadpoles and b) *Lepomis gibbosus* juveniles. Vertical bars correspond to confidence limits at 95% (CL at 95%). * represent statistical differences between LC_x computed before and after short-term exposure to low levels of NaCl, after analysis with a generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$). 69

Fig IV.1: Toxicity endpoints, with the respective 99% confidence limits (error bars), of sodium chloride (dark bars) and natural seawater (light bars), for six clonal lineages of *Daphnia longispina*. a) Median lethal conductivities (LC_{50}), after a 24-h exposure. b) Median effective conductivities (EC_{50}) for juvenile growth rate, after a 72-h exposure. c) Median effect conductivities (EC_{50}) for feeding 90

rate, after a 24-h exposure. Different letters stand for significant differences between NaCl and SW (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$). Note: in figure a) no 99% confidence limits were possible to compute for N35 and E99.

Figure IV.2: Median lethal conductivities (LC_{50}), with the respective 99% confidence limits (error bars), after a 24-h exposure to sodium chloride for the three studied generations (F0, F1 and F2: from light grey to black) of six clonal lineages of *Daphnia longispina*. Different letters (a or b) stand for significant differences between generations, within each clonal lineage, (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$). 90

Figure IV.3: Toxicity endpoints, with the respective 99% confidence limits (error bars), of sodium chloride for three generations (F0, F1 and F2: from light grey to black) of the six clonal lineages of *Daphnia longispina*. a) Median effective conductivities (EC_{50}) for somatic growth, after a 72-h exposure. b) Median effective conductivities (EC_{50}) for feeding rate, after a 24-h exposure. c) Median effective conductivities (EC_{50}) for reproduction, after an exposure until controls released their third brood. d) Conductivities causing a 20% reduction in population growth rate (r) (EC_{20}), after an exposure until controls released their third brood. *no *r* value could be calculated, since this value was below the range of tested concentrations. Generations, within each clonal lineage, sharing the same letter (a or b) represent homogenous groups after comparison with generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$). 92

Figure V.1: Average growth rates (d^{-1}) of *Raphidocelis subcapitata* and *Chlorella vulgaris*, before and after generational acclimation to low levels (respective $EC_{25,72h}$) of sodium chloride (NaCl) (error bars correspond to the standard deviation), under a) single exposure and b) competition exposure to two levels of NaCl and a control. Letters (a, b, c) indicate homogenous groups between different generations. *represent significant differences ($p \leq 0.05$), within each generation, of each NaCl treatment relatively to the control (black: treatments with *R. subcapitata* relatively to control and grey: treatments with *C. vulgaris* relatively to control). 122

Figure V.2: Average growth rates (d^{-1}) of *Raphidocelis subcapitata* and *Chlorella vulgaris*, before and after generational acclimation to low levels (respective 124

$EC_{25,72h}$) of natural seawater (SW) (error bars correspond to the standard deviation), under a) single exposure and b) competition exposure to two levels of NaCl and a control. Letters (a, b, c) indicate homogenous groups between different generations. *represent significant differences ($p \leq 0.05$), within each generation, of each SW treatment relatively to the control (black: treatments with *R. subcapitata* relatively to control and grey: treatments with *C. vulgaris* relatively to control).

Figure VI.1: Schematic representation of the collection procedure of a) Macroinvertebrates (where dashed lines represent the capture trails performed within each mesocosms) and b) Zooplankton. 148

Figure VI.2: Long-term experiment sampling schedule for a) invertebrates and b) water samples. Abbreviations stand for: Z – Zooplankton; M-Macroinvertebrates; C – Chlorophylls; P – measurement of water parameters (pH, conductivity, dissolved oxygen); N – nutrient analysis of the water samples. Black arrows below the XX axis indicating each sampling time (for invertebrates or water samples). Arrow above the XX axis correspond to the saline intrusion moment (dark orange arrow). The pre-I, I, D and post-D denominations correspond to the pre-intrusion, saline intrusion, dilution and post-dilution phases, respectively. 151

Figure VI.3: Conductivity ($mScm^{-1}$) of the water column throughout the mesocosms outdoor experiment within the four established phases: pre-intrusion (pre-I), pulse of saltwater (I phase), periodic dilutions (D phase) and post-dilution (post-D phase). 153

Figure VI.4: Percentage of abundance of organisms belonging to the five major taxonomic groups of Macroinvertebrates (Coleoptera, Heteroptera, Odonata, Ephemeroptera and Diptera) and the four major taxonomic groups of Zooplankton (Copepoda, Branchiopoda, Ostracoda and Rotifera), sampled in outdoor mesocosms at different salinity levels, for the four periods defined during the experiment: pre-intrusion (pre-I), saline intrusion (I), periodic dilutions (D) and recovery post-dilution (post-D). 155

Figure VI.5: Principal component analysis for the five major taxonomic groups of the macroinvertebrate community in each established phase. pre-I: pre-intrusion 156

phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery. Where CTR- control, M1, M2, M3, M4 and M5 correspond to the following conductivities: 15.0, 9.09. 5.51, 3.34, 2.02 $mScm^{-1}$, respectively.

Figure VI.6: Principal component analysis for the major taxonomic groups of the zooplankton community in each established phase. pre-I: pre-intrusion phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery. Where CTR- control, M1, M2, M3, M4 and M5 correspond to the following conductivities: 15.0, 9.09. 5.51, 3.34, 2.02 $mScm^{-1}$, respectively. 158

Figure VI.7: Shannon-Wiener and species richness indexes for a) Macroinvertebrate and b) Zooplanktonic communities exposed to increased salinity levels (mScm-1), during mesocosms experiments. Abreviations stand for: pre-I: pre-intrusion phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery. 159

Figure VII.1: Average aerial biomass (mg) for each of the six plants pairs exposed for a period of 14 days (light grey) and 28 days (dark grey) to a salinity level of 4 mScm-1, either in the absence (Monoculture) or presence of a different species (Polyculture). Ctr corresponds to the control treatment and SW to the salt treatment (4.0 mScm-1). Error bars represent standard deviation. Letters (a to d) represent significant differences (Holm-Sidak, $p<0.05$) between paired comparisons of control and SW treatments, of the different exposure periods, within each scenario (Mono- or Polyculture). 179

Figure VII.2: Average root biomass (mg) for each of the six plant pairs exposed for a period of 14 days (light grey) and 28 days (dark grey) to a salinity level of 4.0 mScm-1, either in the absence (Monoculture) or presence of a different species (Polyculture). Ctr corresponds to the control treatment and SW to the salt treatment (4.0 mScm-1). Error bars represent standard deviation. Letters (a to g) represent significant differences (Holm-Sidak, $p<0.05$) between paired comparisons of control and SW treatments, at the different exposure periods, within each scenario (Mono- or Polyculture). 181

Figure VIII.1: Schematic representation of the measurements made of the minimum, maximum and diagonal diameters of the mycelium of fungus. 197

Figure VIII.2: FTIR-spectra of the mycelia of the studied terrestrial fungi*: Lentinus sajor caju* exposed to a) sodium chloride (NPE-NaCl), b) natural seawater (SW) and c) pre-exposure to low levels of NaCl (PE-NaCl), *Trametes versicolor* exposed to same conditions (d, e and f), as well as *Rhizopus oryzae* (g, h and i) and *Phanerochaete chrysosporium* (j, K and l), respectively. Values at the left of each curve correspond to the salinity level tested (in $mScm^{-1}$). Indentions on the spectra correspond to: i) peak in 3650–3000 cm^{-1} range for O-H bond vibrations from carboxyl, hydroxyl or phenol groups, and from amides N-H vibrations; ii) peak in 3000-2765 cm^{-1} range for lipids with functional groups CH; iii) peak in 1750-1500 cm^{-1} range for proteins with functional groups, amides I and II; iv) peak in 1495–1200 cm^{-1} range for proteins and lipids with functional groups, CH_2 and CH_3 and phosphate compounds with functional group P=O; and v) peak in 1200–805 cm^{-1} range for polysaccharides with functional groups C-O-C and C-O-P. 204

Figure IX.1: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to lethal levels of sodium chloride (NaCl). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Table 1 for further details). Superscripts are as follows: [a]Wallen et al., 1957; [b]Paradise, 2009; [c]USEPA, 2008; [d]Thamer and Abdulsamad, 2005; [e]Zalizniak et al., 2006; [f]Kefford et al., 2009; [g]Ghazy et al., 2009; [h]Loureiro et al., 2013; [i]Gardner and Royer, 2010; [j]Dougherty and Smith, 2006; [k]Gökçe and Turhan, 2014; [l]Patrick et al., 1968. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points. 231

Figure IX.2: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to lethal levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Table 2 for further details). Superscripts are as follows: [a]Ghazy et al., 2009; [b]Mohammed and Agard, 2007. Abbreviations on the table are as follows: HC_x- 232

Hazard Concentration that affect X% of the species; R^2- coefficient of determination of the curve; N-number of data points.

Figure IX.3: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to sublethal levels of sodium chloride (NaCl). Values correspond to median effective conductivities collected at Chapter II, III, IV rom the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Table 1 for further details). Superscripts are as follows: [a]Koeypudsa, 2005; [b]Stanley, 1974; [c]Simmons, 2012; [d]Reynoso et al., 1982; [e]Paradise, 2009; [f]Zalizniak et al., 2006; [g]Kolkmeier, 2013; [h]Loureiro et al., 2013. Abbreviations on the curve are as follows: DW-dry weight; Gr-growth; Em-emergence; R-reproduction; EH-egg hatching; F-feeding; M-morphology; EP-egg production; FI-fluorescence inhibition. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points. 233

Figure IX.4: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to sublethal levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Table 2 for further details). Superscripts are as follows: [a]Roache et al., 2006. Abbreviations on the curve are as follows: Gr-growth; EA-enzymatic activity; Em-emergence; F-feeding; M-morphology. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points. 234

Figure IX.5: Species Sensitive Distribution curve (SSD) assembled for soil species exposed to increased levels of sodium chloride (NaCl). Values correspond to median lethal conductivities collected at Chapter VII and VIII from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 3 for further details). Superscripts are as follows: [a]Ibrahim et al., 2016; [b]Rebey et al., 2017; [c]Jamil et al., 2005; [d]Robidoux and Deslile, 2001; [e]Joutti et al., 2003; [f]Pereira et al., 2015; [g]Gozyte, 2011; [h]Venâncio et al., 2017. Abbreviations on the curve are as follows: Gr-growth; 235

Germ-germination; SP-seed production; W-dry weight; RL-root length; SL-shoot length; FW-fresh weigth; S-survival; R-reproduction. Abbreviations on the table are as follows: HCx-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points.

Figure IX.6: Species Sensitive Distribution curve (SSD) assembled for soil species exposed to increased levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter VII and VIII from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 4 for further details). Superscripts are as follows: [a]Pereira et al., 2015; [b]Venâncio et al., 2017. Abbreviations on the curve are as follows: Gr-growth; Germ-germination; S-survival; R-reproduction. Abbreviations on the table are as follows: HCx-Hazard Concentration that affect X% of the species; R^2: coefficient of determination of the curve; N-number of data points. 236

List of Tables

Table I.1: Examples of functions and services provided by Soil and Freshwater Ecosystems 7

Table II.1: Summary of procedures, range of concentrations and additional information relatively to the test species. Abbreviations: Light (^{L}) dark (^{D}) cycle, natural seawater (SW), sodium chloride (NaCl), after multigenerational exposure to NaCl ($NaCl_{GE}$), conductivity causing 50% of mortality (LC_{50}) or another effect (EC_{50}). 37

Table III.1: Information on the parameters, endpoints and range of concentrations used in tests. Abbreviations: Light (^{L}) dark (^{D}) cycle, natural seawater (SW), sodium chloride (NaCl), after short-term exposure to low levels of salinity ($NaCl_{STE}$), conductivity causing 50% of mortality (LC_{50}) or another effect (EC_{50}). 66

Table V.1: Ecotoxicological endpoints for *Raphidocelis subcapitata* and *Chlorella vulgaris* exposed to increased salinity. EC_{50} – effect concentration causing 50% of reduction of growth rate. NOEC – no observed effect concentration. LOEC – Lowest observed effect concentration. SW- seawater; NaCl – sodium chloride 127

Table VI.1: Resume of long-term studies focusing the effects of salinization on freshwater communities. 143

Table VI.2: Mesocosms sediment characterization: humidity (%), total organic matter (TOM - %) and grain size distribution (expressed in % in relation to the total sediment dry weight). 147

Table VII.1: Conductivities for sodium chloride (NaCl) and natural seawater (SW), provoking 10, 20 and 50% (EC_{10}, EC_{20}, and EC_{50}, respectively) of inhibition on germination and dry weight of four species of terrestrial plants. 95% confidence limits are depicted within brackets. n.d. – could not be determined as no inhibition on germination or growth could be detected at the irrigation water treatment corresponding to 100% SW. 177

Table VIII.1: Description of the conductivities range ($mScm^{-1}$) tested and the duration of the assays (days) for each fungus species. NPE: fungi non-pre-exposed to low levels of salinity; PE: fungi pre-exposed to low salinity levels; SW-natural seawater. The dilution factor used for all serial dilutions was 1.2x. 196

Table VIII.2: Correspondence between profiles absorbance peaks obtained with FTIR-ATR and macromolecules present in fungi mycelia. 199

Table VIII.3: Conductivities ($mScm^{-1}$), with corresponding 95% confidence limits depicted within brackets, causing 10, 20, 25 and 50% (EC_x) of growth inhibition in fungi exposed to sodium chloride [NaCl, for non-pre-exposed (NPE-NaCl) and pre-exposed (PE-NaCl) fungi)] and natural seawater (SW). * indicates differences between EC_x computed for NPE-NaCl and EC_x computed for SW (no overlap of confidence limits at 95%). Letters (a,b) indicate differences between EC_x computed for NPE-NaCl and EC_x computed for PE-NaCl (no overlap of confidence limits at 95%). 201

Table IX.1: Median values of Hazard Concentrations that protect 95% of the species (HC_5) obtained through the Species Sensitivity Distribution curves (SSD) method for natural seawater (SW). MAC – Maximum Acceptable Concentrations. L(E)C_{50}- Median lethal or effective conductivity ($mScm^{-1}$). 238

Table IX.2: Risk quotient for freshwater ecosystems based on the ratio between salinity levels detected in field studies (values considered the Predicted Effect Concentrations – PEC) and PNEC (Predicted Non-Effect Concentrations) computed through the lowest effect concentration (LC or EC_{50}) for seawater (SW) divided by a safety factor of 10. [a]Based on the rCl/rHCO3 ratio – rapid increments on this index is an indication of saline intrusion; Ferreira, 2012. [b]Sampling points with freshwater characteristics but which occasionally present high salinities due to tidal elevations or due to water scarcity; IDAD, 2008. 239

Chapter I

General Introduction

1. Introduction

1.1 Climate Change: human and natural drivers

Climate patterns are among the major drivers shaping Earth since its beginning, altering between colder and warmer periods. They are the result of phenomena such as volcanic eruptions, impacts of meteors, changes in solar outputs, the hydrological cycle, changes on earth's orbital route or their conjunction (e.g., Cane et al., 2006). Simultaneously ice sheets have extended and retreated, and mean sea level decreased and rose several times. During these processes, ecosystem's composition has also change, with many species loss, while others have thrived or even appear. For instance, in the last warming period (end of the Ice Age), about 10,000 years ago, sea level rise lead to the formation of the world´s largest coral reef structure, the Great Barrier Reef, in northeastern Australia (Bowen and Bowen, 2011), which constitutes an important biodiversity hotspot. However, at present, scientific community is concerned on the possibility of recent climate changes being potentiated by anthropogenic activities rather than a consequence of Earth biogeochemical cycles (Solomon, 2007). Global mean temperatures have raised one degree Fahrenheit (corresponding approximately to 0.6 ºC), in the last century (EEA, 2016). A change of one degree Fahrenheit is enough to induce severe changes in climate patterns, like changes in the frequency of El Niño patterns, which influences ecosystems (Riebeek, 2009). For instance, it was reported that an increase in the frequency of El Niño storms is responsible for the decline on the productivity of waters along the South America coast line (Riebeek, 2009). This happens because the warmer surface waters, characteristics of El Niño events, do not allow the upwelling of deep cool waters, rich in essential nutrients. In turn, this has severe effects on phytoplanktonic communities (that depend on these nutrients to proliferate) and on other communities that rely on phytoplankton also to survive (Riebeek, 2009). Furthermore, even at a lower rate, comparing to the terrestrial ecosystems, oceans are also warming up (0.18 ºF or 0.1 ºC) (EEA, 2016) and, since ocean's warming up is a slower process, effects may not be observed immediately but should be projected in a longer timescale.

Human activities have been vastly contributing to the release of greenhouses gases (GHG's, also called heat-trapping gases) in the atmosphere, mainly as the result of fossil fuels combustion (Frumhoff et al., 2015; EEA, 2016b). As an example, in the United States, between 1990 and 2014, the production of energy, transportation and chemical industry accounted for 77% of total GHG emissions, with agriculture having minor contributions, although still accounting for 9% of total emissions (EPA, 2014). Within GHGs, almost 80% of the emissions relate to carbon dioxide and methane while nitrous oxide (predominant greenhouse gases from agriculture) represents 17% and 3% relate to fluorinated gases (commonly used in several industry processes) (EPA, 2010). Oceans have been a major key component in the retention of these gases. They are considered the largest sink for carbon dioxide (CO_2) and can retain much of the emissions produced by human activities (Ciais et al., 2014). However, this has contributed to changes in the chemical composition of seawater, which also endangers several ecosystems. For instance, the bleaching of corals due to changes in water pH interferes with CO_2 storage (Feely and Doney, 2016).

Additionally, land use modifications induced by humans (e.g., to increase soils productivity, to establish human settlements, to discard human-derived wastes), interfere with the terrestrial net production and contribute also to the impairment on the retention of GHGs by soils, exacerbating climate change scenarios (Wu et al., 2014). This trapping phenomenon of GHG's gases by soils occurs due to the extensive exchange of CO_2, either through respiration or photosynthesis, between terrestrial ecosystems (soils and plants) and the atmosphere, making the soil also an important reservoir for this particular gas, additionally to oceans (Wu et al., 2014). When altering forest-land to crop-land or to urbanized areas, this equilibrium between compartments is altered, leading to a decrease in the ability of soils to retain CO_2, which accumulates in the atmosphere compartment, enhancing the greenhouse effect (Imhoff et al., 2004; Houghton et al., 2012). Plus, increasing crop or urban areas brings extra concerns as both may strongly affect the hydrological cycles, either by interfering with soils permeability or altering natural recharge of groundwater supplies (Shi et al., 2013). Bad irrigation practices or the excessive use of fertilizers are among the causes that interfere with the hydrological cycle (Stuart et al., 2014).

Alongside with increasing global temperatures, there are many climate change impacts, such as accelerated sea level rise, frequent wildfires and prolonged drought periods. The National Oceanic and Atmospheric Administration (NOAA) reported 2016 as the hottest year (surpassing the previous records of 2014 and 2015) in Europe since 1925 (NOAA, 2016). In addition, it was also the year where it was registered the lowest annual extent of the Arctic sea ice sheets and the fourth year with higher melting rate record. Extreme weather events were also registered outside Europe back in 2012. For instance, India and the United States recorded several rainfall events while East Africa witnessed their most severe drought period (Faisal et al. 2013; FAO, 2006; Wolf et al., 2016). Storms frequencies with occurrence at the eastern North Pacific (NOAA, 2016) and hurricanes at the North Atlantic (Christensen et al., 2014) have also increased since 1980's, either in frequency, duration and intensity. Europe's precipitation patterns are also shifting and becoming more inconstant, hindering their predictability: while southern Europe has become 20% drier in the last century, northern Europe regions (like the United Kingdom) have recorded a higher number of heavy rainfall events over the past 45 years (EEA, 2016b; Sanderson, 2010). Regarding the Iberian Peninsula, Casanueva et al. (2014) reported that its vulnerability is closely related with the decrease in the number of wet days (rainy periods) but with concomitant increase of extreme precipitation events. So, these sudden, severe and simultaneous hazards, considered above the natural average, are already occurring but are expected to be even more frequent in the future, and since there is a positive feedback between them, even a small increase in any of these events, may trigger unpredictable effects on others (IPCC, 2014).

1.2 Sea level rise

Within the context of climate change, sea level rise may occur not only due to the melting of ice sheets, caps and glaciers (which release water into the ocean), but also because of thermal expansion of seawater (as a result of warmer temperatures), constituting a critical concern for coastal areas. Within these scenarios, low-lying ecosystems near coastal areas, commonly named as Low Elevation Coastal Zones (LECZ - comprised between 0 and 10 km), are of special

vulnerability (Oliver-Smith, 2012). They include small islands, estuaries and continental terrestrial and freshwater ecosystems that comprise 2% of the planet Earth global surface (Oliver-Smith, 2012). Land subsidence's combined with sea level rise are, already, being considered as responsible for major seawater intrusions events (flooding or groundwater, for instance) causing several negative impacts in these LECZ: almost 10% of the world population inhabit these specific areas are at risk of being displaced; salinization of the ecosystems is occurring; there is an increased demand for freshwater in coastal areas (for human consumption: drinking or irrigation) or other anthropogenic activities that destroy natural protection barriers (Small and Nichols, 2003; Neumann et al., 2015). Together with warmer and longer dry periods and lower precipitation periods, these seawater intrusion events result in lower aquifers recharge rates.

Since 1880, global sea level rose 20 cm, mainly due to land ice melting but also because of seawater warming that leads to seawater expansion (Church and White, 2011; Gregory, 2013). In 2001, the Intergovernmental Panel on Climate Change (IPCC) Third Assessment Report predicted a sea level rise of 20 to 70 cm by 2100 (IPCC, 2001). In 2007, the Fourth Assessment Report (IPCC, 2007) projected similar results, with sea level rise comprised between 18 and 59 cm by the end of the century. But the most recent report projections (Fifth Assessment Report on Climate Change; Church et al., 2013; IPCC, 2014) stated that it is very likely that the rate of global sea level rise, during this century, will exceed the scenarios observed within the period 1971-2010, pointing to, in a worst-case scenario, a rise of 52 to 98 cm, with an escalating rate of 8 to 16 $mmyear^{-1}$ during the period of 2081-2100 (scenario RCP8.5). Additionally, this report highlights the increased risk of extinction that several terrestrial and freshwater species will face during the 21^{st} century. This vulnerability is aggravated especially as climate change interacts with several other stressors, namely, habitat modification and/or fragmentation, over-exploitation, pollution caused by anthropogenic activity, introduction of invasive species, among others.

1.3 Salinization of Low Elevation Coastal Zones (LECZ)

The salinization of low-lying freshwater and terrestrial ecosystems is already occurring at several coastal regions in the planet. Salinization may arise from primary and/or secondary processes.

Primary salinization (or natural) is related with natural phenomena. Long periods of time and/or deposition of ocean's salt carried by wind or rain are examples of primary salinization (especially in costal zones, since this occurrence loses magnitude as the distance from the coast increases) but also weathering of soil's parental rock may lead to the accumulation of salts (such as magnesium, calcium, sodium, and chloride). Even so, in both cases, sodium and chloride may be the most influencing ions in this process because, in soils they are considered the most soluble ions and in seawater they compromise about 86% of all seawater component ions (e.g., Pujiastuti et al., 2016). Secondary salinization (or human-induced) is related with anthropogenic activities that may influence or change the hydrological cycle. Agricultural practices can potentiate salinization of soils: land clearing and replacement of perennial grasses (that maintain the fragile equilibrium between water use and transpiration) by annual crops in addition to bad irrigation practices (such as use of salt-rich waters or excessive irrigation in poor drainage soils may lead to the raise of the water table and subsequent mobilization of salts that once were stored in the soil).

Table 1: Examples of functions and services provided by Soil and Freshwater Ecosystems.

	Supporting/Provisioning	**Regulating**	**Culture**
Soils[1,2]	Nutrient cycling Soil formation Food Wood Medicinal resources Biodiversity	Local climate Pollination Erosion Carbon storage	Tourism Aesthetic Spiritual Recreation Health
Freshwater[3,4]	Nutrient cycling Freshwater Food Biodiversity	Local climate Water purification Flood regulation Waste-water treatment	

[1]Lavelle et al., 2006 [2]Adhikari and Hartemink, 2016 [3]De Groot et al., 2002 [4]MEA, 2000

Salinization will, therefore, impact low-lying freshwater and terrestrial ecosystems, as well functions and services that they encompass and are tightly connected with biodiversity and human

well-being, social and economic aspects (e.g., Table 1). Regarding soils, it has major implications in fertility, since many crops, forage and livestock feeding plants are glycophytes (designation attributed to plants that cannot withstand with high salinity conditions). All around the world, major agricultural land losses have been reported, mainly due to permanent submersion or frequent flooding that causes salinization of the water table. According to the European Commission (2012), over 3 million of hectares (ha) of soils were considered inappropriate for use due to its high salt content. The same report stated a total loss of over 700 ha of arable and fertile land for Spain, Greece, Portugal, France, and Italy (European Commission, 2012). Salinization of soils (with consequent loss of the services that they provide) is the main cause of wealth loss, for instance, of coastal human settlements, forcing populations to migrate and search for better resources (Science for Environment Policy, 2015). The aspects aforementioned reinforce the economic and societal impacts of soil salinization, since the most likely scenario points to an increase up to 1,000 million in human population by 2050 and it is also expected that food demand will increase almost 50% by 2030 and 70% by 2050, according to the Food and Agriculture Organization of the United Nations (FAO, 2006). But there still exists a big gap in knowledge regarding the risks that increased salinity may pose to soils and its biota. Moreover, soil is characterized by huge spatial heterogeneity (chemical and physical) that makes such assessment even more complex. Furthermore, mobilization of salts through soils may pose an increased risk to freshwater systems, such as aquifers or lakes or other freshwater systems.

Likewise soils, freshwater or wetland ecosystems near coastal areas face the same threat - salinization. These ecosystems are important reservoirs of freshwater and are involved in several natural cycles (e.g., carbon, nutrients and hydrological cycle). Even more, these ecosystems have recreational, ecological, cultural, and aesthetic value, playing a very important role at both economic and social levels (Morris et al., 2011; Russi et al., 2013). Many of them are considered hotspots of biodiversity because not only they are habitat for several aquatic organisms like fish, plants and younger life stages of some species (e.g., amphibians) but also provide shelter, refuge, food for many other forms of life (like birds or mammals). In 2012, New Jersey, located at northeastern corner and mid-Atlantic region of the United States, was shaken by the super storm

Sandy. Along its coast, several freshwater and other ecosystems (like forest) related to it were severely damaged. For instance, in some lakes the seawater intrusion and the excessive amount of debris killed entire fish populations and, in certain areas, up to a quarter of standing trees were dead because of high amount of salt in the soils (e.g., Middleton, 2016).

There is an increasing concern on the adverse effects that increased salinity may cause either on terrestrial and freshwater organisms. Although data has already been generated for other regions in the globe, it may not be representative of what is happening in the Mediterranean regions (where Portugal is partially located). For instance, many reports point to the effects of salinization to aquatic biota in a global perspective or comparing regions from tropical regions, such as Australia or Africa (e.g., Kefford et al., 2012; Dittmann et al., 2015; Castillo et al., 2017). However, temperate and polar regions are predicted to be the regions were the greatest differences in temperature and severe weather occurrences are likely to occur (Root et al., 2003). Therefore, impacts may change according to regions, species and ecosystems characteristics demanding the urgent generation of information on this matter. For Portuguese coastal ecosystems, the amount of information on salinity tolerance by autochthonous species is poor. Its location makes of it home of a very unique biodiversity (fauna and flora), with a very high number of endemism's (when a species inhabits a very specific region) (e.g., Sundseth, 2009; Honrado et al., 2014). Thus, it is urgent to assess salinity tolerance of organisms inhabiting these Portuguese coastal systems because Portugal has an extensive coastline and reports on climate change have pointed Southern Europe as one of the most vulnerable areas to sea level rise and drought, with medium confidence levels (IPCC, 2014) and the data generated may be transposed for other regions sharing these same characteristics of clime and biodiversity (as Spain). Plus, the produced information, although focused in this particular region and in a reduced number of taxa, may be integrated with data already available for other regions of the globe and run meta-analysis studies. Such approach is helpful in order to improve our knowledge on the responsiveness of the ecosystems to such changes and to employ adaptation or mitigation strategies to preserve these ecosystems (e.g., Zhou et al., 2016).

1.4 Assessment of Salinization effects

Much of the information available to estimate the effects caused by salt stress is based on standard toxicity tests that assess mortality (e.g., Hammer, 2012; Loureiro et al., 2012; Gökçe and Turhan, 2014). Considering a scenario of extreme weather events, sudden increases in salinity levels are most likely to occur and effects at the ecosystem level expected to be irreversible. Cladocerans and freshwater cnidarians are likely to be severely affected taking this scenario in account, as salinity values reported in the literature to cause mortality are very low for these taxa, in some cases not reaching 1 $mScm^{-1}$ (taking conductivity as a measure of salinity and values for sodium chloride salt as a surrogate for natural seawater; e.g., Santos et al., 2007; Oliveira-Filho et al., 2008; Gökçe and Turhan, 2014). Though, it should not be disregarded that adverse effects may be exerted at much lower salt concentrations (non-lethal levels), affecting organism's performance. For instance, assessment of feeding rates or juvenile growth rates should be also considered. They are sensitive endpoints to detect early effects and do not implicate organism death, thus considering animal welfare practices. Such endpoints may also be translated later in other organisms and/or population traits as they can impact reproduction (e.g., Paradise, 2009). For example, Santos et al. (2007) verified that the medium lethal toxicity at 96 hours ($LC_{50,96h}$) caused by NaCl to *Hydra attenuata* was of 0.246 $mScm^{-1}$, but cnidarians started to present body abnormalities (that may interfere with normal feeding behavior) at a concentration almost half of the one computed for mortality ($EC_{50,96h}$: 0.147 $mScm^{-1}$). Young life stages of several species of insects or frogs seem also to be very vulnerable to increased levels of salinity, which in turn might have consequences on future populations of these organisms (e.g., Dougherty and Smith, 2006; Zalizniak et al., 2006). Producers (e.g., algae that also consists on food source for zooplanktonic grazers) may be also affected at low salinity levels: approximately a value of 3 $mScm^{-1}$ may be sufficient to reduce their photosynthetic mechanisms and consequently growth rates (e.g., Simmons, 2012). On the other hand, fish species seem able to tolerate much higher salinity levels, above 10 $mScm^{-1}$ (e.g., Zuanon et al., 2009). Many of these species are potentially invasive while others have already established populations at these salinity-impacted freshwater systems. This wide tolerance to salinity exhibited by freshwater species, comes to highlight the need to generate

information that might to help set protection measures based on their most sensitive ecological receptors.

Regarding soils, information on the effects of salinization is even scarcer, so the need to gather information on this specific compartment it's fundamental. Like freshwater ecosystems, data reported in the literature suggests that it exists a large difference in tolerance to salinity among soils species. Between producers and invertebrates, producers present the highest tolerance towards salinity (e.g., Robidoux and Delisle, 2001; Ibrahim et al., 2016) but salinities below 1 $mScm^{-1}$ were already reported to be enough to induce effects in invertebrate's life traits (Pereira et al., 2015). For instance, *Triticum aestivum* (var. Yang) total dry weight was found to be reduced by half after 21 days ($IC_{50,21d}$) of exposure to a salinity level of almost 16 $mScm^{-1}$ (Ibrahim et al., 2016) but, according to Pereira et al. (2015) the effective conductivity value causing 50% of reduction in *Folsomia candida* reproduction ($EC_{50,28d}$) was of only 0.99 $mScm^{-1}$.

The comparison of tolerance values reported so far in the literature for cladocerans, freshwater cnidarians and soil microinvertebrates (values of or below 1 $mScm^{-1}$ might be enough to induce sublethal effects) with the conductivity of natural seawater (correspond to approximately to a salinity of 52 $mScm^{-1}$ at 20 ºC (roughly because seawater composition and salinity may change according to the location around the globe) highlights how vulnerable these ecosystems can be to scenarios of salinization.

It is hypothesized that if salinity levels increase is gradual, rather than abrupt, it is possible that organisms can cope and even persist on the impacted sites. Therefore, risk assessment on salinization effects must also integrate such aspects, as these responses may be a key process to the resilience of freshwater and terrestrial ecosystem to these changes and may help in the development of protection strategies and decision-making (e.g., Angeller et al., 2014). Within such responses, avoidance and acclimation should be considered. The ability of organisms to detect an unfavorable environment is called avoidance. Retreating or escaping of organisms to prevent exposure to contaminants is referred as spatial avoidance (e.g., da Luz et al., 2004; Lopes et al., 2004; Takahashi, 2007; Araújo et al., 2016) while the production of dormant structures, like ephippia, to avoid harsh conditions is denominated as temporal avoidance (e.g., Radzikowski,

2013). Soils are characterized by a high spatial heterogeneity and can provide shelter for organisms facing some kind of stress, but also in aquatic systems the establishment of gradients between non-contaminated and contaminated waters may trigger this behavior. For instance, Owojori and Reinecke (2009) have found that earthworms were able to avoid natural saline soils and Araújo et al. (2014) showed that tadpoles of tropical and of European species were able to avoid copper contamination. Even sessile organisms may show this feature. For instance, plants have evolved a strategy of avoidance at the root level designated as halotropism. They can redirect (circumventing) their root growth in order to avoid saline environments (Galvan-Ampudia et al., 2013). Either for terrestrial and freshwater systems, the avoidance behavior may be of special interest because this response may be comparable to that of mortality, since in both cases there is a decreased in the number of organisms at the salinity-impacted population. Furthermore, its relevance increases since it can be detected at much lower concentrations and only within few hours than those reported by standardized methodologies (e.g., Beketov and Liess, 2008; Araújo et al., 2016). In case of disturbance of an ecosystem, organisms may avoid it but, later, if it returns to more favorable conditions, organisms can migrate and re-colonize it (James et al., 2003).

Whenever organisms cannot escape or avoid and must persist in these saline conditions, other processes may be triggered as a response to stressful conditions. Acclimation, maternal transfer and inheritance of epigenetic traits are examples of such responses. But the triggering of such responses is advantageous in scenarios of gradual and/or sub-lethal levels of salinization. Otherwise, if high salinization occurs abruptly organisms will most probably perish. This strategy to cope with low levels of contamination trough acclimation has already been documented for fish (e.g., Harper et al., 2008) and for cladocerans (e.g., Lopes et al., 2005) and through epigenetics in plants (e.g., Demirkiran et al., 2013; Sani et al., 2013). If organisms are indeed able to trigger these responses, ecotoxicological studies may overestimate the risks that increased salinity may pose to natural freshwater ecosystems.

Overall, increased salinity may exert pressure over several aspects. It may be considered as a driving force, exerting selective pressure in populations and communities because increased salinity levels may: i) shift species ranges and limits, ii) influence species and populations

densities, since only the most tolerance species will be able to thrive under increasing salinity scenarios, iii) facilitate the entrance or spread of alien species, and iv) induce phenotypic alterations (e.g., body size, behavior) and possibly induce shift in the genetic diversity of natural populations (Bakkenes et al., 2002; Root et al., 2003).

It is also mentioned that freshwater wetlands are more vulnerable to extreme weather conditions and may take longer to recover from such damages when compared to saltwater ecosystems, which are considered more resilient (Tahsin et al., 2016). Assessment of the impacts of salinization both in the aquatic and terrestrial compartments is important since they are providers and storage of many ecological services and functions. Alongside, the quality services and functions may also depend on the inhabitants that constitute these compartments (e.g., Table 1).

2. Goals and Thesis Structure

As mentioned previously in this introductory chapter, salinization due to sea level rise is already occurring in several coastal regions of the globe causing severe adverse effects in ecosystems worldwide. Additionally to this global perspective, it is also relevant to consider this problematic issue at a more regional scale. In Portugal, this issue is extremely relevant since half of the Portuguese border is in contact with the Atlantic Ocean and along its coastal line many ecosystems are considered as hotspots of biodiversity and protected by national and international laws (e.g., Andresen and Curado, 2005). Furthermore, considering that Portugal is located at the southern Europe (abovementioned as one of the most affected regions by climate change), that its northwest coast is considered a highly energetic region (Cruz, 2008), being subjected normally to intense storm, erosion and seawater intrusion phenomena, its vulnerability to extreme weather events is high. These factors associated with the increase of sea level rise, drought periods and freshwater scarcity propitiates the risk of seawater intrusion in Portuguese coastal low-lying ecosystems. Thus, the gap knowledge existing on the potential effects of salinization in these Portuguese ecosystems is, as well, of major concern.

Therefore, both within an international and national framework of seawater intrusion in coastal ecosystems, the present work intended to evaluate the adverse effects that salinization may cause

to coastal ecosystems. For this, several specific goals were identified: (i) to determine if sodium chloride (NaCl) may be used as a surrogate of SW at early stages of ecological risk assessment frameworks. This possibility would be advantageous since many toxicity data exist for NaCl and, therefore, it would reduce the number of toxicity assays needed to be carried out; (ii) to identify the ecological receptors most sensitive to salinization, by using standard approaches; (iii) to establish if biota is capable of acquire an increased tolerance to low levels of salinization through mechanisms phenotypic plasticity; (iv) to assess the effects of increased salinity on interspecies relationships; and (v) to identify the effects of salinization for freshwater and soil communities under realistic exposure scenarios. These specific objectives were addressed in the present thesis along seven chapters, described below.

The overall structure of this thesis is divided into the following chapters:

[Chapter I] General introduction, that consists of a brief contextualization of the natural and human drivers that potentiate salinization and on its ecological impacts in low elevation coastal zones.

[Chapter II] In chapter II, three main objectives were tackled: i) to assess the suitability of sodium chloride (NaCl) as a possible surrogate to evaluate natural seawater (SW) toxicity; ii) to identify the most sensitive freshwater ecological receptors to salinity, and iii) to determine if increased tolerance to salinity was acquired after multigenerational exposure to low levels of salinization. For this, standard monospecific assays were performed with several short-life cycle species - the green algae *Chlorella vulgaris* and *Raphidocelis subcapitata*, the macrophytes *Lemna minor* and *L. gibba*, the cyanobacterium *Cylindrospermopsis raciborskii*, primary consumer species as *Brachionus calyciflorus*, *Heterocypris incongruens* and *Theodoxus fluviatilis*, and the decomposer *Chironomus riparius*. The selected organisms were subjected to lethal and sublethal salinity levels of NaCl and SW. Sodium chloride was studied as a possible surrogate for SW because SW is mainly composed by sodium and chloride and facilitates the comparison of data gathered on tolerance to NaCl with other data sets. To assess the effects of salinization through generations, the same standard toxicity assays were carried out after exposing each species for two generations to low levels of salinization.

[Chapter III] The objective of this chapter was to evaluate the effects of salinization on two freshwater vertebrate species: the fish *Lepomis gibbosus* (juveniles) and the amphibian *Pelophylax perezi* (eggs and tadpoles). Three main goals were addressed: i) to evaluate if NaCl may be used as a safe surrogate for risk assessment of SW regarding freshwater vertebrate species; ii) to assess the sensitivity of the two freshwater vertebrate species to salinity (either caused by NaCl or SW), and iii) to determine if these two-vertebrate species were able to acclimate to low levels of salinization. For that, juveniles of *L. gibbosus* and embryos and tadpoles of *P. perezi* were exposed to serial concentrations of NaCl or SW dilutions, and mortality and somatic growth (only for *P. perezi* tadpoles) were monitored. The capacity of organisms to acclimate was assessed by exposing eggs of *P. perezi* during their embryonic development or juveniles of *L. gibbosus* for a two-month period to low levels of salinity.

[Chapter IV] This chapter focused on multigenerational lethal and sub-lethal effects of increased salinity on six clonal lineages of *Daphnia longispina*. These clonal lineages were selected because they represent a natural population and may give information on the multiplicity of responses that organisms from the same population may present when confronted with stressful conditions. Accordingly, three specific objectives were delineated: i) to compare the lethal and sublethal toxicity of NaCl and SW, in order to understand if the former one could be used as a safe surrogate for the later one; ii) to evaluate possible multigenerational effects after exposure to low levels of salinization, and iii) to evaluate if an association may exist between tolerance to lethal and sublethal levels of salinity and lethal levels of metals (based on previous works focusing on the tolerance of these clonal lineages to metals).

[Chapter V] The main goal of this chapter was to assess the effects of multigenerational exposure to low levels of salinization on the competitive outcome between two species of freshwater microalgae (*C. vulgaris* and *R. subcapitata*). Accordingly, this chapter aimed at: i) comparing the toxicity of NaCl and SW to the two microalgae, under long-term exposure; ii) determining the ability of the two microalgae to acclimate to low levels of salinization, and iii) evaluating the influence of long-term exposure to low levels of salinization on the competitive outcome of the two microalgae.

[Chapter VI] This chapter main objective was to explore the long-term effects of a pulse of seawater intrusion. For that, an outdoor mesocosms (a more realistic scenario of exposure) was assembled and colonized with natural communities of macroinvertebrates and zooplankton. The structure and composition of both communities were monitored during three distinct periods: i) a saltwater intrusion phase, where mesocosms were pulsed-contaminated with natural seawater (33 days); ii) recovery of the mesocosms with sequential dilutions to restore the conductivity levels before seawater intrusion (58 days), and iii) recovery from the end of the dilutions onward, where mesocosms were left intact (126 days).

[Chapter VII] This chapter focused on the effects that increased salinity level of irrigation waters may induce in four species of terrestrial plants: *Trifolium pratense*, *Vicia sativa*, *Festuca arundinaceae,* and *Lolium perenne*. The effects on seeds germination and growth were assessed under the following exposure scenarios: i) exposure to increased salinity induced with NaCl, in monoculture; ii) exposure to increased salinity induced by SW, in monoculture, and iii) exposure to a salinity threshold level of SW (4.0 $mScm^{-1}$) under a mixed culture scenario.

[Chapter VIII] The main goal of this chapter was to assess the effects of salinization on four species of terrestrial fungi: *Lentinus sajor caju*, *Trametes versicolor*, *Rhizopus oryzae*, and *Phanerochaete chrysosporium*. Fungi play an important role in nutrient recycling in soils and they are reported to be very tolerant to contamination. Accordingly, their growth rates and biochemical composition were determined under three exposure scenarios: i) exposure to serial dilutions of SW; ii) exposure to serial dilutions of NaCl, and iii) exposure to serial of NaCl after a period of pre-exposure to low levels of NaCl.

[Chapter IX] General discussion and Conclusions. In this final chapter, information obtained within this study is discussed in an integrated way. The obtained data is assembled in Species Sensitive Distribution curves (SSD curves) as an attempt to establish safety values of salinization for coastal zones in risk of salinization.

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Chapter II

Sea level rise as a threat to coastal freshwater ecosystems: How sensitive are ecological receptors?

Sea level rise as a threat to coastal freshwater ecosystems: How sensitive are ecological receptors?

Venâncio C, Castro BB, Ribeiro R, Antunes SC, Abrantes N, Soares AMVM, Lopes I

Abstract

Salinization of coastal freshwater ecosystems due to sea level rise is already occurring in some regions of the world. This phenomenon raises serious concerns on the protection of coastal ecosystems, since many of them support and shelter a large number of species and are considered hotspots of biodiversity. This work intended to assess the adverse effects that salinization, caused by the intrusion of seawater, may pose to freshwater organisms. For this, three specific goals were addressed: (i) to assess if sodium chloride (NaCl) may be used as a surrogate of natural seawater (SW), (ii) to identify the most sensitive ecological receptors to salinity, and (iii) to determine if increased tolerance to salinity may be acquired after multigenerational exposure to low levels of salinization. Standard monospecific bioassays were carried out by exposing organisms, before and after multigenerational exposure to low levels of salinity, from different taxonomic groups (from producers to secondary consumers) to gradients of salinity obtained with NaCl or with SW. In general, NaCl tended to exert similar or higher toxicity than SW, both at lethal and sub-lethal levels, suggesting that it may be proposed as a protective surrogate of SW for first tiers of ecological risk assessment. Among all tested species, the cyanobacterium, the daphnid and the rotifer were the most sensitive taxa to salinization ($EC_{50} \leq 4.38$ $mScm^{-1}$). Given their position at the basis of the food web, it is suggested that small increments of salinity may be sufficient to impair energy balance in trophic webs, and, ultimately, disrupt the natural functioning of threatened coastal freshwater ecosystems. Furthermore, no evidences of increased tolerance after multigenerational exposure to low levels of salinity were found: in most cases, reassessment of lethal and sublethal endpoints of organisms that had been pre-exposed to low salinity levels for several generations did not show a significant increased tolerance to salinity.

Keywords: salinization; freshwater ecosystems; surrogate; zooplankton; multigenerational exposure

1. Introduction

Seawater intrusion, due to sea level rise, in coastal freshwater ecosystems is already occurring in several regions of the globe (e.g., Barlow and Reichard, 2010; Picado et al., 2013). Following the projections on climate change regarding an increase on the frequency of extreme events (e.g., storms, droughts), it is expected that further coastal areas will likely be affected in a near future (IPCC, 2014). These seawater intrusions are causing an increase in the salinity of such coastal freshwater ecosystems (e.g., IDAD, 2008; Martins et al., 2011). Depending on the intensity and duration, increased salinity events may cause several adverse effects in freshwater organisms, including mortality (e.g., Lob and Silver, 2012; Struewing et al., 2014) or alterations in life cycle traits that impair fitness, like reduced growth or reproduction rates (Ghazy et al., 2009; Kearney et al., 2012; Simmons, 2012; Carneiro et al., 2013) or metabolic costs (Peña-Villalobos et al., 2016; Tyree et al., 2016). These effects, observed at the individual level, may translate into adverse consequences at higher levels of biological organization, including the disruption of ecosystem functions and, consequently, the services it provides (Piscart et al., 2005; Flöder et al., 2010; Van Meter et al., 2012; González-Ortegón et al., 2015).

An aquatic ecosystem holds a complex net of organisms, which exhibit differential sensitivity to environmental perturbations. It is then necessary to identify the most sensitive group of organisms to a stressor in order to set protective environmental levels to that particular stressor. Beatty et al. (2011) reported salinity values of 8.2 or 14.6 g L^{-1} of NaCl (approximately 16.3 and 28.9 $mScm^{-1}$ at 20ºC, respectively) to induce mortality in three south-western australian endemic freshwater fish. Yilmaz (2007) reported a significant decrease in growth (expressed as dry mass) for the freshwater macrophyte *Lemna gibba* exposed to salinities above 22 gL^{-1} NaCl (approximately 43.6 $mScm^{-1}$ at 20ºC, respectively). Other freshwater species have revealed a higher sensitivity to salinization. For example, the growth rates of *Pseudokirchneriella subcapitata* and *Cylindrospermopsis raciborskii* were reduced in 50% at 0.87 and 1.46 g L^{-1} of NaCl (1.72 and 2.89 $mScm^{-1}$ at 20ºC), respectively (Santos et al., 2007). This wide tolerance to salinity, exhibited

by freshwater species, highlights the awareness in protect these coastal ecosystems through the identification of theirs most sensitive ecological receptors, to establish salinity safe levels.

Many works have addressed the effects of salinity to freshwater biota. But, the majority focused mostly on the effects of specific salts (NaCl, $MgCl_2$, among others) using monospecific standard toxicity tests (e.g., Lewis and Analysts, 1999; Kefford et al., 2002; Kennedy et al., 2005; Leitão et al., 2013; Jones et al., 2015; Kotalik et al., 2017). Whether the toxicity data generated by exposure of organisms to these salts may be safely used for risk assessment of salinization due to seawater intrusion still holds uncertainties, since the information available in the literature for toxicity induced by seawater is still rather scarce (e.g., Kefford et al., 2004; Ghazy et al., 2009). One of the few examples was reported by Ghazy et al. (2009): these authors have compared the median lethal concentration obtained after exposing *Daphnia magna*, for 48h ($LC_{50,48h}$) and for 21d ($LC_{50,21d}$), to increased salinity levels using NaCl, synthetic seawater (SS) and filtered natural seawater (FNS). At the end of the 48-h exposure period, it was observed, in decreasing order of toxicity, that NaCl>SS>FNS, with $LC_{50,48h}$ values of 5.92, 7.76 and 9.54 $mScm^{-1}$, respectively; while at the end of the 21-d exposure period, it was found that the salt NaCl was not the one causing higher toxicity: in decreasing order of toxicity it was observed that SS>NaCl>FNS, with $LC_{50,48h}$ values 4.43, 5.03 and 6.89 $mScm^{-1}$, respectively. These results highlight the need to make comparisons between toxicity data obtained for salts and seawater in order to purpose salts, like NaCl, as possible surrogates for early phases of risk assessment of salinization due to seawater intrusions in coastal regions.

Adding to the above, standard monospecific assays involve one-generational exposure to salinity, but, in the field exposure may occur over generations. In fact, either exposure to pulsed seawater intrusions (e.g., extreme phenomena) or continued exposure (e.g., through groundwater exposure) are relevant scenarios that must be considered, especially for short life cycle and typically multivoltine biota. This way, it is important to understand if organisms are capable to increase their tolerance to salinity through acclimation mechanisms [*i.e.*, promote short-term compensatory physiological/osmoregulatory changes within their life-cycle; for example, frogs (Wu et al., 2014) or algae (Venâncio et al., 2017a)], and/or through other types of phenotypic

plasticity (e.g. developmental plasticity, epigenetics) that are transferred to the descendants, allowing the organisms from later generations to be better fitted to live in salinized conditions [as reported for algae (Venâncio et al., 2017a) or daphnids (Coldsnow et al., 2017)]. For instance, working with two species of freshwater green algae, Venâncio et al. (2017a) observed that the algae growth rates decreased when algae were exposed to increased salinity levels induced by NaCl; though, after multigenerational exposure to low levels of salinity, these species were able to increase their tolerance to salinization (*Chlorella vulgaris* after one generation and *Raphidocelis subcapitata* after two generations). Neglecting this type of responses in toxicity assays may lead to the overestimation of risk, therefore, when planning experimental designs to assess the risk of salinization, they should take into consideration longer exposure periods, involving several generations. Among the mechanisms that organisms use to deal with increased salinity levels, at the individual level, some authors have discussed the vacuolar compartmentalization or ion sequestration in producers (Bazihina et al., 2014) and the triggering of an osmoregulatory reaction (i.e., to maintain homeostasis organisms may adjust, internally, their water levels and minerals contents so they became like their external environment) in cladocerans (Heine-Fuster et al., 2010). Adding to this, it has been discussed more recently, the possible role of epigenetic phenomena (e.g., DNA methylation) as a possible process to acquire increased tolerance through multigenerational exposure (e.g., Ho and Burggren, 2010; Roberts and Gavery, 2011).

Following the above mentioned, this work aimed at addressing the effects of salinization on coastal freshwater ecosystems, within scenarios of sea level rise, by: i) evaluating the suitability of sodium chloride (NaCl) as a surrogate to assess toxicity of natural seawater (SW); ii) identifying the most sensitive freshwater ecological receptors and iii) determining if increased tolerance to salinity may be acquired after multigenerational exposure to low levels of salinization.

2. Materials and Methods

2.1 Test solutions

The salt (NaCl) was supplied by Merck (St Louis, MO, USA) and seawater (SW) was collected at the NW Atlantic Ocean (40°38'33"N 8°44'55"W, Aveiro, Portugal). The collection site was located in front of a Natural Reserve (São Jacinto dunes, created in 1979) and its waters are subjected to periodic monitoring programs according to the European Union Directive 2006/7/CE. The waters from this location are acknowledged as being good-excellent quality and for the past 28 years has received the prestigious Blue Flag award granted by the Foundation for Environmental Education (please see Venâncio et al., 2017a).

The NaCl concentrations were prepared by the addition and direct dissolution of the salt in the control medium used for each tested species (please see section 2.3 of Materials and Methods and Table 1). Only fresh solutions were used to perform the toxicity assays. Collected SW was always filtered through cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain) before being used in toxicity assays to remove particles in suspension and organisms. The SW dilutions were made directly by diluting SW with the control medium used for each tested species (please see section 2.2 of Materials and Methods).

2.2 Culturing conditions of tested species

Laboratory cultures of microalgae species, *Raphidocelis subcapitata* (Chlorophyta, Sphaeropleales), *Chlorella vulgaris* (Chlorophyta, Chlorellales) and *Cylindrospermopsis raciborskii* (Cyanophycae, Nostocales), were maintained in MBL in 250 ml erlenmeyers flasks, under controlled conditions of light (100 µE m^{-2} s−1) and temperature (23ºC), according to OECD guideline 201 (OECD, 2006). Only *C. raciborskii* was maintained at a lower light intensity (≈ 40 µE m^{-2} s^{-1}). Cultures were renewed once per week.

Cultures of the macrophyte *Lemna minor* and *Lemna gibba* (Tracheophyta, Arales) were maintained in 250 mL glass vessels with Steinberg medium (OECD, 2006b), at 23ºC with a light intensity of 100 µE m^{-2} s^{-1}. Cultures were renewed once per week.

Neonates of the freshwater rotifer *Brachionus calyciflorus* (Rotifera, Ploimida) and of the ostracod *Heterocypris incongruens* (Arthropoda, Podocopida) were obtained after the hatching of cysts available in commercial kits (MicroBioTests, Gent, Belgium). For the rotifer, the cysts were hatched at 23ºC, for 24 hours, at a constant light intensity of 3000-4000 lux. For the ostracod, the cysts were left to hatch at 25ºC, for 52 hours, also at a constant light intensity of 3000-4000 lux.

Six clonal lineages of *Daphnia longispina* (Arthropoda, Cladocera) were maintained in laboratory under room temperature of 20±2°C and 16 h^L :8 h^D photoperiod, in ASTM hard water medium (ASTM, 2002). Cultures were renewed every other day, fed with *R. subcapitata* (1.5 x 10^5 $cells^{-1}$ ml^{-1} day^{-1}) and medium supplemented with an organic additive (Baird et al., 1989). Organisms used for experiments were born between the 3rd and the 5th broods and were less than 24-hours old.

The culture of *Chironomus riparius* (Arthropoda, Diptera) was maintained with an inorganic fine sediment layer and ASTM hard water medium (ASTM, 2002) (proportion 1:4), at room temperature of 20±2°C and 16 h^L :8 h^D photoperiod cycle. Organisms were fed three times a week with a suspension of commercial fish food TetraMin® (Tetrawerke, Melle, Germany) (Azevedo-Pereira et al., 2011).

Regarding the snail *Theodoxus fluviatilis* (Mollusca, Cycloneritimorpha) individuals were collected at the Anços River spring (for further details on this location see Feio et al., 2010 and Graça et al., 2012), where they are abundant in stream-bed stones. The organisms were transported to the laboratory in refrigerated chambers, containing water from the collection site, to reduce organism's stress. Also, prior to use in ecotoxicity assays, organisms were acclimated to laboratorial conditions (room temperature of 20±2°C and 16 h^L :8 h^D photoperiod cycle) and to the test medium (ASTM; ASTM, 2002), at least for one week (please see Correia et al., 2013).

2.3 Toxicity Assays

Lethal and sublethal toxicity assays were carried out with ten freshwater species, representing different taxonomic and functional groups. Details on the procedures to perform the assays are described in Table 1 and in the text below.

During the toxicity assays, the following parameters were measured: salinity/conductivity, pH and dissolved oxygen with WTW (Weilheim, Germany) portable meters 440i, pH330i and OXI 330i, respectively.

2.3.1 Toxicity assays with microalgae/phytoplankton

The 72-hour growth inhibition assay was carried out with *R. subcapitata* and *C. vulgaris.* The assays were performed according to the OECD guideline 201 (OECD, 2006) adapted for 24-well plates, as described in Moreira-Santos et al. (2004). Experiments were run for 72 hours under continuous light (100 μE m^{-2} s^{-1}) and controlled temperature (23ºC). For each microalgae, a concentration of 10^4 cells ml^{-1} was set up in each replicate at the beginning of the assay. Three replicates, each of 1 ml, were performed for each treatment. Experimental treatments included a control (MBL medium; Nichols, 1973), 9 NaCl concentrations and 10 SW dilutions (Table 1). Each replicate was resuspended twice a day using a sterile pipette to avoid settlement of algae and subsequent shading effects on their growth. The growth of algae was determined by measuring the absorbance (*ABS*) at 440 nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, VT, USA) at the end of the 72-hour period, and converted into cell density (*Conc*, cell ml^{-1}) according to equations 1 or 2.

$Conc\,(\text{cell ml}^{-1}) = -17107.5 + (ABS * 7925350)$ (R^2= 0.99), for *R. subcapitata* (equation 1)

$Conc\,(\text{cell ml}^{-1}) = -155820 + (ABS * 13144324)$ (R^2= 0.98), for *C. vulgaris* (equation 2)

Average specific growth rate (μ, day^{-1}) was determined for each test concentration and control according to OECD (2006):

$\mu = \frac{\ln D_b - \ln D_a}{t_b - t_a}$ (equation 3), where D_b is the cell density at the end of the assay, D_a is the initial cell density and t_b-t_a is the exposure time interval (72 hours).

The growth inhibition assay with *C. raciborskii* was conducted following OECD guideline 201 (OECD, 2006) with adaptations in light conditions and time of exposure (Table 1; please see Nunes et al., 2014). Exposure was done in 100 ml sterilized Erlenmeyer flasks filled with 50 ml of test solution or control medium (MBL medium; Nichols, 1973), under 40 μE m^{-2} s^{-1} of

continuous light intensity (approximately 3000 lux) and 23ºC. Three replicates were carried out for each treatment. Experimental treatments consisted of a control, 10 NaCl concentrations and 10 SW dilutions (Table 1). The initial cell concentration in each replicate was 10^4 cells ml^{-1}. At the end of the assay, cell density (*D*, cell ml^{-1}) was calculated by counting sample aliquots using a Neubauer Improved Counting Chamber, and average specific growth rate (μ, day^{-1}) was calculated as for green algae (OECD guideline 201; OECD, 2006), with equation 3. The assay duration was 10 days.

2.3.2 Toxicity assays with macrophytes

A 7-day growth assay was run with the macrophytes *Lemna minor* and *L. gibba* according to OECD guideline 221 (OECD, 2006b). For both species, at the beginning of the assay three colonies of similar size, totalizing 12 fronds, were introduced per replicate flask. This consisted in a 150-ml sterilized Erlenmeyer flask filled with 100 ml of test solution or Steinberg medium (control) (Table 1). Experimental treatments consisted of a control, 9 NaCl concentrations and 10 SW dilutions for *L. minor* (Table 1) and consisted of a control, 6 NaCl concentrations and 6 SW dilutions for *L. gibba* (Table 1). Three replicates were performed per treatment. Exposures were carried out at 23ºC and continuous light (100 $\mu E\ m^{-2}\ s^{-1}$) (Table 1). At the end of the assay, the total number of fronds was counted in each vessel and the dry weight of the macrophytes was estimated after drying at 60ºC for 24 hours. Average growth rates for both species (μ, day^{-1}) were calculated according to equation 3.

2.3.3 Toxicity assays with consumers

Mortality (24 hours) and reproduction (48 hours) assays were performed with the rotifer *B. calyciflorus*. Both assays were performed according to the standard operation procedure for the Rotoxkit F (MicroBioTests, Gent, Belgium) in multiwell plates, in total darkness at 23ºC. For the mortality assay, five replicates with five newly hatched rotifers were assigned to each treatment: control (ASTM medium), 5 NaCl concentrations and 5 SW dilutions (Table 1). An organism was considered dead if did not exhibit any movement within five seconds of observation after gentle

agitation of the medium. Reproduction assay were carried out under the same conditions as the mortality assays, eight replicates were performed per treatment: control (ASTM medium), 10 NaCl concentrations and 9 SW dilutions (Table 1). At the beginning of the assay one rotifer was introduced per well. After 48 hours of incubation of the test plates the total number of swimming organisms was counted.

The 6-day mortality and somatic growth assay with the ostracod *H. incongruens* followed the standard operation procedure for the Ostracodtoxkit F (MicroBioTests). Assays were run in total darkness at 25ºC. Four replicates with ten ostracods each were set per treatment: control, 8 NaCl concentrations and 8 SW dilutions (Table 1). After the 6^{th} day of incubation of the test plates, all swimming organisms were retrieved and measured. Mortality was calculated by subtracting the initial number of organisms to the final number of organisms retrieved, considering that this difference corresponded to the number of dead organisms. Somatic growth of the ostracods was determined through measurement of the length of the organisms at the beginning and end of the test. Measurements were performed under the stereomicroscope (MS5; Leica Microsystems, Houston, TX, USA), using a calibrated eyepiece micrometer and values converted to length considering that smallest subdivisions of the micrometer lines were of 50 µm.

For *D. longispina* were selected six clonal lineages. The choice of these particular clonal lineages was based on the fact that they were already target of many studies, which provided much knowledge on their sensitivity to chemicals, including NaCl (e.g., Lopes et al., 2004; Martins et al., 2007; Silva et al., 2010; Saro et al., 2012; Leitão et al., 2013). For each of the six clonal lineages, the following assays were performed: 24-h mortality (according to OECD guideline 202; OECD, 2004), 24-hour feeding inhibition (according to Allen et al., 1995; McWilliam and Baird, 2002); and 3-day somatic growth inhibition assay (according to Burns, 2000). All procedures adopted in each toxicity assay are described at chapter IV (please see *Multigenerational effects of salinity in six clonal lineages of* Daphnia longispina, *Section 2.2 and 2.3 of Materials and Methods*). At this chapter, only the median lethal (mortality) and median effective conductivities (feeding and somatic growth) of the six clonal lineages were presented. In table 1 are presented the range of conductivities of SW or NaCl at which the organisms were exposed.

Ecotoxicity assays with *T. fluviatilis* were carried out with organisms with a shell height and length of approximately 4 and 5 mm, respectively. A 48-hour lethal toxicity assay was followed by a 3-hour feeding inhibition test. To assess mortality, organisms were exposed, at 20ºC in a 16^{L}:8^{D} photoperiod cycle, to a range of NaCl concentrations and SW dilutions plus a control (only ASTM medium) (Table 1). Four replicates with five organisms each were assembled. Each replicate consisted in a 50-ml vessel filled with the respective solution and covered with a net, tied with a rubber band (to avoid organisms from escaping). Each of the 50-ml vessels was also introduced inside larger vessels (150 ml) filled with the respective solutions to assure that the smaller vessel was completely full of the respective concentration/dilution. Mortality was verified at 24 and 48 hours. At the end of the mortality test, surviving organisms were transferred individually to 24-well plates. Each well was previously filled with 2 ml of clean medium and 150 brine shrimp nauplii (*Artemia salina*). After the 3-hour feeding assay snails were immediately removed and the remaining food items counted. Feeding assay ran at 20ºC in total darkness. The feeding procedure was previously developed and validated by Correia et al. (2013).

2.3.4 Toxicity assays with detritivores

To conduct mortality, growth and emergence assays with *C. riparius*, 2-day old larvae (1st stage) were used. The lethal assays followed the OECD guideline 235 (OECD, 2011), using neither food nor sediment. Four replicates, with five organisms each, were established per treatment or control (ASTM medium; ASTM 2002) (Table 1). For growth and emergence, a set of six concentrations replicated eight times was prepared (Table 1). Each replicate contained ten organisms. Vessels contained a 2-cm high layer of sediment and 300 ml of respective test solutions (OECD, 2004b, Guideline 219). At day ten, half of the replicates were sacrificed to evaluate growth. Larvae were removed from the sediment and preserved in 70% ethanol and, afterwards, the body and head length (mm) of each larva were determined using a stereomicroscope (Leica MS5, Leica Microsystems, Houston, TX, USA) fitted with a calibrated eyepiece micrometer. Growth (mm) was evaluated by subtracting the average initial length of the organisms from the final length of

each organism. The remaining replicates were maintained until the 28th day and emergence of adults was recorded every day.

2.4 Multigenerational exposure to low levels of salinity

In order to understand if multigenerational exposure to low levels of salinity increased the tolerance of organisms to this stressor, the studied species were exposed for two generations to low levels of NaCl. The salt NaCl was used instead of SW because it showed to be a safe surrogate of SW in the assays described in the previous section. These generations were designated as F0 (with no previous exposure to low levels of salinity) and F1 and F2 (generations with previous exposure to low levels of salinity). After exposure for two generations to low levels of salinity, the lethal and sublethal endpoints mentioned above were reassessed by performing the assays previously described (see 2.3 Toxicity Assays). This allowed comparing the sensitivity of organisms before (F0) and after multigenerational exposure (F2) to low levels of salinity.

Conductivities set for multigenerational exposure are described in Table 1 for each species. The conductivity values were selected based on the LC_{50} for each tested species divided by a factor of 2. This approach intended to expose the organisms to sublethal salinity levels though close to those inducing mortality, to simulate high stress levels and promote responses that would allow later generations to cope better with increased salinity levels. Whenever high mortality was observed during multigenerational exposure, this factor was adjusted in order to lower the conductivity (LC_{50}/3, LC_{50}/4, LC_{50}/5 and so on) until no significant mortality was observed during the multigenerational exposure (Table 1).

Regarding the period of multigenerational exposure, was considered one generation whenever the culture reached the exponential phase. Each new generation was set from the previous one when cultures were at their exponential growth phase (in case of *R. subcapitata* and *C. vulgaris* this period was of 4/5 days, for *C. raciborskii* was of 10 days).

Regarding the macrophytes, only the macrophyte *L. minor* was tested to assess increased tolerance after multigenerational exposure to low levels of salinity. No tests were possible to perform with *L. gibba* since no healthy fronds (green, with no signs for necrosis) were possible to obtain after

multigenerational exposure to a salinity levels corresponding to $EC_{50\ growth}/6$. A generation period was considered whenever the macrophytes fronds cover the whole medium surface of a 250 ml Erlenmeyer, filled with 100 ml medium. Each generation lasted for 7/8 days.

For daphnids and rotifers this period corresponded to two generations of asexual reproduction and for chironomids corresponded to two generations of sexual reproduction. Regarding daphnids and chironomids, cultures with medium spiked with NaCl (Table 1) were maintained as previously described in section 2.3 Toxicity Assays. For rotifers, a culture was implemented from rotifers hatched from cysts. The rotifers were collected within 6 hours after hatching (to avoid much discrepancy in organisms age) and individualized in 24-well plates. Each well was filled with 1 ml of the solution of NaCl at the conductivity level desired (Table 1). Organisms were fed and kept under control conditions of darkness and temperature as described in the procedure Rotoxkit F (MicroBioTests, Gent, Belgium). At 24 hours, organisms were checked for the release of first brood, and then every 12 hours. This allowed to follow the organisms until the release of third or fourth broods. To carry on the next generation, only third or fourth brood individuals were used as well for the reassessment of endpoint by the end of the second generation.

For *H. incongruens* and *T. fluviatilis* it was not possible to assess their capacity to acquire an increased tolerance to salinity after multigenerational exposure because the maintenance of cultures under standard laboratorial conditions at low levels of salinity was unsuccessful.

Table 1: Summary of procedures, range of concentrations and additional information relatively to the test species. Abbreviations: Light (^{L}) dark (^{D}) cycle, natural seawater (SW), sodium chloride (NaCl), after multigenerational exposure to NaCl ($NaCl_{GE}$), conductivity causing 50% of mortality (LC_{50}) or another effect (EC_{50}).

Test species	Endpoint	Concentration for multigenerational exposure	Dilution water	Test conditions	Test water conductivity (mS cm^{-1})	Geometric factor
Microalgae / Phytoplankton						
Chlorella vulgaris	Growth[1] $EC_{50\ growth}$	$EC_{50\ growth}$/4	MBL	24 h ^{L} : 0 h ^{D} 23ºC 72 h	SW: NaCl: $NaCl_{GE}$:	5.15 – 19.0 7.10 – 21.8 4.10 – 21.8	1.15
Raphidocelis subcapitata	Growth [1] $EC_{50\ growth}$	$EC_{50\ growth}$/4	MBL	24 h ^{L} : 0 h ^{D} 23ºC 72 h	SW: NaCl: $NaCl_{GE}$:	5.15 – 19.0 7.10 – 21.8 4.10 – 21.8	1.15
Cylindrospermopsis raciborskii	Growth[1] $EC_{50\ growth}$	$EC_{50\ growth}$/3	MBL	24 h ^{L} : 0 h ^{D} 23ºC 10 d	SW: NaCl: $NaCl_{GE}$:	2.72 – 14.1 1.90 – 9.81 2.72 – 14.1	1.2
Producers						
Lemna minor	Growth [1] $EC_{50\ growth}$	$EC_{50\ growth}$/6	Steinberg medium	24 h ^{L} : 0 h ^{D} 23ºC 7 d	SW: NaCl: $NaCl_{GE}$:	4.93 – 52.3 2.80 – 22.8 2.80 – 22.8	1.3
Lemna gibba	Growth [1] $EC_{50\ growth}$	-	Steinberg medium	24 h ^{L} : 0 h ^{D} 23ºC 7 d	SW: NaCl: $NaCl_{GE}$:	14.1 – 52.3 6.15 – 22.8 –	1.3
Primary Consumers						
Brachionus calyciflorus	Mortality [2] LC_{50}	LC_{50} /4	ASTM	0 h ^{L} : 24 h ^{D} 25ºC 24 h	SW: NaCl: $NaCl_{GEi}$:	3.50 – 13.4 2.74 – 10.5 2.74 – 14.7	1.4
	Reproduction [2] $EC_{50\ reproduction}$	LC_{50}/2	ASTM	0 h ^{L} : 24 h ^{D} 25ºC 48 h	SW: NaCl: $NaCl_{GE}$:	1.70 – 7.26 0.95 – 4.9 2.64 – 7.26	1.2

[1] OECD 201 (2006)

[2] Rotoxkit F *acute* (MicroBioTests Inc., Belgium)

[3] OstracodToxKit F *chronic* (MicroBioTests Inc., Belgium)

[4] OECD 202 (2004)

[5] Allen et al (1995)

[6] Correia et al (2013)

[7] OECD 235 (2011)

[8] OECD 219 (2004)

Table 1 (Cont.): Summary of procedures, range of concentrations and additional information relatively to the test species. Abbreviations: Light (^{L}) dark (^{D}) cycle, natural seawater (SW), sodium chloride (NaCl), after multigenerational exposure to NaCl ($NaCl_{GE}$), conductivity causing 50% of mortality (LC_{50}) or another effect (EC_{50}).

Test species	Endpoint	Concentration for multigenerational exposure	Dilution water	Test conditions	Test water conductivity (mS cm^{-1})	Geometric factor
Primary Consumers						
Heterocypris incongruens	Mortality [3] LC_{50}	-	ASTM	0 h ^{L} : 24 h ^{D} 25ºC 48 h	SW: NaCl: $NaCl_{GE}$:	1.78 – 18.2 1.40 – 14.7 –	1.4
	Somatic growth [3] $EC_{50\ growth}$	-	ASTM	0 h ^{L} : 24 h ^{D} 25ºC 6 d	SW: NaCl: $NaCl_{GE}$:	6.0 – 10.5 2.43 – 4.87 –	1.4
Daphnia longispina	Mortality [4] LC_{50}	≈ LC_{50}/8	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 48 h	SW: NaCl: $NaCl_{GE}$:	2.47 – 13.3 1.67 – 14.6 2.71 – 14.6	1.4
	Feeding [5] $EC_{50\ feeding}$	≈ LC_{50}/8	ASTM	0 h ^{L} : 24 h ^{D} 20ºC 24 h	SW: NaCl: $NaCl_{GE}$:	1.53 – 8.54 1.32 – 5.42 1.76 – 6.41	1.2
	Somatic growth [4] $EC_{50\ growth}$	≈ LC_{50}/8	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 72 h	SW: NaCl: $NaCl_{GE}$:	2.6 – 6.27 1.96 – 4.76 1.87 – 4.88	1.1
Theodoxus fluviatilis	Mortality [6] LC_{50}	-	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 48 h	SW: NaCl: $NaCl_{STE}$:	16.5 – 44.0 6.04 – 14.0 –	1.15
	Feeding post-exposure [6] $EC_{50\ feeding}$	-	ASTM	0 h ^{L} : 24 h ^{D} 20ºC 3 h	SW: NaCl: $NaCl_{STE}$:	6.0 – 10.5 2.43 – 4.87 –	1.15
Detritivores						
Chironomus riparius	Mortality [7] LC_{50}	$EC_{50emergence}$/6	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 48 h	SW: NaCl: $NaCl_{GE}$:	10.1 – 21.0 8.44 – 17.5 8.44 – 17.5	1.2
	Growth [8] $EC_{50\ growth}$	$EC_{50emergence}$/6	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 10 d	SW: NaCl: $NaCl_{GE}$:	7.23 – 18.0 4.89 – 12.2 4.89 – 12.2	1.2
	Emergence [8] $EC_{50\ emergence}$	$EC_{50emergence}$/6	ASTM	16 h ^{L} : 8 h ^{D} 20ºC 28 d	SW: NaCl: $NaCl_{GE}$:	6.02 – 12.5 4.08 – 10.1 4.08 – 10.1	1.2

[1] OECD 201 (2006)

[2] Rotoxkit F *acute* (MicroBioTests Inc., Belgium)

[3] OstracodToxKit F *chronic* (MicroBioTests Inc., Belgium)

[4] OECD 202 (2004)

[5] Allen et al (1995)

[6] Correia et al (2013)

[7] OECD 235 (2011)

[8] OECD 219 (2004)

3. Data analysis

Lethal conductivities (as a measure of salinity, comparable across NaCl and SW) provoking 50% of mortality (LC_{50}) were computed with the PriProbit software (Sakuma, 1998). Estimation of median effective conductivities (EC_{50}) for other endpoints was made by using nonlinear regressions, fitting the data sets to a three-parametric logistic/sigmoid curve (the one that fit better), using the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

To evaluate the hypothesis of NaCl as a possibly surrogate for SW, lethal and sub-lethal conductivities between NaCl and SW were compared through a generalized likelihood test. Lethal/sublethal conductivities between NaCl and SW were considered statistically different whenever it was verified $\chi^2_{(1)} \geq 3.84$; $p<0.05$ (Sokal and Rohlf, 1995). No statistical analysis using ANOVA was possible to apply because the range of conductivities tested was not the same for NaCl and SW for most of the studied species.

In order to evaluate the hypothesis that freshwater organisms were able to increase their tolerance to salinity after a two-generation period of exposure to low levels of salinity, the same method described previously was used (a generalized likelihood test). Effective conductivities before and after generational exposure to low levels of salinity were considered statistically different whenever $\chi^2_{(1)} \geq 3.84$; $p<0.05$ (Sokal and Rohlf, 1995). This approach was followed because for the majority of the species the range of conductivities tested before and after multigenerational exposure to low levels of salinity was not the same.

4. Results

4.1 NaCl vs SW toxicity

The conductivities of diluted SW inducing 50% of mortality (LC_{50}) were always higher than the ones of NaCl (Fig. 1a). Nevertheless, NaCl was only proved to be significantly more toxic than SW in two cases: for the cladoceran *D. longispina* ($\chi^2_{(1)} = 23.9$; $p<0.05$) and the snail *T. fluviatilis* ($\chi^2_{(1)} = 169.8$; $p<0.05$). Among the tested species, the decomposer *C. riparius* and the freshwater snail *T. fluviatilis* were the most tolerant species to SW with LC_{50} (95% CL) values of 17.86

$mScm^{-1}$ (17.1-18.8) and 26.4 $mScm^{-1}$ (25.8-27.2), respectively. The rotifer *B. calyciflorus* and the cladoceran *D. longispina* were the most sensitive groups both to SW and NaCl (LC_{50} of 5.09 and 4.01 $mScm^{-1}$ for *B. calyciflorus* and 5.99 and 2.49 $mScm^{-1}$ for *D. longispina*, respectively).

Regarding sublethal endpoints (Fig. 1b), a similar tendency was observed, with SW being less toxic than NaCl for most of the tested endpoints and species. In 6 out of 12 cases, NaCl showed to be significantly more toxic than SW ($\chi^2_{(1)} \geq 3.84$; $p<0.05$; Fig. 1b). Exceptions, where SW was more toxic than NaCl, were recorded for *C. vulgaris* (growth EC_{50}, of 21.8 and 12.2 $mScm^{-1}$ for SW and NaCl, respectively), *B. calyciflorus* reproduction (reproduction EC_{50}, of 1.96 and 3.88 $mScm^{-1}$ for SW and NaCl, respectively) and *C. riparius* emergence (emergence EC_{50}, of 6.99 and 8.17 $mScm^{-1}$ for SW and NaCl, respectively). At sublethal levels (Fig. 1b), the most sensitive organisms were the producer *C. raciborskii* and primary consumers (cladoceran and rotifer), being the reproductive output of *B. calyciflorus* the most sensitive endpoint (Fig 1b). The macrophytes *L. minor* and *L. gibba* were the most tolerant species, namely to SW (EC_{50} of 51.9 $mScm^{-1}$ *L. minor* and EC_{50} of 39.5 $mScm^{-1}$ for *L. gibba*). The macrophyte *L. minor*, the ostracod *H. incongruens* and the freshwater snail *T. fluviatilis* were the species presenting the highest difference in their sensitivity to SW and NaCl (2.7, 2.6 and 2.14-fold difference, respectively), with EC_{50} (95% CL) of: 51.9 (42.8-61.1) and 19.5 (16.7-22.2) $mScm^{-1}$ for growth; 12.4 (10.6-14.3) and 4.86 (4.38-5.34) $mScm^{-1}$ for somatic growth, and 24.6 (21.6-27.6) and 11.5 (9.79-13.2) for feeding rate, for SW and NaCl, respectively.

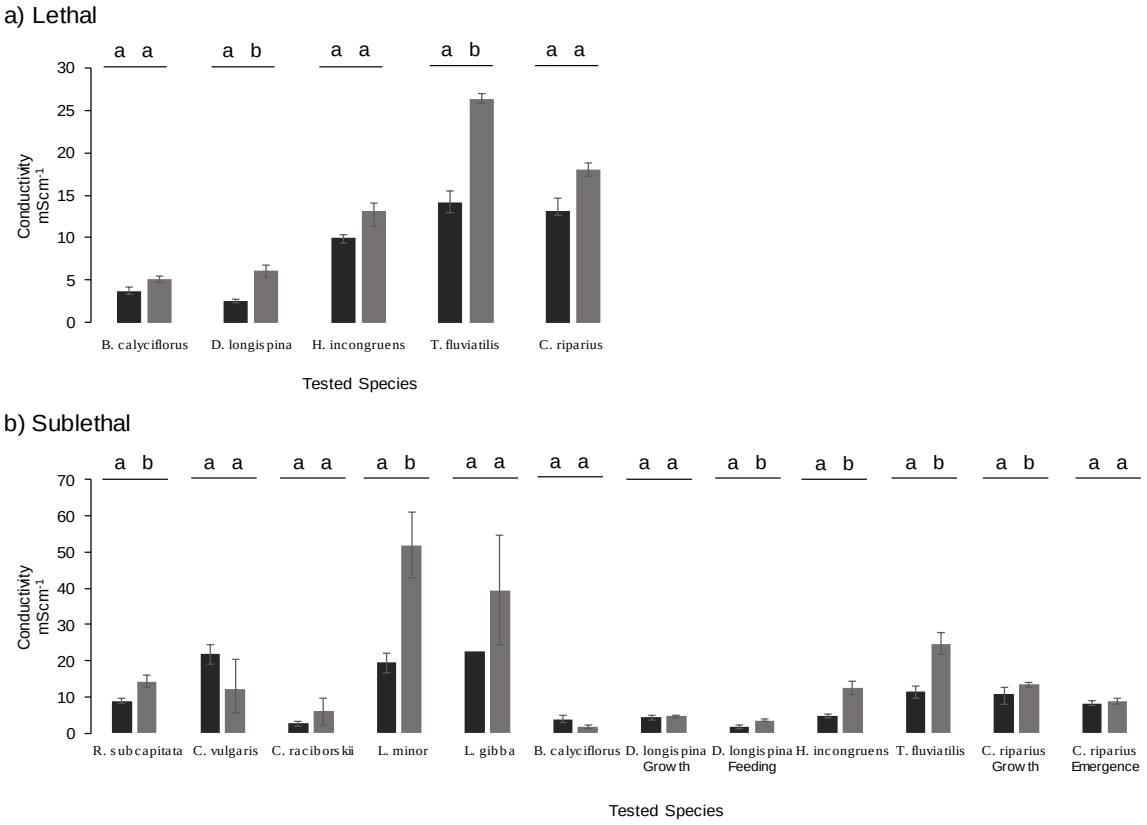

Figure 1. a) Lethal (LC_{50}) and b) Median effective conductivities (EC_{50}) of sodium chloride (NaCl, dark grey bars) and natural seawater (SW, light grey bars), for all the studied species. Vertical bars correspond to the 95% confidence limits. Different letters (a,b) indicate significant differences (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$).

4.2 Multigenerational exposure to increased salinity

Multigenerational exposure to low levels of salinity did not significantly change the lethal sensitivity of *B. calyciflorus* and *C. riparius,* but increased the lethal tolerance of *D. longispina* to salinization (Fig. 2a). For the latter species, the LC_{50}s were, before and after multigenerational exposure, 2.49 and 6.28 $mScm^{-1}$, respectively (Fig. 2a). Analysis of the sublethal effects showed that in 4 out of the 7-tested species, organisms were able to withstand low levels of salinity, although no clear increase of tolerance occurred ($\chi^2_{(1)} < 3.84$, $p>0.05$) (Fig. 2b). The only significant increases were registered for the producer *C. raciborskii* (cyanobacteria) ($\chi^2_{(1)} = 20.2$; $p<0.05$) and the primary consumer *D. longispina* ($\chi^2_{(1)} = 28.3$; $p<0.05$ for feeding and $\chi^2_{(1)} = 53.2$; $p<0.05$ for reproduction). However, their tolerance only increased by a factor of 1.6-fold (for *C. raciborskii* growth) and 2.5 and 1.6-fold (for *D. longispina* feeding and reproduction, respectively), (Fig. 2b). The most evident decreases in tolerance to salinity after multigenerational

exposure to low salinity levels occurred for the growth of *R. subcapitata* and *L. minor* and for the growth and emergence of *C. riparius*. For *R. subcapitata*, the EC_{50}s for growth rate were of 8.92 and 6.88 $mScm^{-1}$ before and after multigenerational exposure, respectively ($\chi^2_{(1)}$= 47.4; p<0.05) and for *L. minor*, the EC_{50}s for dry weight were 19.5 and 10.9 $mScm^{-1}$ before and after multigenerational exposure, respectively ($\chi^2_{(1)}$ = 6.89; p<0.05). For *C. riparius* the growth EC_{50}s were of 11.0 and 7.90 $mScm^{-1}$ and the emergence EC_{50}s were 7.60 and 6.99 $mScm^{-1}$, before and after multigenerational exposure, respectively, but no significant differences were observed in this case ($\chi^2_{(1)}\leq$ 3.84, p>0.05) (Fig. 2b).

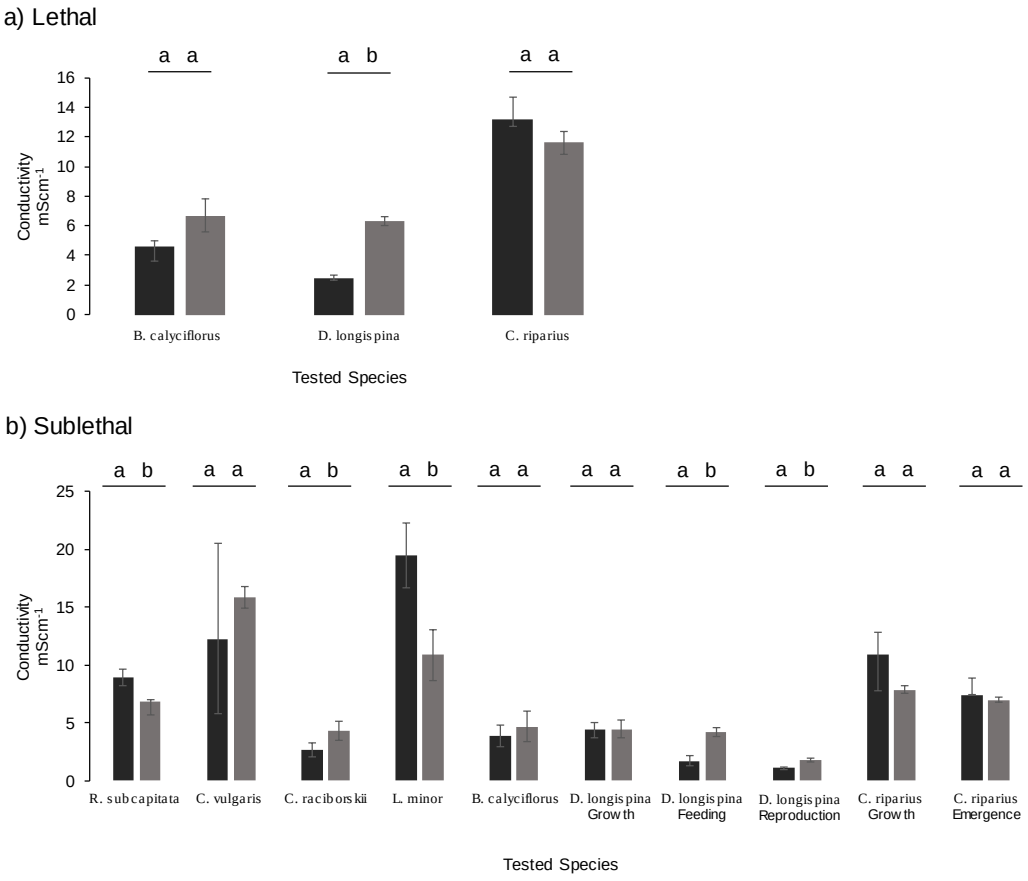

Figure 2. a) Lethal (LC_{50}) and b) Median effective conductivities (EC_{50}) of NaCl before (dark grey bars) and after multigenerational exposure to low levels of NaCl (light grey bars) for the studied species. Vertical bars correspond to the 95% confidence limits. Different letters (a,b) indicate significant differences (generalized likelihood test: $\chi^2_{(1)}\geq$3.84; p<0.05).

5.Discussion

5.1 Sodium chloride as a surrogate of natural seawater

The results obtained with the lethal and sublethal toxicity assays revealed that the salt NaCl exhibited a higher or similar toxicity than SW to the tested species, which may be related with the fact that SW has a more complex chemical composition. Previous works indicated that natural or artificial SW exerts less harmful effects than NaCl, for instance, for aquatic macrophytes (Rout and Shaw, 2001), daphnids (Ghazy et al., 2009) and macroinvertebrates (Kefford et al., 2004). The SW is composed by two major ions (Na^+ and Cl^-) but also includes ions in minor concentrations, such as calcium (Ca^{2+}), magnesium (Mg^{2+}) or potassium (K^+) (Lee and Ray, 2007). These ions have important roles in cellular functions. The ion Ca^{2+} is an important mediator in cell signaling (Stael et al., 2011); boron (B^{3+}) has as well functions in cell signaling and also in cell wall stabilization (Goldbach and Wimmer, 2007) and Mg^{2+} intervenes in the control of ion channels, enzymes or even in metabolic pathways (Romani, 2011). So, the presence of these ions can constitute an advantage and help to explain the lower toxicity of SW when compared to NaCl to the majority of tested endpoints and species. In this work, the highest differences in lethal tolerance to NaCl and SW were registered for *L. minor*, *H. incongruens* and *T. fluviatilis*. The presence of extra ions (e.g., Ca^{2+}, Mg^{2+}, K^+) in SW and not in NaCl solution may constitute an advantage for these species. For instance, regarding the macrophyte *L. minor*, Mg^{2+} may intervene in chlorophyll synthesis helping the plant to continue performing photosynthesis under stress conditions or the presence of K^+ ions may help in very specific cellular processes, namely in cytosol and chloroplasts, as this particular ion cannot be replaced by other cations (Rubio et al., 2003). So, the enrichment of the medium with extra ions (e.g., K^+ and Mg^{2+}) may act as buffers against the effects that Na^+ or Cl^- can cause when solely. The ostracods, more specifically, hold a heavily calcified exoskeleton, so the presence of an external source of Ca^{2+} may favor the molting process and therefore their growth (Turpen and Angell, 1971) and, in the case of the snail species, they not only also possess an external structure rich in calcium but this species is known to have preference for calcium-rich waters and is considered euryhaline (Fretter and Graham, 1962;

Carlsson, 2000; Zettler et al., 2004; Symanowski and Hildebrandt, 2010). Although, considering that these less represented ions are essential, which in part may explain why SW is less toxic than a simple solution of NaCl as aforementioned, the hypothesis that in complex mixture ions can interact should also be considered. Actually, Mount et al. (1997) showed that the interaction between ions could diminish their toxicity: namely Cl^-, SO_4^{2-} and K^+ ions toxicity was reduced in the presence of other cations. For instance, for *D. magna* the NaCl $LC_{50,48h}$ was of 4.77 mgL^{-1} while in a mixture of $NaCl/Na_2SO_4$ was of 5.7 mgL^{-1}, also KCl $LC_{50,48h}$ for *D. magna* was 660 mgL^{-1} while in a mixture of KCl/K_2SO_4 it was 740 mgL^{-1} (Mount et al., 1997). Still, these disparity between toxicity values for NaCl and SW draws attention to the need to include as many endpoints as possible to derive safe conclusions.

Furthermore, at high levels of salinity the differences between ionic compositions of NaCl solutions and SW may not be reflected in differences in lethal responses but at low levels of salinity, differences in sublethal responses may be observed. The difference in the response to the salt or to SW may even be clearer under sublethal exposure scenarios, as results shown for the macrophyte *L. minor*, the ostracod *H. incongruens* and the snail *T. fluviatilis*. This pattern of response has already been reported in the literature, for instance Zalizniak et al. (2009) found that despite the lack of differences between survival rates of *Physa acuta* exposed to media with different ionic composition, a reduction in feeding rates, movement, weight and growth rate was observed in media lacking the ion calcium.

As seawater is mostly composed by Na^+ and Cl^- ions and in most of cases NaCl salt induced similar or higher toxicity than natural SW, results of the present work suggest that NaCl can be used as a protective surrogate of natural sea water for early stages of preliminary risk assessment of salt stress on freshwater organisms. Nevertheless, the disparity between tolerance displayed towards SW and tolerance towards NaCl in some cases may lead to overestimation of the risk. Still, even if overestimated, NaCl-based derived safety values would be protective for the most sensitive species but, this highlights the need to integrate as much information as possible covering different trophic levels, to achieve a broader picture of the risks of salinization that freshwater ecosystems are subjected to.

5.2 Most sensitive freshwater ecological receptors

The daphnid, the rotifer and the cyanobacterium were the most sensitive ecological receptors to increased salinity. The $EC_{50,NaCl}$ for feeding and somatic growth of *D. longispina* were 3.21 and 3.48 $mScm^{-1}$, respectively; the $EC_{50,NaCl}$ for reproduction of *B. calyciflorus* was 1.7 $mScm^{-1}$ and the $EC_{50,NaCl}$ for growth rate of *C. raciborskii* was 2.65 $mScm^{-1}$. These values are above the salinity threshold for freshwaters (considered to be within the range 0.15-0.5 $mScm^{-1}$, U.S. EPA, 2012; Kemker, 2014), which suggests that even the most sensitive ecological receptors may be able to cope with some levels of salinization. Though, these effect conductivity values are more than 15 (*D. longispina* sub-lethal endpoints) and 30 times (*B. calyciflorus* reproduction) smaller than SW conductivity (approximately 52 $mScm^{-1}$, in Atlantic Ocean; e.g., Wang et al., 2010) and are likely to be very easily achieved or surpassed in case of seawater intrusion, making freshwater biota vulnerable under such scenarios. Jeppesen et al. (2007) refer 2‰ (approximately 3.8 $mScm^{-1}$) as the threshold salinity for freshwater zooplankton, which is in line with the results obtained in the present work. Furthermore, salinity values that may provoke sub-lethal effects in some species may already be toxic (lethal) to other species, denoting the importance of studying sub-lethal parameters rather than mortality. For instance, within the present study, the conductivity value that induced 50% of growth inhibition in chironomids was twice higher than the conductivity value that induce 50% mortality in daphnids and rotifers.

The sodium (Na^+) and chloride (Cl^-) uptake may counteract the loss of ions to the medium, as freshwaters are hypo-osmotic compared to the intracellular medium of organisms. In order to easy this exchange, in daphniids and rotifers, the structures at which ionic regulation occurs are rather simple. In rotifers is carried out by a rudimentary system, the protonephridia system (Thorp and Covich, 2009) while in daphnids it is assigned to epipodites: thin-walled and lamellar modifications in the gills (Aladin, 1991; Aladin and Potts, 1995). More, on daphniids this process seems to evolve with the growth of the organisms: being mediated by a Na^+/K^+-ATPase and a Na^+/Cl^- exchanger in neonates, and by a $Na^+/K^+/2Cl^-$ co-transporter in adults, which may be related with the molt cycle along daphniids life (Lucu and Devescovi, 1999; Bianchini and Wood, 2008). But, by facilitating ion exchange in poor media, allowing these organisms to uptake some

essential ions from the surrounding environment, these structures also facilitate the entrance of ions when these organisms are exposed under conditions of high salinity, which may contribute for their low tolerance to salinity. Actually, the sensitivity of *Daphnia* and *Brachionus* genus towards contamination made them relevant and widely used ecotoxicological models (e.g., Altshuler et al., 2011; Won et al., 2017). Furthermore, as ionic regulation is a high energetic metabolic process, the shift on energy investment to osmoregulation may impair other life traits, such as reproduction rates (as in *B. calyciflorus*) and growth and feeding (as in *D. longispina*) (e.g., Smolders et al., 2005). Despite being among the most sensitive group within this study, the cladoceran and the rotifer are potentially able to respond to stressful conditions, by, for instance, producing dormant or resistance eggs. These egg banks are an important strategy of survival and resilience of these species during stressful conditions. This may probably be a useful strategy to withstand increased salinity, although, it may not assure the resilience of the initial composition of the population. For instance, Santangelo et al. (2014) verified that dormant eggs did not hatch at salinities of 16 and 32 gL^{-1} of NaCl. But, when returned to freshwater some were still viable and were able to hatch. In accordance, Bailey et al. (2004) verified that, although time to hatching was delayed at salinity levels of 8 for *B. calyciflorus*, emergence ability was not compromised. On the other hand, Bailey et al. (2004) went further and verified that, at the same salinity level, other species did not display the same behavior: *Daphnia* and *Bosmina* embryos development always stopped before hatching. These species were neither able to hatch in brackish water, nor even when were transferred to reference water. So, increased salinity may not only delay hatching processes in some species as it may also totally impair it in other species. Rotifers and daphnids are the groups with high representativeness within zooplankton (e.g., García et al., 2009). Their role in energy transfer between trophic levels and in ecosystems productivity (Kerfoot and Sih, 1987) is very important; not only performing a role in top-down effects, for being important and efficient grazers (Lampert et al., 1986), but also in bottom-up, as they constitute food for other species, such as fish (Carpenter and Kitchell, 1984; Lacerot et al., 2013).

Together with zooplanktonic species above mentioned, the cyanobacterium *C. raciborskii* showed also to be one of the most sensitive species to increased salinity levels. Other studies performed

with this species have already stated similar evidences (e.g., Moisander et al., 2002). This species growth may start to be reduced at salinity levels above 2 gL^{-1} of NaCl (approximately 3.96 $mScm^{-1}$). Above this level, respiration rates decreased possibly due to the reductions on carbon dioxide (CO_2) fixation and nitrogenase activity (NA). In fact, the same authors verified that at a concentration of 10 gL^{-1} (approximately 19.8 $mScm^{-1}$), CO_2 fixation totally ceased.

5.3 Multigenerational exposure to low levels of salinity

After the multigenerational exposure to low levels of salinity, most of the studied species did not exhibit an increased tolerance to salinization. Previous studies have shown that continuous exposure to a stressor could trigger mechanisms that allow the maintenance of fitness along generations (e.g., Chen and Stillman, 2012). In the present study, most species exhibited a similar tolerance or a higher sensitivity to NaCl after being exposed for a two-generation period to low levels of salinity. With *D. magna* exposed to zinc, Vandegehuchte et al. (2010) observed that the reproductive success in the F0 generation (meaning that there was no previous exposure of the mothers to the contaminant) decreased, but in the subsequent generations (F1 and F2), this decline was not observed when organisms were collected and maintained, again, in control medium. Yet, when organisms were continuously exposed to zinc, the authors registered reduction in reproduction not only in F0 but also in F1. However, the absence of a decrease in reproduction in F2 during this multigenerational exposure, suggests that *D. magna* was able to deal with stress by possibly triggering physiological mechanisms in response to the chemical stress from F1 to F2. Nonetheless, it is important to note that this probable increase to tolerance was associated to fitness costs, such as reduced metabolic rates and decreased lethal tolerance to salt throughout the experiment. In other species (as algae) it is suggested that the development of coping mechanisms may be triggered quickly. For instance, algae can increase their tolerance to salinity within three generations when exposed to sublethal levels of salinity (e.g., Venâncio et al., 2017a). According to Hart et al. (1991), the period provided for organisms to acclimate is essential: the longer the period organisms have been exposed to a particular salinity level, the higher their ability to later cope with salt stress. In the present work, the multigenerational exposure period seemed not

enough to trigger such response. Actually, in a few cases, within this study (*R. subcapitata*, *C. riparius* and *L. minor*) it was possible to observe that organisms become more sensitive after multigenerational exposure to low levels of salinity. Though a very small decrease in tolerance was observed, it is hypothesized that if the exposure period was extended and/or the salinity level was increased, the observed effects could be aggravated over generations. For instance, Paradise (2009) studies with macroinvertebrates showed that salinity values that caused minimal effects on short-term survival, provoked severe impacts on reproduction, actually in some cases, no reproduction was observed. As an example, this author observed that for *C. tepperi*, the recruitment potential to the second generation was of only half (50%), when the parental generation was exposed to salinity level 80% lower than the salinity level that provoke 50% mortality after four days (96-hours LC_{50}). An increase in sensitivity or similar sensitivity of the organisms from before to after multigenerational exposure to low levels of salt may have repercussions at other levels. If similar effects, as those reported by Paradise (2009), will happen to the species here studied (i.e. become more sensitive to salinization after multigenerational exposure), shifts on availability of food sources (*R. subcapitata*), shelter (that might be provided by *Lemna* sp.) or even energy flow and productivity imbalances may be expected even at freshwater ecosystems suffering from low salinization levels.

On the other hand, in a few cases, it was observed an increase in tolerance after multigenerational exposure to low levels of salinity (for instance, *C. raciborskii* growth and *D. longispina* feeding and growth). Relatively to *Daphnia*, previous studies have already reported their ability to increase its tolerance in a few generations, for instance, between 5 to 10 generations (*D. pulex* in Coldsnow et al., 2017) or in a 3-generation period (in *D. longispina* clonal lineages in Venâncio et al., 2017b). The higher values of conductivity effects for *D. longispina* reported in this work after multigenerational exposure to low levels of salinity, may be related with energy expenditure in osmoregulation functions. In order to maintain homeostasis, organisms spend energy that may be replenished by the ingestion of more food (e.g., Lukas and Wacker, 2014). Furthermore, in *D. longispina* it was observed a maintenance in effect conductivities for somatic growth before and after multigenerational exposure but an increase in tolerance in reproductive output. This may be

indicative that, under salt stress, organisms may also invest in different strategies to assure the maintenance of population at impacted sites, for instance, not investing in their own growth and divert the energy for reproduction. This strategy has already been saw for other chemicals: Knops et al. (2001) verified that *D. magna* individuals exposed to CTAB (cetyltrimethylammonium bromide) where smaller when compared to non-exposed ones, but with no repercussion on neonate production. Regarding the cyanobacterium (*C. raciborskki*) it was observed, first, that this species was one of the most sensitive ecological receptors to increased salinity (please see section 5.2 Most sensitive freshwater ecological receptors), but after multigenerational exposure to low levels of salinity, the effect conductivities computed for this particular species more than doubled. Salinity may be a limiting factor on the expansion or possible bloom formation of this species (e.g., Moisander et al., 2002), though it should not be discarded that, in more complex scenarios in conjunction with other factors, its fast ability to increased tolerance towards salinity may lead to severe and irreversible changes in freshwater ecosystems. Moreover, there might be a risk also of toxin production and release by this species, with consequences for other organisms inhabiting freshwaters but also possibly to human health (Pomati et al., 2004).

6. Conclusions

Within a scenario of climate change, sea level rise is a threat to many coastal freshwater ecosystems. In the present work, when assessing the possible effects of increased salinity on aquatic biota, NaCl proved to be a safe substitute of SW for early stages of risk assessment frameworks, since the former induced similar or higher toxicity than the later for most of the studied species. Further, from a battery of standard bioassays comprising several ecological groups, it was observed that the cyanobacterium, the daphnids and the rotifers were the most sensitive ecological receptors ($LC_{50} \leq 4.98$ mScm^{-1} and $EC_{50} \leq 4.38$ mScm^{-1}, both for NaCl). These tolerance values are very low comparatively to natural seawater conductivity ($\approx$ 52 mScm^{-1}), being easily attained under SW intrusion scenarios and since both groups occupy the basis of the trophic chain, it is expected that such effects may reverberate on to other trophic levels.

Furthermore, no evidences of increased tolerance were found after multigenerational exposure to low levels of salinity. Most of the studied species presented similar tolerance before and after multigenerational exposure to low levels of salinity but it should be pointed that the producers *R. subcapitata* and *L. minor* presented increased sensitivity after multigenerational exposure to low levels of salinity. Despite being a small increase in sensitivity, the effects may be intensified in future generations, with continuous exposure or if there were increments on the salinity level. Therefore, populations resilience in salt-disturbed freshwater environments may be compromised and induce changes at the community level.

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Chapter III

May amphibians and fish acclimate to low levels of salinization?

May amphibians and fish acclimate to low levels of salinization?

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Abstract

Considering the predictions of the International Panel for Climate Changes on sea level rise, it is foreseen that the number of coastal regions worldwide impacted with salinization will increase in a near future. In this context, the present work intended to evaluate the sensitivity and the capacity to acclimate to salinization in two freshwater vertebrate species. For this, three specific objectives were targeted: (i) to assess if NaCl may be used as a safe surrogate for risk assessment of seawater (SW) intrusion for freshwater vertebrate species; (ii) to evaluate the sensitivity of two freshwater vertebrate species to increased salinity (both due to NaCl or SW); (iii) to determine the capacity of the studied species to acclimate to low levels of salinization. The following freshwater species were studied: the green frog *Pelophylax perezi* and the pumpkinseed fish *Lepomis gibbosus*. Embryos and tadpoles of *P. perezi* and juveniles of *L. gibbosus* were exposed to serial concentrations of NaCl or SW dilutions, and mortality and somatic growth (only for *P. perezi*) were monitored. To assess their capacity to acclimate, eggs of *P. perezi* and juveniles of *L. gibbosus* were exposed to low levels of salinity during the embryonic development or for a period of two months, respectively.

Results have shown that between both species, the fish was the most tolerant species. The $LC_{50,96h}$ estimated for *L. gibbosus* were 21.3 and of 23.6 $mScm^{-1}$ for NaCl and SW, respectively. Eggs and tadpoles of *P. perezi* exhibited the highest sensitivity to salinization: the estimated $LC_{50,96h}$ for SW were of 11.5 and 8.96 $mScm^{-1}$ (embryos and tadpoles, respectively) and for NaCl were of 10.60 and 13.0 $mScm^{-1}$ (embryos and tadpoles, respectively). The two-fold difference between the sensitivity of the two-tested species reinforces the idea that ecological risk assessment for amphibians based on fish toxicity data may underestimate risk to the former taxon. Pre-exposure to low levels of salinity caused a slight increase in tolerance to salinization by the tadpoles of *P. perezi*: $LC_{50,96h}$ obtained before and after pre-exposure to low salinity levels were of 13.0 and 16.5

mScm^{-1}, respectively. *Lepomus gibbosus* did not significantly increase its tolerance to salinization after a period of 2-months of exposure to low levels of salinity. However, it was able to cope with high salinity levels (almost half of seawater conductivity), which may facilitate, later, their spread over brackish waters.

Keywords: salinization; life stage; acclimation; short-term exposure; seawater

1. Introduction

Lowland coastal freshwater ecosystems are becoming salinized mainly because of seawater intrusion as a consequence of sea level rise (IPCC, 2014). Such salinization may be exacerbated in a near future due to land subsidence, changes in hydrological cycles and climate conditions (e.g., Da Lio et al, 2015; Eriksson, 2017; Hoover et al., 2017). Specifically, climate-driven changes are expected to become short-term but more severe, which may not only increase salinization of these systems but as well provoke frequent fluctuation in salinity levels (IDAD, 2008; IPCC, 2014). So far, risk assessment frameworks, regarding increased salinity at freshwater environments, are commonly performed based on toxicity data generated by standard assays that have addressed the effects of salinity on freshwater biota. These studies usually deal with salts (e.g., NaCl, $MgCl_2$), artificial seawater salts, among others (e.g., Christy and Dickman, 2002; Chinathamby et al., 2006; Dougherty and Smith, 2006; Sanzo and Hecnar, 2006), rarely studying the effects of natural seawater in biota. However, under the scenario of seawater intrusions in coastal freshwater ecosystems, it is necessary to address the effects of such natural seawater since it holds a complex composition, including the presence of several salts. Venâncio et al. (2017a, 2017b) reported SW to be equally or less toxic to microalgae and cladocerans, which were in accordance with previously presented works addressing this same issue in macroinvertebrates and daphnids (e.g., Kefford et al., 2004; Ghazy et al., 2009). But if information available regarding natural SW toxicity is limited, when considering vertebrate species, the number of studies assessing the toxicity of seawater on such taxa is quite low, namely for amphibians.

Amphibians, in their majority, are strictly dependent on water to complete the life cycle. This might prove to be an additional disadvantage when considering species that inhabit low-lying

coastal systems facing seawater intrusion, since literature suggest that amphibians may be very sensitive to increased salt content. For instance, Sanzo and Hecnar (2006) found out that salinity levels around 5.23 $mScm^{-1}$ might be enough to induce 50% of mortality after 4 days of exposure ($LC_{50,4d}$) to increased salinity levels in *Rana sylvatica* tadpoles, while for *Xenopus laevis* and *R. clamitans*, Dougherty and Smith (2006) reported $LC_{50,7d}$ values of just 2.61 and 0.8 $mScm^{-1}$, respectively. But, effects of salinity may also be reflected in behavioral patterns (e.g., distance travelled, reaction capacity; e.g., Denoël et al., 2010), impairing organism's ability to escape from predators or even foraging for food. On the other hand, freshwater fish seem able to withstand with much higher salinity levels. Values of $LC_{50,96h}$ in the literature range from around 12 $mScm^{-1}$ for the fathead minnow, *Pimephales promelas* (Beatty et al., 2011) to values as high as 40 $mScm^{-1}$ ($LC_{50,96h}$) reported for *Lepomis macrochirus* (Waller et al., 1996).

However, biota may have the capacity to acclimate to low levels of chemical stress, a perspective that usually is not taken into consideration in traditional risk assessment frameworks. Some studies reported the ability of freshwater biota to be capable of persisting and flourish at these saline-disturbed environments. For instance, *Pelophylax perezi* was found to reproduce in coastal ponds with salinity values of 3 and 11 (approximately 5.94 and 21.8 $mScm^{-1}$ at 20ºC, respectively) (Sillero and Ribeiro, 2010). When challenged with such increased salinity scenarios, the rapid activation of stress response mechanisms may be decisive for the organism's survival and persistence under salinized conditions. Among other strategies, some relate with the accumulation of internal solutes that protect cells against osmotic stress, hormonal regulation and increased expression of ionic channels that regulate the concentrations of ions going in and out of cells (Treberg et al., 2006; Uchiyama and Konno, 2006; Wu et al., 2014). For instance, in fish compensatory mechanisms of osmoregulation were positively related with enzymatic activity of Na^+/K^+-ATPase complexes of and of succinic dehydrogenase (e.g., Subramanyam, 1974) but also increased production and release of compounds (such as sugars or steroids) to the blood stream to increase osmotic pressure (e.g., Fontaínhas-Fernandes et al., 2003; Treberg et al., 2006; Sherwani and Parwez, 2008). In larval stages of anurans regulation of increased ionic content may be mediated by mitochondria-rich cells present in the gills (Uchiyama and Yoshizawa, 1992) but

in adults, the increased content of free amino acids in muscle or blood stream or even urea accumulation, seem to be more common mechanisms to cope with the effects of increased salinity (e.g., Grundy and Storey, 1994; Wright et al., 2004). Not only is important the ability to trigger such mechanisms to deal with increased salinity, but the time to activate them may be as well critical for the organism's maintenance and survival under salinized environments. In tadpoles, mechanisms of water content regulation may be rapidly triggered in response to increased ion content. As an example, Wu et al. (2014) observed that a 48-hours exposure to 7 ppt of salt (approximately 13.8 $mScm^{-1}$ at 20ºC) was sufficient to induce an efficient osmoregulation response in tadpoles of the Indian rice frog (*Fejervarya limnocharis*) when compared to non-pre-exposed tadpoles. In fish, the induction of regulatory mechanisms to deal with increased salinity may also be triggered quickly. For instance, Riar et al. (2003) found out that the rainbow trout was able to acclimate to salinity levels of 8 and 17 g/L (artificial sea salt; corresponding to 13.9 and 27.7 $mScm^{-1}$, approximately), within 14 days but for the teleost fish *Heteropreustes fossilis* it was showed that acclimation to 30% of SW may start within only just 3 days (Sherwani and Parwez, 2008). But, if organisms are indeed able to acclimate after a short-term exposure to salinity, their tolerance to salinization will be higher than that predicted by standard assays, which will overestimate the risk.

In the context of the above mentioned, the present work aimed at: i) assessing the suitability of NaCl as a safe surrogate for risk assessment of seawater (SW) intrusion for freshwater vertebrate species; ii) evaluating the sensitivity of two vertebrate species (a fish and an amphibian species) to increased salinity levels induced either by NaCl and SW and ii) determining the capacity of the studied species to acclimate to low levels of salinization.

2. Materials and Methods

2.1 Test solutions

Sodium chloride (NaCl), used as a proxy of natural seawater (SW), was supplied by Merck (St Louis, MO, USA). All NaCl concentrations were prepared fresh at the beginning of each toxicity

assay by the addition and direct dissolution of the salt in the medium according to each tested species (Table 1).

Natural seawater was collected from the Atlantic Ocean, from the north region of Portugal, at a reference site (40°38'33"N, 8°44'55"W, Aveiro, Portugal). The quality of these waters is monitored on a regular basis according to the European Union Directive 2006/7/CE, being classified as waters of good to excellent quality. Additionally, the waters at this location hold the Blue Flag award, assigned by the Foundation for Environmental Education. Prior to its use in toxicity assays, SW was always filtered through cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain). All tested dilutions were made directly from SW by using the culture media of each studied species.

2.2 Test species

Egg masses of the green frog *Pelophylax perezi*, were collected in a reference freshwater pond, located near the city of Aveiro (40°36′16″N, 8°41′48″W), during spring (please see Santos et al., 2013 for detailed information). The egg masses were transported to the laboratory under refrigerated conditions in plastic vessels filled with water from the collection site, to minimize possible perturbations. The collected eggs masses were at Gosner stage 10-11 (age < 24 hours) characterized by a clear cleavage between the vegetal pole and the animal pole.

Individuals of *Lepomis gibbosus*, commonly known as pumpkinseed sunfish, were captured in the Braças pond (Portuguese littoral; 40º14'37''N, 8º48'31''W) following the same procedure of Rodrigues et al. (2012). Transportation was also done in refrigerated containers, equipped with aeration pumps. Captures were sorted for selection of juveniles with 4 to 5 cm of length and 1 to 2 g of weight; at this stage, there is not distinction on sex gender.

2.3 Toxicity assays

A portion of the eggs of the green frog *P. perezi*, at Gosner stage 10–11 (Gosner, 1960), were immediately used to carry out the mortality assay with embryos. Eggs were exposed for 96 hours to lethal salinity levels of NaCl and SW and a control (FETAX medium; Dawson and Bantle,

1987) (Table 1) in 50-cm diameter Petri dishes filled with 10 ml of test solution. The test dilutions and control treatments were changed at 48 hours and mortality checked every day with removal of dead individuals. Also, individuals were checked for abnormalities caused by increased salinity during the experiment. Four replicates were assigned for each concentration, with ten eggs per replicate.

The remaining portion of the eggs were left to hatch and maintained in FETAX medium until reaching Gosner stage 25 (defined by the gills still being covered by the operculum closure; Gosner 1960). At this stage tadpoles were used to perform 96-h lethal toxicity assays (ASTM, 1998). Tadpoles were introduced in 120-ml plastic vessels filled with 100 ml of test solution, with four replicates assigned per concentration/dilution (NaCl/SW, respectively), each replicate with five organisms. Mortality was checked every day and the medium changed at 48 hours of exposure. The body length (mm) of the tadpoles was recorded at the beginning and at the end of the assay using a stereomicroscope (MS5; Leica Microsystems, Houston, TX, USA), fitted with a calibrated eyepiece micrometer. Growth was assessed by subtracting the average initial length of the organisms from the final length of each organism. All toxicity assays were performed at 20ºC with a 16^{L}:8^{D} photoperiod cycle.

To perform the lethal assays with *L. gibbosus*, fish were first selected according to their size and weight to achieve some uniformity (please see section 3.2.2 of Material and Methods). The NaCl or SW concentrations/dilutions were made with aged tapwater (Table 1). Exposures were conducted with seven replicates per concentration/dilution and one fish per replicate. The test lasted 96 hours, at 20ºC with a 16^{L}:8^{D} photoperiod cycle and continuous aeration. Medium renewal was performed at 48 hours (Rodrigues et al., 2012).

Salinity (Wissenschaftlich Technische Werkstätten-WTW conductivity 440i, Weilheim, Germany), conductivity (WTW conductivity 440i), pH (WTW pH330i) and dissolved oxygen (WTW OXI 330i) were measured at the beginning and end of each test and whenever the medium was changed.

2.4 Short-term exposure to low levels of salinity

Concentrations to perform short-term exposures to low levels of salinity (expressed in conductivity, $mScm^{-1}$) are presented in Table 1. Short-term exposures to low levels of salinity were only performed with NaCl, as this salt previously proved to be of similar or higher toxicity than SW and, thus, constituting a protective surrogate for early stages of salinization risk assessment (please see section 3.2 of Materials and Methods).

Eggs from *P. perezi* at Gosner stage 10-11 (age < 24 hours) (please see section 3.2.2 of Material and Methods) were exposed to a conductivity level corresponding to the median lethal conductivity value (LC_{50}) obtained before short-term exposure divided by a factor of 2, until they reach the Gosner stage 25 (G25) (Table 1). Developing embryos were checked every day for dead individuals (to avoid fungi propagation to health individuals) and medium was renewed every other day. After reaching G25 state, the tadpoles were exposed to a range of NaCl conductivities (Table 1) to reassess mortality and growth of the tadpoles as described previously (please see section 3.2.2 of Materials and Methods).

Regarding the fish, *L. gibbosus*, juveniles were selected according to the characteristics above described (please see section 3.2.2 of Materials and Methods) and exposed for two months to a conductivity level also corresponding to the median lethal conductivity value (LC_{50}) obtained before short-term exposure divided by a factor of 2 (Table 1). During this period, fish were fed with frozen chironomids larvae every other day and medium was partially (half) changed in every feeding day. After the short-term exposure period, mortality was reassessed as described before (section 3.2.3 of Materials and Methods).

Table 1: Information on the parameters, endpoints and range of concentrations used in tests. Abbreviations: Light (^{L}) dark (^{D}) cycle, natural seawater (SW), sodium chloride (NaCl), after short-term exposure to low levels of salinity ($NaCl_{STE}$), conductivity causing 50% of mortality (LC_{50}) or another effect (EC_{50}).

Test species	Endpoint	Acclimation concentration	Dilution water	Test conditions	Test water conductivity (mS cm^{-1})		Geometric factor
	Mortality [1] (embryos) LC_{50}	-	FETAX	16 h ^{L} : 8 h ^{D} 23ºC 96 h	SW: NaCl: $NaCl_{STE}$:	4.10 – 19.8 3.51 – 17.0 -	1.3
Pelophylax perezi	Mortality [1] (tadpoles) LC_{50}	$LC_{50}/2$	FETAX	16 h ^{L} : 8 h ^{D} 23ºC 96 h	SW: NaCl: $NaCl_{STE}$:	4.10 – 19.8 3.51 – 17.0 3.51 – 17.0	1.3
	Somatic growth $EC_{50\ growth}$	$LC_{50}/2$	FETAX	16 h ^{L} : 8 h ^{D} 23ºC 96 h	SW: NaCl: $NaCl_{STE}$:	4.10 – 19.8 3.51 – 17.0 3.51 – 17.0	1.3
Lepomis gibbosus	Mortality [2] LC_{50}	$LC_{50}/2$	Deionized Tap water	16 h ^{L} : 8 h ^{D} 20ºC 96 h	SW: NaCl: $NaCl_{STE}$:	12.5 – 16.8 18.7 – 21.8 18.7 – 21.8	1.05 (SW) 1.03 (NaCl, $NaCl_{STE}$)

[1] Gosner, 1960

[2] Rodrigues et al., 2011

3. Data analysis

Computation of conductivities ($mScm^{-1}$, as a measure of salinity) causing X% of mortality (LC_x) was performed with the PriProbit software (Sakuma, 1998). Effective conductivities (EC_x) were estimated by fitting the logistic model to the data, using the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

Data obtained from exposure to NaCl and SW could not be analyzed using ANOVA since the conductivity ranges for NaCl and SW did not match. To test the hypothesis of NaCl as a safe surrogate for SW and to check if tolerance increased after a short-term exposure to low levels of salinity, it was employed a generalized likelihood test, considering statistical differences whenever verified $\chi^2_{(1)} > 3.84$; $p < 0.05$ (Sokal and Rohlf, 1995). All calculations were performed using the software package SPSS Statistics 20.

4. Results

4.1 NaCl *versus* Seawater

At lethal salinity levels, both stages of *P. perezi* (embryos and tadpoles) displayed the highest sensitivity to salinity, either when exposed to SW or to NaCl (when compared to *L. gibbosus*). The 96-hours LC_{50s} (95% CL) for SW and NaCl were 11.5 (10.3-12.8) and 10.6 (7.18-14.9) $mScm^{-1}$ for embryos, respectively, and 8.96 (8.94-8.98) and 13.0 (9.17-13.2) $mScm^{-1}$ for tadpoles, respectively (Fig. 1a, 1b). For embryos, results showed that SW and NaCl started to induce significant effects at very similar levels, with 96-hours LC_{20s} of 6.17 and 7.60 $mScm^{-1}$ (Fig. 1a), respectively; while for tadpoles, significant differences were found between toxicity caused by SW and NaCl (96-hours LC_{20s} of 8.72 and 12.6 $mScm^{-1}$, respectively; $\chi^2_{(1)} = 83.6$; $p<0.05$; Fig. 1b). Also, exposure to lethal levels of NaCl and SW caused reductions of approximately 10% of the total body length of the tadpoles: the computed $EC_{10,96h}$ (95% CL), for NaCl and SW, were very similar: 5.95 (5.65 – 6.25) and 6.78 (5.79 – 7.77) $mScm^{-1}$, respectively ($\chi^2_{(1)}<3.84$; $p>0.05$). This pattern of response remained similar with the increase of the intensity of salinity level, both for eggs and tadpoles.

The juveniles of the fish species, *L. gibbosus*, showed to be more sensitive to lethal levels of NaCl than to lethal levels of SW: $LC_{50,96h}$ (95% CL) were 23.6 (23.1-24.1) and 21.3 (20.8-21.8) $mScm^{-1}$, for SW and NaCl, respectively ($\chi^2_{(1)} = 99.9$; $p<0.05$; Fig. 1c). As for *P. perezi*, the response of the fish remained very similar with increasing level of salinity.

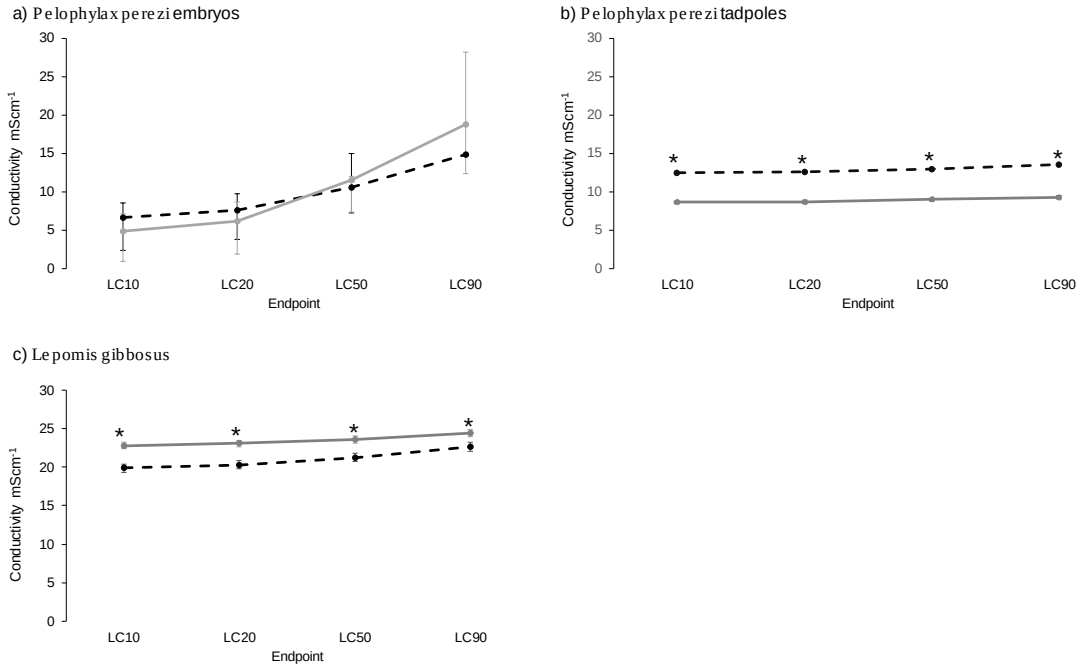

Figure 1. Lethal conductivities ($mScm^{-1}$) causing X% of effect (LC_x) after exposure to natural seawater (SW; light gray lines) and to sodium chloride (NaCl; dashed black lines) for a) *Pelophylax perezi* embryos, b) *Pelophylax perezi* tadpoles and c) *Lepomis gibbosus* juveniles. Vertical bars correspond to confidence limits at 95% (CL at 95%). * represent statistical differences between LC_x computed for NaCl and LC_x computed for SW after analysis with a generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$).

4.2 Short-term exposure to low levels of salinity

Comparing the tolerance of organisms to salinization before and after pre-exposure to low levels of salinity, results showed that *P. perezi* tadpoles increased their tolerance to NaCl: LC_{50s} (95% CL) were 13.0 (9.17-13.2) and of 16.5 (16.3-16.8) $mScm^{-1}$ before and after the pre-exposure, respectively (Fig. 2a). Statistical analysis showed significant differences at all computed lethal conductivities between before and after pre-exposure ($\chi^2_{(1)} \geq 77.1$; $p<0.05$; Fig. 2a). Regarding total body length of tadpoles, it was observed a slight increase on the conductivity causing 10% of effect on growth after pre-exposure but, still with statistical difference: computed $EC_{10,96h}$ (95% CL) were of 5.95 (5.65-6.25) and of 6.13 (5.58-6.67) $mScm^{-1}$ before and after pre-exposure, respectively ($\chi^2_{(1)}<3.84$; $p>0.05$). Pairwise comparison showed significant differences ($p<0.05$) between all tested conductivities before and pre-exposure.

For the fish *L. gibbosus*, the $LC_{50,96h}$ (95% CL) for NaCl computed before and after pre-exposure to low levels of salinity were of 20.4 (19.8-20.8) and of 21.3 (20.8-21.8) $mScm^{-1}$, respectively

(Fig. 2b). No significantly differences (p>0.05) were found in tolerance of *L. gibbosus* to salinization before and after pre-exposure to low levels of NaCl.

For both species, and as observed before pre-exposure to low levels of salinity, the pattern of response to increased levels of salinity remained very similar after pre-exposure to low levels of salinity.

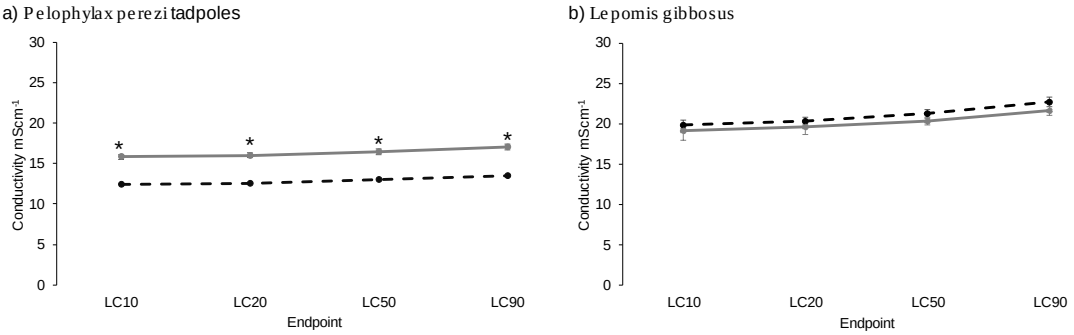

Figure 2. Lethal conductivities ($mScm^{-1}$) causing X% of effect (LC_x) computed for sodium chloride (NaCl) before (dashed black lines) and after pre-exposure to low levels of salinity (light grey lines) for a) *Pelophylax perezi* tadpoles and b) *Lepomis gibbosus* juveniles. Vertical bars correspond to confidence limits at 95% (CL at 95%). * represent statistical differences between LC_x computed before and after short-term exposure to low levels of NaCl, after analysis with a generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$).

5. Discussion

5.1 NaCl *versus* Seawater

In the present work, natural seawater (SW) presented similar or higher toxicity comparatively to NaCl for both tested species. This similar toxicity of NaCl and SW can be attributed to SW composition, which is almost 86% constituted by sodium (Na^+) and chloride (Cl^-) ions. Other ions, in smaller percentages, are also present in SW and they may decrease Na^+ and Cl^- ions toxicity. For instance, in a study performed with a freshwater snail *Amerianna cumingi*, it was found that this species presented lower lethal tolerance when exposed to a single salt ($MgSO_4$) that when they were exposed to a mixture of salts (magnesium and calcium - Mg^{2+}:Ca^{2+}) in a 9:1 proportion (Van Dam et al., 2010).

The fish (*L. gibbosus*) showed to tolerate higher conductivity levels, either caused by NaCl or SW (median mortality value around 20 $mScm^{-1}$). The literature reports also high tolerance values, for other fish species. For instance, almost 60% of the juveniles of the freshwater fish *Colossoma macroponum* were able to survive at a salinity level of 15.0 gL^{-1} (of disinfected seawater; approximately 30.0 $mScm^{-1}$) (Fiúza et al., 2015). Also, the LC_{50} at 96 hours for *Poecilia reticulata* and *Danio rerio* were of 21.7 and 10.4 gL^{-1} of NaCl (approximately 42.9 and 20.6 $mScm^{-1}$; Dolezelova et al., 2009). Regarding specifically the genus *Lepomis*, information available is very scarce, though existing data suggests that individuals form this genus can withstand high salinity levels. For instance, 20 gL^{-1} of NaCl (approximately 39.6 $mScm^{-1}$) were reported for *Lepomis macrochirus* as the concentration provoking 47% of mortality (Waller et al., 1996). In some freshwater cartilaginous fish, such as the elasmobranchs, it is discussed that the present lower ability to accumulate urea is an adaptation to freshwater. This means that the ancestors of these organisms were able to accumulate urea (as an osmoprotectant), to prevent osmotic stress caused by saltwater (Treberg et al., 2006). This ability may not be completely absented from freshwater species (Treberg et al., 2006), presenting an advantage when organisms are exposed to salt stress. Between the two-tested species, the amphibian *P. perezi* showed to be the most sensitive species, in both life stages: eggs and tadpoles. Amphibians are very important in energy transfer from the aquatic to the terrestrial compartment. Although adults can avoid saline environments, they are still dependent on aquatic systems to carry out reproduction. Actually, in the literature even lower salinity values are reported to induce mortality in tadpoles. For instance, Dougherty and Smith (2006) have reported that 0.41 gL^{-1} of NaCl (0.80 $mScm^{-1}$) were sufficient to induce 50% of mortality in *Rana clamitans* tadpoles after 7 days. Sanzo and Hecnar (2006), reported a NaCl $LC_{50,96h}$ of 2.64 gL^{-1} (approximately 5.23 $mScm^{-1}$) for *Rana sylvatica* tadpoles. Low salinity levels may interfere with other life cycle traits. For instance, seawater concentrations of 8 - 9 (15.8 - 17.8 $mScm^{-1}$) were enough to greatly reduce and delay growth in tadpoles of the white-lipped frog *Leptodactylus albilabris*. This delay inhibited organisms to metamorphose (Ríos-López, 2008). Gomez-Mestre and Tejedo (2003) detected morphological abnormalities (such as tail bending or ventral edemas) in *Bufo calamita* tadpoles at concentrations of 8 gL^{-1} of NaCl

(approximately 15.8 $mScm^{-1}$). These abnormalities, in natural conditions, would increase susceptibility to predation, most probably reducing the number of the individuals in the population that could reach adulthood. However, this may be species-specific, as in the present study no abnormalities were detected and delays on growth detected within the range of the tested concentrations did not surpass 20% (considered a threshold for effect). But this reduction on growth may not be persistent if organisms have the opportunity to return to favorable conditions. In a study with tadpoles of the brown tree frog *Litoria ewingii*, it was shown that when exposed at seawater concentrations ranging from 5 to 15 (approximately 8.96 – 24.7 $mScm^{-1}$) tadpoles significantly grew slower than control tadpoles. However, if exposed organisms were returned to freshwater conditions, the differences in size between salt-exposed and control tadpoles disappeared within only 8 days (Squires et al., 2010). Moreover, exposure to saline stress may trigger other kind of responses. For instance, at a threshold salinity value, *Litorea tasmaniensis* and *L. aurea* were able to accelerate metamorphosis (Kearney et al., 2012). However, this kind of investment can rebound in individual body size at metamorphosis and have consequences in other features, such as competition for food, shelter or mates. Furthermore, in the present study, it was found that tadpoles were more sensitive to SW than eggs. This can be because eggs are surrounded by a jelly coat. This structure is highly permeable to hydrophilic substances (like nitrates, sodium or chlorine) but its thickness and constitution provides protection (Salthe, 1963; Seymour, 1995). Also, tadpoles skin is very permeable and highly sensitive to external stressors (Hillyard et al., 2007).

Finally, in the previous chapter (Chapter II), where a battery of standard monospecific bioassays was performed in order to identify the most sensitive freshwater ecological receptors to increased salinity levels, it was observed that the daphnid, the rotifer and the cyanobacterium were the most sensitive taxa, namely conductivities levels ≤ 3.48 $mScm^{-1}$ could induce 50% of effect at sublethal levels and ≤ 4.01 $mScm^{-1}$ could reduce survival of the zooplanktonic species by 50%. Therefore, the setting of safety or protection values based on the results obtained before, could also be protective for the young life forms of the amphibian *P. perezi* here tested, that showed to be the most sensitive to salinity when compared to the fish.

5.2 Short-term exposure to low levels of salinity

In the present study, the ability to cope with low levels of salinity was also analyzed. Freshwater fish are reported to be able to increase their plasma electrolytes in order to increase their internal osmolarity, preventing the entrance of ions from the external media. With *Galaxias maculatus*, Urbina Foneron (2013) verified that exposure to seawater could disrupt the osmoregulatory capacity, through increasing Na^+ and Cl^- concentrations in the plasma and by water loss. Nevertheless, this disruption only persisted for 24 hours, which might be explained by the fact that this is an amphidromous species. But when fish were exposed to an acclimation period of 14 days to salinities ranging from freshwater to 43 (approximately 8.51 $mScm^{-1}$), such steep adaptations were not observed. Rather, acclimation involved minor plasma osmolyte changes, accompanied with molecular changes, such as increased expression of Na^+/K^+ATPase pump units that are correlated with increased osmoregulatory capacity, to restore the membrane ion gradients and cellular homeostasis. According to this and since the literature suggest that members of this genus are able to cope with higher salinity levels (e.g., Waller et al., 1996), it was expected that after short-term exposure to NaCl this fish would increase its tolerance towards salinity. Still, effect concentrations obtained before and after short-term exposure to low levels of salinity were very similar. It is possible that time established for the short-term exposure to low levels of salinity was too long [two months when compared with 14 days in Urbina Foneron study, (2013)], enabling organisms to reach again the homeostatic state. But despite this species did not increase its tolerance towards salinity, the obtained values are still high when compared with the values obtained for daphnids, rotifers and cyanobacterium (previous chapter, Chapter II). It must also be considered that this North American species has already an impact on freshwater ecosystems, namely in Europe where it is considered an invader (Almeida et al., 2014). As an invasive species, it can induce structural and functional alterations, by changing the surrounding environment or changing trophic interactions between native species (e.g., predation over cladocerans and even young life stages of frogs) (Reshetnikov, 2003; Leunda, 2010; Almeida et al., 2014). Allying this behavior with its high tolerance to salinity, it should be considered its high potential for thrive in

freshwater systems affected by saltwater intrusion or even its potential to spread from freshwater ecosystems to estuarine zones.

On the present study, tadpoles of the green frog *P. perezi* increased their tolerance after a short-term exposure to low levels of salinity. Still, if steep increments of conductivity occur in natural freshwater ecosystems or if exposure occurs for a long period of time, this increase may not be enough to assure their survival. Gomez-Mestre and Tejedo (2003) comparing one freshwater population of *B. calamita* with another one from a brackish environment, verified that, although the brackish population presented an initial higher tolerance towards salinity, this advantage disappeared over time. Nevertheless, it is important to remark that the organisms were still able to cope with salinity for the same time, which is inline with Kearney et al. (2012) results. Kearney et al. (2012) showed that *Litorea aurea* tadpoles had the highest survival rates at salinity levels above 4 (approximately 7.92 $mScm^{-1}$) when compared to freshwater. With *Fejervarya limnocharis* tadpoles, it was demonstrated that the direct transfer to a saline solution (11 ppt; 21.8 $mScm^{-1}$) induced death within 12 hours, whereas if tadpoles were allowed to acclimate previously to a 7 ppt solution (13.8 $mScm^{-1}$), they could survive at least 48 hours (Wu et al., 2014). Salt stress may cause immediate water loss and interfere with respiratory functions. Regarding gills, major alterations can be detected, such as morphological (mucous secretion or alterations of the surface epithelium) (Bernabò et al., 2013) or molecular (increased enzymatic activity for instance of branchial Na^+/K^+-ATPase) ones (Bernabò et al., 2013; Wu et al., 2014).

6. Conclusions

The present study has shown that the possible use of NaCl as surrogate of SW must be considered with caution since it may underestimate risk for some biota, since, for tadpoles of *P. perezi* natural SW caused higher toxicity than its surrogate.

Both life stages of the amphibian species here studied have shown to be more sensitive than the fish. For instance, fish were able to cope with high salinity levels (> 20 $mScm^{-1}$), while these levels showed to be already detrimental for both amphibian's life stages. Salinity levels of ≥ 20

$mScm^{-1}$ may actually cause high mortality percentages for amphibian's eggs and tadpoles, which suggests that they might be highly vulnerable to present and future sea level rise, when compared to other freshwater vertebrate species.

Amphibians presented increase tolerance to salinity after short-term exposure to low levels of salinization, which may suggest that this species might be able to cope with slight or gradual increases in salinity under natural conditions. In the fish, despite acclimation was not observed, they could still tolerate high salinity levels (almost half of natural seawater conductivity). Being this species a non-native in Europe, its high tolerance to salinization may confer it a further competitive advantage over native species under salinization scenarios of coastal freshwater ecosystems, potentiating its invasive capacity, replacing native species.

7. Acknowledgments

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Chapter IV

Multigenerational effects of salinity in six clonal lineages of *Daphnia longispina*

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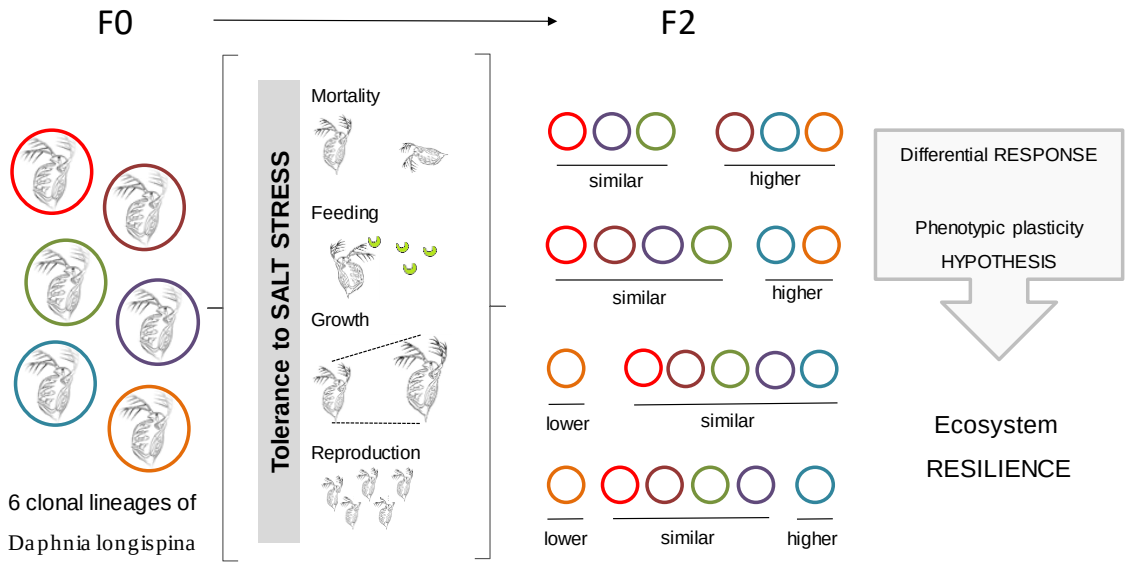
Multigenerational effects of salinity in six clonal lineages of *Daphnia longispina*

Venâncio C, Ribeiro R, Soares AMVM, Lopes I

Graphical abstract



Abstract

Sea level rise, as a consequence of climate changes, is already causing seawater intrusion in some freshwater coastal ecosystems worldwide. The increase in salinity at these freshwater coastal ecosystems may occur gradually (through groundwater) or abruptly (through extreme weather events). Moreover, many of them are also being altered and threatened by anthropogenic activities. Accordingly, the present study aimed at assessing the multigenerational lethal and sublethal effects caused by increased salinity in six clonal lineages of the freshwater cladoceran *Daphnia longispina* differing in their sensitivity to lethal levels of copper. Three specific objectives were delineated: i) to compare the lethal and sublethal toxicity of sodium chloride (NaCl) and natural seawater (SW); ii) to evaluate possible multigenerational effects after exposure to low levels of salinity, and iii) to evaluate if an association exists between tolerance to lethal and sublethal levels of salinity and tolerance to metals. Overall, NaCl was found to elicit sublethal

effects at lower or similar concentrations than SW, suggesting its use as a protective surrogate of SW in early phases of ecological risk assessment schemes. Multigenerational exposure to conductivities between 0.73 ± 0.015 mScm^{-1} led to dissimilar responses by the six clonal lineages. Significant associations were found neither between lethal and sublethal endpoints nor between salinity and metals, possibly indicating the absence of common mechanisms responsible to confer metal tolerance and salt stress. However, some clonal lineages presented an inverse sensitivity to lethal levels of NaCl and of copper. These results suggest that natural populations of *D. longispina*, by exhibiting clonal lineages with differential tolerance to increased salinity, may cope with long-term exposure to small increases of this stressor. However, over time those populations may face the occurrence of genetic erosion due to the loss of the most sensitive genotypes before or after a multigenerational exposure.

Keywords: zooplankton; salinization; long-term exposure; life history; ecotoxicology

1. Introduction

Considering the projections of the Intergovernmental Panel on Climate Change (IPCC, 2014) for the coming years, low-lying coastal areas constitute quite vulnerable ecosystems. Within this scenario, sea level rise allied with extreme weather events are considered as main drivers for coastal freshwater ecosystems salinization. Besides social and economic losses all around the globe, salinization of coastal freshwater ecosystems entails several environmental implications and the loss of several services provided by them. Increased salinity will certainly affect biota inhabiting these freshwater ecosystems. Within the most sensitive groups towards increased salinity levels are the freshwater zooplanktonic species, which is possibly related with their poor osmoregulatory capacity (Aladin and Potts, 1995). This particular group constitutes a key intermediate in energy transfer of trophic chains in freshwater systems, which increases the need to understand their responses when submitted to salt stress (e.g., D'Alelio et al., 2016). For instance, conductivities of just 3.6 mScm^{-1} can reduce the survival of the freshwater cladoceran *Daphnia pulex* by 50% (Gardner and Royer, 2010). Several other works provided similar lines of

evidence; Sarma et al. (2006) referred that salinities above 2 (approximately 3.45 $mScm^{-1}$) may severely affect natural populations, and other authors reported that salinity values ten times lower than the salinity value of natural seawater may be enough to interfere with normal reproduction rates and lead to severe decline of natural populations of cladocerans (e.g., Grzesiuk and Mikulski, 2006; Gonçalves et al., 2007). Nonetheless, these organisms could be able to cope with higher levels of salinity; if in contact with this stressor for a sufficient period of time, they may be able to develop strategies (e.g., activating detoxification metabolic pathways) to increase or maintain their fitness under salinization (e.g., acclimation). This acclimation to low-levels of stress has already been documented for microalgae (Venâncio et al., 2017), cladocerans (Lopes et al., 2005) and fish (Adeyemi and Klerks, 2013; Cheng et al., 2013) and may be related with several tolerance mechanisms, such as production and storage of lipids or fats for osmotic protection or even changes at the molecular composition of cellular membranes (e.g., Singh et al., 2002; Wu et al., 2014). For instance, Chen and Stillman (2012) successfully acclimated *Daphnia pulex* to salinity (up to 5; approximately 9.0 $mScm^{-1}$) and temperature (15-30ºC) different from optimal. If acclimation of organisms to increased salinity levels occurs then it may lead to risk overestimation, which is not envisaged by traditional approaches (i.e., risk calculation based only on these standard methodologies would be greater than that possibly observed in a real case scenario, as organisms would be able to cope with higher levels of the stressor); yet, most of the studies do not consider that organisms may be exposed to salinity stress for several generations. Furthermore, alongside with toxic effects that may be exerted immediately at the individual level, (like examples above mentioned), responses to stress may also be transferred vertically, i.e., to organisms of later generations (e.g., Crean et al., 2013), which increases the need not only to assess the effects at the individual level but to integrate that information with that obtained from subsequent generations. This multigenerational transfer of new phenotypic characteristics without modification of gene sequence may occur through epigenetic changes (Berger et al., 2009), which were already reported for weed plants (*Arabidopsis* sp.) exposed to salt stress (Sani et al., 2013; Kim et al., 2015). More, the acquisition of tolerance (e.g., to salt) may occur within a very tight window of time, which may constitute an advantage for organisms when living in ecosystems

under rapid change and this ability is of great importance when considering organisms with a rapid life cycle as daphnids. For instance, Coldsnow et al. (2017) have found that the acquisition of tolerance towards salinity by *D. pulex* was achieved within a period of 5 to 10 generations of exposure to salinity levels between 15 and 1000 mgL^{-1} of Cl^- (corresponding approximately to 0.05 to 3.45 $mScm^{-1}$). Furthermore, Coldsnow et al. (2017) suggested that a pre-exposure to different salinity levels induced different salinity tolerance levels, leading, latter, to the appearance of two distinct populational groups within the community: populations tolerating moderate salinity levels and populations tolerating high salinity levels. This development of differential tolerance may originate from the activation of different physiologic mechanisms that later may translated in differences at the genetic level (e.g., Latta et al., 2012).

Another factor to take into consideration when trying to understand the effects that salinization may pose to ecosystems facing seawater intrusion is that many of them are already suffering from other sources of contamination due to human activities (e.g., release of cobalt and chromium from hard metal industries or copper and zinc commonly found as components of herbicides and fertilizers used in agricultural practices). Depending on the extent and intensity of the contamination, some populations inhabiting such already chemically-impacted ecosystems may have undergone genetic erosion, through for example the elimination of the most sensitive genotypes to that particular contamination (Lopes et al. 2005; Ribeiro and Lopes, 2013), which will influence its capacity to respond to future stress. If co-tolerance exists with the new stressor then the population will be able to persist in that ecosystem. In cladocerans such co-tolerance could be observed since there are evidences that respiratory organs, where ionic regulation occurs (such as regulation of sodium ions) also controls for some metals uptake (e.g., Henry et al., 2012). However, if an inversion in tolerance exist between the former and the latter contaminants, then the risk of extinction of the population will increase (since the most tolerant genotypes to the first contaminant are the most sensitive to the later one; Schat and Vooijs, 1997; Lopes et al., 2005; Knauer et al., 2010; Venâncio et al., 2016). For example, Leitão et al (2013) found a significant negative correlation between the lethal tolerance to copper and to sodium chloride in five clonal lineages of *D. longispina*, thus suggesting that populations of *D. longispina* that were genetically

eroded due to exposure to copper or increased salinity may be more susceptible to an ulterior exposure to the other contaminant.

Within the above context, this study intended to investigate multigenerational effects of salinity in the life-history of six clonal lineages of the freshwater cladoceran *Daphnia longispina*. To attain this major goal, the following specific objectives were addressed: a) to determine if sodium chloride can be used as a proxy of natural seawater (SW) when assessing the effects of increased salinity in freshwater ecosystems. For similar conductivities, a slightly lower toxicity of SW was expected, since NaCl contribute by only 86% to the total salinity of SW, although other SW salts may cause additive affects. If confirmed, then the former can be used as an easy-to-use surrogate of the latter in early stages of ecological risk assessment schemes of increased salinity in freshwater ecosystems; b) to investigate possible multigenerational effects of an exposure to low levels of salinity (a NaCl solution with a conductivity between 0.73 ± 0.015 $mScm^{-1}$) on the six clonal lineages, looking at three generations: F0 (neonates from non-exposed mothers), F1 (neonates from salinization-exposed mothers) and F2 (neonates from salinization-exposed mothers and grandmothers). Differential tolerance among generations would indicate multigenerational effects, either positive or negative; c) to assess if, among clonal lineages, there is an association between tolerance to lethal and sublethal levels of salinity (NaCl or SW) and of metals (copper, cobalt, chromium, and zinc; Venâncio et al., 2016). Although not a principal objective, the possible association between responses to increased salinity and other chemicals (in this case metals) was studied since it is expected that many coastal lagoons predicted to suffer future salinization are already impacted with metal contamination.

2. Materials and Methods

2.1 Test solutions

Natural seawater (SW) was collected in the North Atlantic Ocean, at a sampling site located in front of a natural reserve (São Jacinto dunes, created in 1979; 40°38'33.94"N, 8°44'55.91"W; Aveiro, Portugal) that holds, for more than 25 years, the eco-label of blue flag conferred by the

Foundation for Environmental Education (a non-governmental and non-profiting foundation acknowledge by UNESCO to have expertise in Environmental Education and Sustainable Development). The site of collection is routinely monitored with a classification of good to excellent in water quality, according to the Directive 2006/7/CE of the European Union, (please see Venâncio et al., 2017).

In the laboratory, SW was filtered through cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain), prior to its use in toxicity assays, to remove particles in suspension and possible organisms. All tested dilutions were made by diluting directly SW with ASTM hardwater medium (American Society for Testing and Materials; ASTM, 2002; conductivity around 490 – 511 μScm^{-1}) (Supplementary Table S1).

Sodium chloride (NaCl; supplied by Merck, St Louis, MO, USA) was tested as a possible surrogate of SW when conducting ecological risk assessments of SW intrusions. A stock solution of 50 gL^{-1} of NaCl (with a final conductivity of approximately 82.9 $mScm^{-1}$) was prepared by the addition and direct dissolution of the salt in the culture media of the daphnids (ASTM hardwater). This solution was made fresh for each assay. All posterior solutions (for test purposes) were made through dilution of this stock solution with ASTM.

2.2 Test organisms

Six clonal lineages of *Daphnia longispina* O.F. Müller, known to exhibit differential genetically determined tolerance to lethal levels of acid mine drainage and copper (Lopes et al., 2004; Saro et al., 2012), were selected to perform this study. *Daphnia* is a genus commonly used in ecotoxicological studies because it reproduces by cyclic parthenogenesis, allowing the maintenance of exactly the same genotype for several generations under laboratorial conditions. Also, these clones of *D. longispina* have long been studied, with their allozyme and microsatellite genotypes previously characterized by Martins et al. (2007) and Silva et al. (2010), respectively. Cultures of each clonal lineage were maintained in laboratory (for more than 500 generations) under controlled conditions of temperature (19 to 21ºC) and photoperiod (14:10 hours L:D) in ASTM hardwater (conductivity values between 564 – 571 μScm^{-1}), with the addition of vitamins

and the organic additive Marinure 25 (an extract from the algae *Ascophyllum nodosum*; Pann Britannica Industries Ltd., Waltham Abbey, UK) (Baird et al., 1989). Changes in the sensibility of the tested clonal lineages was periodically checked during the long-term culture by performing toxicity assays with copper. Medium was changed every other day and organisms were fed daily with the green algae *Raphidocellis subcapitata* (Korshikov) F. Hindák (formerly known as *Selenastrum capricornutum*) (1.5×10^5 $cells^{-1}mL^{-1}day^{-1}$). All clonal lineages were maintained by asexual reproduction, being neonates from third, fourth or fifth broods selected to maintain laboratory cultures and to carry out the toxicity assays.

2.3 Toxicity assays

Lethal and sublethal toxicity assays were carried out by exposing neonates (from the 3rd or 4th brood) to a range of NaCl concentrations and SW dilutions. The neonates used at this stage were born from laboratorial cultures of *D. longispina* (considered as the generation F0). These experiments intended to address the first specific objective, i.e. establish if NaCl could be used as a surrogate of SW when assessing the risk of increased salinity to freshwater ecosystems. Since, the results obtained from assays performed with F0 revealed NaCl to be a good surrogate of SW for ecological risk this salt. Therefore, to determine the effects of increased salinity after multigenerational exposure, neonates (from the 3rd and 4th brood) from the F0, of each clonal lineage, were exposed for two generations (F1 and F2) to ASTM medium spiked with NaCl (with a conductivity of 0.73 ± 0.015 $mScm^{-1}$) and maintained in the same conditions as the original cultures (please see Test Organisms, section 2.2 of Materials and Methods). The sensitivity of F1 and F2 to increased salinity was then assessed by exposing the respective neonates (from 3rd and 4th brood) to a range of lethal and sublethal concentrations of NaCl and then compared with the results obtained for F0 generation.

The concentration of NaCl selected for multigenerational exposure (with a final conductivity between 0.73 ± 0.015 $mScm^{-1}$) corresponded to the highest concentration of NaCl that allowed survival and clonal lineages.

2.4 Lethal toxicity assays

All lethal assays were performed according to the OECD guideline 202 (OECD, 2004). Definitive ranges of conductivity (as a measure of salinity) were set for each clonal lineage and for SW and NaCl after performing range-finding assays. For the three-generation tested (F0, F1 and F2), each clonal lineage was exposed to a control (ASTM hardwater) and to at least five concentrations/dilutions of NaCl and SW (for further details please see Supplementary Table S1). Assays were carried out for 48 hours at 19 to 21ºC and a photoperiod of 16:8 hours L:D, with neither food addition nor medium renewal. Five neonates (6 to 24-hours old) were introduced in 50-ml glass vessels containing 30 ml of test solution, four replicates were performed per concentration and control. Mortality at 24 and 48 hours was monitored. An organism was considered dead if remained immobile during 15 seconds after gentle prodding. Results obtained from these assays were used to identify sublethal concentrations ranges to perform sublethal assays (feeding and growth responses). During the assays, the following parameters were measured: salinity (Wissenschaftlich Technische Werkstätten-WTW conductivity440i, Weilheim, Germany), conductivity (WTW conductivity 440i), pH (WTW pH 330i) and dissolved oxygen (WTW OXI 330i) (Supplementary Table S2).

2.5 Sub-lethal assays

2.5.1 Feeding inhibition assays

Feeding inhibition (FI) was assessed by exposing the organisms to sublethal concentrations/dilutions of NaCl (for each clonal lineage and generation – F0, F1 and F2) and to SW (only for F0). Newborns (from 3rd or 4th broods) were collected and maintained in the same medium as their mothers and left to growth for 4 days, prior to the assays. Groups of 5 neonates were randomly introduced in 50-ml glass vessels filled with 20 ml of test solution. At least five concentrations/dilutions (Supplementary Table S1) plus a control were tested, with four replicates each. Organisms were allowed to feed for 24 hours on the green algae *R. subcapitata* that was added in a concentration of 1.5 x 10^5 cells^{-1}mL^{-1}day^{-1} to each test vessel. Experiments were

conducted at 19 to 21º C and in total darkness to avoid algal growth. For each test, four blanks were used to guarantee that initial algal concentrations did not increase significantly during the exposure period. After removing the organisms, final cell density (*Conc*, cellsmL^{-1}) was estimated by measuring absorbance (*ABS*) at 440 nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, VT, USA). Conversion was made recurring to a previously established calibration curve for this specific green alga, as follows in Equation 1 (regression coefficient):

Conc = -17,107.5 + (*ABS* x 7,925.350), (R^2=0.99) Equation 1

The conversion to feeding rates (*FR*, cellshour^{-1}) was made considering $\ln_f$ the final number of algae cells, $\ln_i$ the initial number of algae cells and t_f - t_i (hours) the time interval (Allen et al., 1995; Equation 2). Parameters were monitored as described in section 2.3.1.

$$FR = \frac{\ln_f - \ln_i}{t_f - t_i}$$ Equation 2

2.5.2 Juvenile growth rate

A 3-day somatic growth inhibition assay was carried out using sublethal concentrations/dilutions of NaCl (for each cloned lineage and generation – F0, F1 and F2) and SW (F0 generation). Newborns (from 3rd or 4th broods), with 6 to 24 hours, were exposed individually in 50-ml glass vessels filled with 20 ml of test solution. At least five concentrations/dilutions (Supplementary Table S1) were tested plus a control, each with ten replicates. Medium was supplied with algae (*R. subcapitata* at a concentration of 1.5 x 10^5 cellsmL^{-1}day^{-1}) and the above-mentioned organic extract. After 48 hours of exposure the medium was renewed. Organisms were measured at 0 and 72 hours and values converted to daily growth (*JGR*, mmday^{-1}) considering l_f the length of organisms at the end of the assay (mm), l_i the initial length of organisms (mm) and t_f - t_i (days) the time interval (Burns, 2000; Equation 3). Parameters were monitored as described in section 2.3.1.

$$JGR = \frac{l_f - l_i}{t_f - t_i}$$ Equation 3

2.5.3 Reproduction assays

Concerning the toxicity assessment of NaCl on reproductive parameters, the standard protocol OECD 211 (OECD, 1998) was followed. Reproduction was assessed only for NaCl and in all three generations. Ten neonates (6 to 24 hours old), from the 3rd or 4th brood were exposed individually, at 19 to 21ºC and a 16:8 hours L:D photoperiod, to NaCl and control composed solely of ASTM hardwater (Supplementary Table S1). Fifty-mL glass vessels were used, containing 20 mL of the test solution with the addition of Marinure 25 and of the green algae *R. subcapitata* (1.5×10^5 $cells^{-1}mL^{-1}day^{-1}$). Organisms were fed every day and medium was renewed every other day. Assays ended when all ten individuals from control vessels released the third brood (from 21 to 23 days). The following endpoints were assessed: time to release the first brood, total number of neonates released per female, body size and intrinsic population growth rate (r, day^{-1}). The latter is a demographic parameter that integrates age (x), probability of survival (l_x) and fecundity of the females until age x (m_x). Calculations were based in Euler-Lotka equation and standard errors of r were estimated by jackknifing (Meyer et al., 1986: Equation 4).

$$1 = \sum_{x=0}^{n} e^{-rx} \times l_x \times m_x \qquad \text{Equation 4}$$

Additionally, all females were measured at the end of each test and their growth compared to their initial length. The total growth (TG, $mmday^{-1}$) calculation was made considering l_f the final length of organisms (mm), l_i the initial length (mm) and t_f -t_i (days) the time interval (Burns, 2000; Equation 5). Parameters were monitored as described in section 2.3.1.

$$TG = \frac{l_f - l_i}{t_f - t_i} \qquad \text{Equation 5}$$

3. Data analysis

The estimation of conductivities provoking 50% of mortality (LC_{50}), for each clonal lineage, were performed using the software Priprobit (Sakuma, 1998). Computation of sub-lethal parameters (effective conductivity, EC_x) were made by fitting the logistic model to the data with the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA) and Pearson correlations (R) analysis were computed through the software Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

In order to assess if NaCl was significantly different from SW, the two data sets for each clonal lineage were compared through a generalized likelihood test, because the ranges of concentrations used for each tested substance were not the same, and therefore a statistical analysis using ANOVA was not possible. Data set from NaCl was considered different from that of SW whenever $\chi^2_{(1)} \geq 3.84$; $p<0.05$.

Regarding multigenerational exposure data, analysis of data of lethal toxicity, feeding growth assessments, total reproduction and intrinsic population growth (*r*) was done using a generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$).

The data sets were tested for normality (Kolmogorov-Smirnov test) and for homocedasticity (Levene test).

4. Results

4.1 Toxicity of NaCl and SW

In general, NaCl provoked significant adverse effects at concentrations similar or lower than those of SW for all *D. longispina* clonal lineages and assessed endpoints (Fig. 1). The lethal sensitivity of clonal lineages to SW was similar to that to NaCl, except for E89 and N91, which showed to be significantly more tolerant to SW than to NaCl ($\chi^2_{(1)}=14.3$, $p<0.05$ and $\chi^2_{(1)}=10.6$; $p<0.05$, respectively; Fig. 1a). Equally, at sublethal levels of salinity, most clonal lineages exhibited similar sensitivity to NaCl and SW. Exceptions were observed for clonal lineage N35 (for juvenile growth rate and feeding inhibition) and N91 (for feeding inhibition), which exhibited a higher sensitivity to NaCl relatively to SW ($\chi^2_{(1)}=87.3$, $\chi^2_{(1)}=13.8$, and $\chi^2_{(1)}=36.0$, respectively, all with $p<0.05$) (Fig. 1b and 1c).

No significant correlations between tolerance to NaCl and SW were found for mortality (R=0.62, p=0.19), somatic growth (R=0.32, p=0.53) or feeding inhibition (R=0.74, p=0.09) (Supplementary Fig. S1). However, some clonal lineages that were tolerant to lethal levels of NaCl were also tolerant to SW (e.g., N35; Supplementary Fig. S1a) and the most sensitive clonal

lineages to NaCl were also the most sensitive to SW in what concerns feeding inhibition responses (e.g., N91 and E99; Supplementary Fig.S1c).

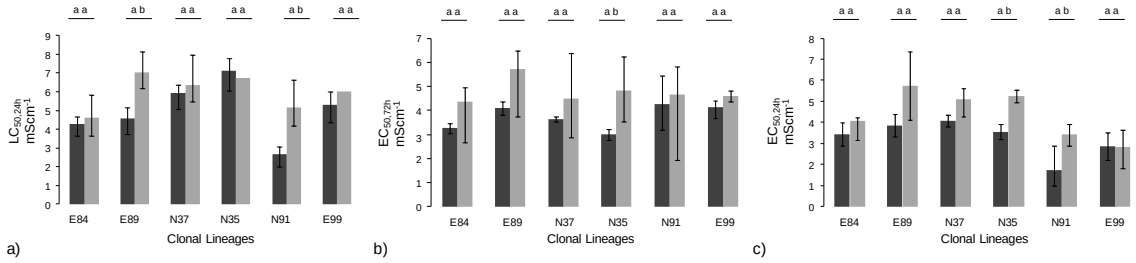


Figure 1: Toxicity endpoints, with the respective 99% confidence limits (error bars), of sodium chloride (dark bars) and natural seawater (light bars), for six clonal lineages of *Daphnia longispina*. a) Median lethal conductivities (LC_{50}), after a 24-h exposure. b) Median effective conductivities (EC_{50}) for juvenile growth rate, after a 72-h exposure. c) Median effect conductivities (EC_{50}) for feeding rate, after a 24-h exposure. Different letters stand for significant differences between NaCl and SW (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$). Note: in figure a) no 99% confidence limits were possible to compute for N35 and E99.

4.2 Multigenerational Effects

4.2.1 Lethal Assays

Two patterns of multigenerational lethal effects of NaCl in the six clonal lineages of *D. longispina* were observed: (i) no changes in sensitivity to lethal levels of NaCl, suggested by similar values of $LC_{50,24h}$ among the three generations (E84, E89, N37, N35 and E99; $\chi^2_{(1)}<3.84$, $p>0.05$; Fig. 2), and (ii) increased tolerance to NaCl, indicated by an increase in $LC_{50,24h}$ from F0 and F1 and a maintenance from F1 to F2 (N91; $\chi^2_{(1)}>26.6$, $p<0.05$; Fig. 2).

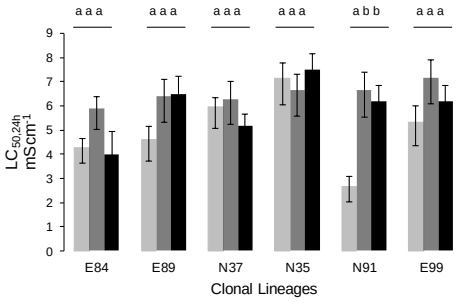


Figure 2 - Median lethal conductivities (LC_{50}), with the respective 99% confidence limits (error bars), after a 24-h exposure to sodium chloride for the three studied generations (F0, F1 and F2: from light grey to black) of six clonal lineages of *Daphnia longispina*. Different letters (a or b) stand for significant differences between generations, within each clonal lineage, (generalized likelihood test: $\chi^2_{(1)} \geq 3.84$; $p<0.05$).

4.2.2 Sub-Lethal Assays

Results obtained after multigenerational exposure; of the six clonal lineages of *D. longispina*, to sublethal concentrations of NaCl showed some differences from those observed at lethal levels (Fig. 3). For the juvenile somatic growth rate (JGR), a similar sensitivity to NaCl was observed throughout the three generations (F0 to F2) in all clonal lineages except E99, which showed a decrease in tolerance to NaCl after multigenerational exposure to this salt (generation F0 significantly different from F1 - $\chi^2_{(1)}$=205.2, p<0.05 – and F0 significantly different from F2 - $\chi^2_{(1)}$=105.3, p<0.05; Fig. 3a).

Regarding feeding inhibition, two patterns of response were registered: (i) a similar sensitivity among the three generations (F0 to F2; $\chi^2_{(1)}$<3.84, p>0.05) (E84, E89, N37 and N35, though for the later clonal lineage F1 was significant different from F2, but both were equal to the F0, thus indicating no change in sensitivity) and ii) an increase in tolerance to salinity from F0 to F1, but with no changes in sensitivity to NaCl from F1 to F2 (N91 and E99; $\chi^2_{(1)}$=33.3, p<0.05 and $\chi^2_{(1)}$=4.96, p<0.05, respectively) (Fig. 3).

For total reproduction, three different patterns of response were observed: (i) similar sensitivity in all tested generations (E84, N37 and N35; $\chi^2_{(1)}$<3.84, p>0.05); (ii) increased tolerance in F1 and F2 relatively to F0 (E89 and N91; $\chi^2_{(1)}$>9.5 , p<0.05 and $\chi^2_{(1)}$>23.1, p>0.05, respectively) and (iii) similar tolerance in F0 and F1, with a decrease in F2 (E99; $\chi^2_{(1)}$>9.83 , p<0.05) (Fig. 3c).

For *r*, several patterns of response were also found: (i) similar sensitivity in all tested generations (E84, E89); (ii) similar tolerance in F0 and F1, with a decrease in F2 relatively to F0 (N37; $\chi^2_{(1)}$=9.18 , p<0.05); (iii) decreased tolerance from F0 to F1 and to F2 but only statistically different F0 from F2 (N35; $\chi^2_{(1)}$=8.27 , p<0.05); iv) increased tolerance from F0 to F1 maintaining the increased tolerance to F2 (N91; $\chi^2_{(1)}$>27.1 , p<0.05), and iv) sensitivity to NaCl significantly different in F0, F1 and F2, with a sharp decrease in F2 (E99; EC_{20} < 0.53 $mScm^{-1}$; $\chi^2_{(1)}$>4.61, p<0.05) (Fig. 3d).

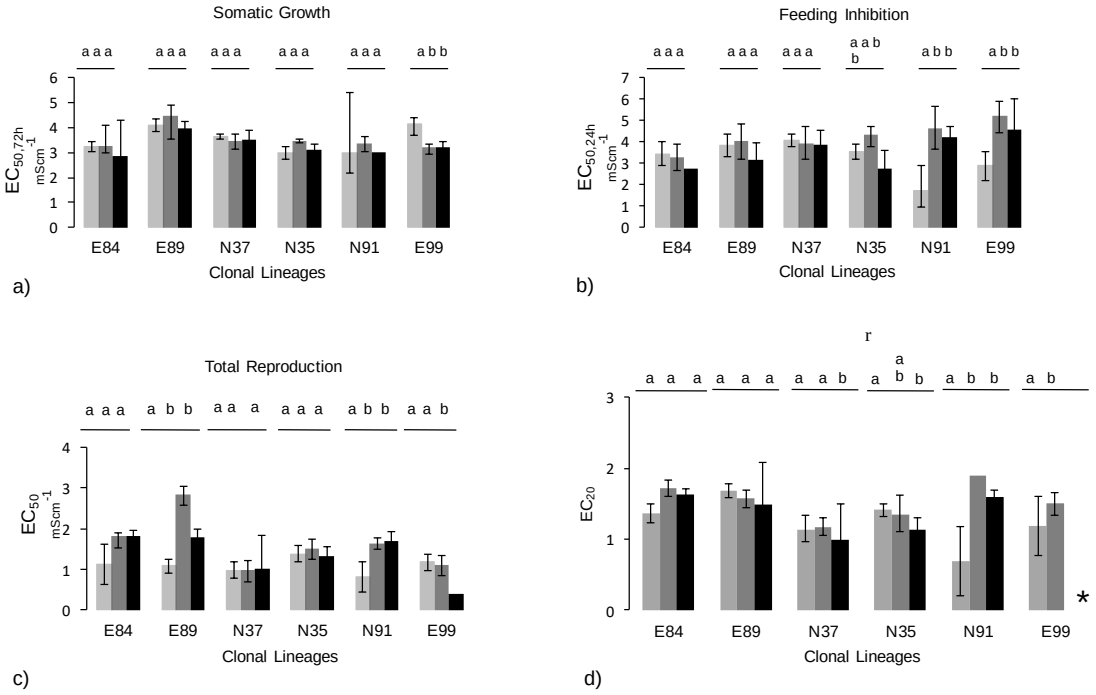


Figure 3 – Toxicity endpoints, with the respective 99% confidence limits (error bars), of sodium chloride for three generations (F0, F1 and F2: from light grey to black) of the six clonal lineages of *Daphnia longispina*. a) Median effective conductivities (EC_{50}) for somatic growth, after a 72-h exposure. b) Median effective conductivities (EC_{50}) for feeding rate, after a 24-h exposure. c) Median effective conductivities (EC_{50}) for reproduction, after an exposure until controls released their third brood. d) Conductivities causing a 20% reduction in population growth rate (r) (EC_{20}), after an exposure until controls released their third brood. *no *r* value could be calculated, since this value was below the range of tested concentrations. Generations, within each clonal lineage, sharing the same letter (a or b) represent homogenous groups after comparison with generalized likelihood test ($\chi^2_{(1)} \geq 3.84$; $p<0.05$).

4.3 Association between tolerance to metals and to NaCl

Considering the six clonal lineages of *D. longispina*, neither significant associations among NaCl lethal and sublethal toxicity endpoints, nor with these endpoints and median lethal concentrations of copper, cobalt, zinc, and chromium were found ($p>0.05$) (Supplementary Table S3). Nevertheless, some specific cases of inverse tolerance between lethal tolerance to NaCl and lethal tolerance to metals were found: N37, one of the most sensitive lineages to lethal levels of copper and zinc, was among the most tolerant to lethal levels of NaCl, and E99, the most sensitive lineage to lethal levels of cobalt and one of the most sensitive to lethal levels of copper, was among the most tolerant to NaCl (Supplementary Fig. S3). Between sublethal levels of NaCl (feeding

inhibition) and lethal levels of metals some inversions were also observed: lineages sensitive to copper (e.g., E89 and N37) or to chromium (e.g., E89) were the most tolerant to sublethal levels of NaCl (Supplementary Fig. S3).

Also, as for NaCl, associations neither between SW lethal and sublethal toxicity endpoints, nor between these endpoints and median lethal concentrations of copper, cobalt, zinc, and chromium were found ($p>0.05$) (Supplementary Table S4). Still, some inversions in tolerance were found. At lethal levels of SW and metals, E89 and N37 showed to be very tolerant to salinity while sensitive to copper and zinc (Supplementary Fig. S1). Regarding sublethal (feeding inhibition) levels of SW, E89 and N37 showed to be very tolerant and sensitive to copper and zinc (Supplementary Fig. S4). Still in SW, E89 showed to be very tolerant to SW (in somatic growth assessment) while being sensitive to copper and zinc (Supplementary Fig. S4).

Regarding total reproduction, no correlations were found between this endpoint and lethal levels of the four metals (Supplementary Fig. S5).

5. Discussion

5.1 Toxicity of NaCl and SW

The choice of NaCl as a possible surrogate of SW is related with the fact that the latter one is composed mainly by chloride (Cl^-) and sodium (Na^+) ions. However, the salinity of SW is also influenced by several other ions, which may also be beneficial for organisms and possibly contribute to lower its toxicity when compared to that of NaCl. For example, the supply of small dosages of other ions, like calcium, are important for molting (e.g., Smirnov, 2013) and may have a protective role against other stress sources as other cations (like magnesium) (e.g., Ha et al., 2016). In the present work, the results of exposure to lethal and sublethal salinity levels indicated that, in general, the toxicity of NaCl was equal or higher than that of SW, which is inline with results obtained by previous studies. For instance, Ghazy et al. (2009) reported for *D. magna* $LC_{50,48h}$ values of 4.82 and 2.99 gL^{-1}, for SW and NaCl respectively. The same trend was reported by Kefford et al. (2004) when comparing NaCl to an artificial ocean salt in freshwater organisms.

Namely for the macroinvertebrate *Micronecta annae* reported $LC_{50,72h}$ values were of 13 and 11 $mScm^{-1}$ for artificial salt ocean and NaCl, respectively; for the cladoceran *D. carinata* reported $LC_{50,48h}$ values were of 11 and 4.5 $mScm^{-1}$ for artificial salt ocean and NaCl, respectively and for decapod *Caridina nilotica* reported $LC_{50,72h}$ values were of 36 and 18 $mScm^{-1}$ for artificial salt ocean and NaCl, respectively (Kefford et al., 2004). And also, Venâncio et al. (2017) verified that natural SW was less toxic to two species of freshwater green algae (the $EC_{25,72h}$ values for *R. subcapitata* were of 10.3 and 4.6 $mScm^{-1}$ for SW and NaCl, respectively and the $EC_{25,72h}$ values for *Chlorella vulgaris* were of 15.4 and 6.9 $mScm^{-1}$ for SW and NaCl, respectively). These results suggest that NaCl can be used as an easy and safe surrogate of SW in early stages of ecological risk assessment schemes (ERA) of saline intrusion in coastal freshwater ecosystems, since it will constitute a protective approach. In addition, since many toxicity data has been generated for this salt, within the context of its use as a road deicer (e.g., Mahrosh et al., 2014; Jones et al., 2015), those available datasets may be used as preliminary assessments of salinization risk assessment avoiding the performance of further unnecessary assays. Furthermore, although a correlation was not observed between the sensitivities of clonal lineages to NaCl and SW, overall the lineages most sensitive to NaCl were also the most sensitive to SW, with a few exceptions (e.g., regarding feeding inhibition, N35 was the most sensitive clonal lineage to NaCl and one of the most tolerant to SW).

Still, either for NaCl or SW, effective concentrations obtained within this study did not surpass 9 $mScm^{-1}$ (about 17% of SW conductivity). Considering the extreme weather event scenarios foreseen by the IPCC (2014), these values can be easily achieved and/or surpassed in coastal freshwater ecosystems. Therefore, results here obtained suggest that natural populations of cladocerans present at low-lying coastal freshwater ecosystems can be severely affected. In fact, this particular group has already been reported as the least tolerant group towards salinity when compared to other invertebrates, such as rotifers and copepods (e.g., Schallenberg et al., 2001). This is certainly related with the maximum optimal salt concentration reported for freshwater cladocerans. This threshold value is defined as nearly 2 (roughly 0.05 $mScm^{-1}$) (Jeppesen et al., 1994), so the increment of ions in the external medium beyond this value may result in a

deregulation of the homeostatic and/or osmoregulatory states. For instance, salinity values above 5 (corresponds approximately to 8.0 $mScm^{-1}$ at 20 ºC) are known to reduced metabolic rates in *D. magna* (Arner and Koivisto, 1993; Schuytema et al., 1997) but lower salinity values (concentrations equal or above 3.0 and 4.0 gL^{-1} of NaCl, corresponding approximately 5.0 and 6.5 $mScm^{-1}$, respectively) have already been reported to induce smaller sizes at first reproduction, delayed maturity and, consequently, the onset of reproduction in *D. magna* (Grzesiuk and Mikulski, 2006). Also for *D. magna*, concentrations of 5.0 and 1.7 gL^{-1} of NaCl (approximately 8.6 and 2.8 $mScm^{-1}$, respectively) were reported to reduce by half the neonate production (Gonçalves et al., 2007; Ghazy et al., 2009). Nevertheless, cladocerans can withstand higher values, as observed also within this study and which are in agreement with other published works (Arner and Koivisto, 1993; Martinez-Jerónimo and Martinez-Jerónimo, 2007).

In addition to the low effective conductivities, in the present work, the levels of salinity inducing lethal or sublethal effects revealed to be very close, suggesting all-or-nothing responses to this type of chemical stress. Therefore, even if small increases in salinity occur, they may result in severe effects on daphnids' life traits and, possibly, culminate in profound changes at the population level.

5.2 Multigenerational Effects

The study of multigenerational exposure scenarios has thus gained increased attention to complement standard assays based on one single generation (e.g., Li et al., 2015; Prud'homme et al., 2016). This multigenerational approach may provide a more realistic assessment of what happens to natural populations. In the present study, in general most clonal lineages maintained their tolerance to salinization through generations. This pattern of response was also reported by other authors that studied the multigenerational effects of chemicals in daphnia's (e.g., *D. magna* reproductive output did not change significantly along eight generations - except in F6 - of exposure to propranolol when compared to non-exposed organisms; Jeong et al., 2015). However, clonal lineage N91 was able to increase and maintain its tolerance after generational exposure to low levels of salinity (clonal lineage N91 when assessing mortality, feeding, reproduction and

intrinsic rate of natural increase). It is interesting to notice that in this clonal lineage the multigenerational tolerance for growth did not change over generations, while that for feeding, reproduction and *r* increased, which may suggest a strategy of a higher input of algae (energy) that is allocated to reproduction while maintaining a basal somatic growth. As well clonal lineage E99 increased its tolerance to salinization from F0 to F1 maintaining it to F2 (feeding and intrinsic rate of natural increase). However, this clonal lineage decreased its tolerance when looking at the growth rate, which may constitute a different strategy to cope with salinization and maintain or increase its fitness. These results suggest that *D. longispina* may acclimate to salinization after a long-term exposure to low levels of salinity; following up on what was already reported namely for cladocerans. For example, for *D. magna*, it was already found that an intermediate salinity level (around 4) was indeed favorable to several life traits of this species, such as individual and population growth rates (Arner and Koivisto, 1993). Furthermore, very recent work with natural *Daphnia* populations have showed that pre-exposure to low levels of salinity induced by road salt can result in increased tolerance when these natural populations are submitted again to increased salt concentrations and, furthermore, that the acquisition of tolerance to salinity can be achieved within a very narrow window of time (2.5 months) (Coldsnow et al., 2017). But if we consider that, in the present work, tolerance towards salinity in clonal lineage N91 and E99 increased within one-generation (from F0 to F1), it might be suggested that acquisition of saline tolerance may be achieved within a tighter time interval (in *D. longispina* three generations correspond to approximately 1.5 months). These results are promising regarding the persistence of these organisms in coastal lagoons with small seawater intrusions. Although, here only two generations were studied, and we cannot predict if tolerance is maintained or not in subsequent generations, thus further studies should be done to clarify this point.

Adding to the above, the multigenerational exposure to salinity, in a few cases, led to a decrease in tolerance to salinity (e.g. N35 tolerance, assessed by intrinsic rate of natural increase, decreased significantly at F2). Other studies with cladocerans have also reported the acquisition of a higher sensitivity after generational exposure to chemicals (Chen and Stillman, 2012; Chen et al., 2014; Silva et al., 2016).

The mechanisms behind salt tolerance are not yet well explained and may account for the multiplicity of responses here obtained. The tolerance by individual freshwater organisms to an increased external ion content could be due to changes in fatty acid/lipid composition and concentration (Luvizotto-Santos et al., 2003; Ghazy et al., 2009) or in the levels of heat-shock proteins, as reported for daphnids (Werner and Hinton, 2000), plants and mussels (Werner, 2004; Podlipaeva and Berger, 2012). These acclimation changes conferring increased tolerance at the individual level, which obviously are not due to genetic alterations, may be transferred to the next generation. For instance, an increased survival of neonates from 7-day exposed mothers to microcystins, when compared to neonates from non-exposed mothers, was putatively related to the maternal transfer of enzymatic activation factors (Ortiz-Rodriguez et al., 2012). Other mechanisms may also play a role on increased tolerance to stress, with epigenetics traits being increasingly investigated. Despite the lack of knowledge on invertebrates, it is already reported in the literature that salt stress affects mechanisms associated with epigenetics, such as global DNA methylation (e.g., Verhoeven et al., 2010; Al-Lawati et al., 2016). These traits, when transferred to later generations, enable organisms to subsist in disturbed environments. These alterations may persist even when the stress source is removed, which may account as an advantage if the population may suffer from future stresses. As an example, global DNA methylation levels, in *D. magna*, were significantly different under a 5 gL^{-1} of NaCl (approximately 9.9 $mScm^{-1}$) exposure when compared to the control (no salt) (Asselman et al., 2015). In the same study, at least a two-fold difference in global DNA methylation, during exposure to saline stress, was observed between two different *D. magna* genotypes, while no differences were detected under control conditions (Asselman et al., 2015). Similar mechanisms, responsible for inducing a large variety of responses among the clonal lineages of *D. longispina* (used as a model in the present study) may have also occurred, as this species shares many characteristics with the *D. magna* (used as a model species by Asselman et al., 2015). This multiplicity of phenotypic responses may contribute to the resilience of populations under real scenarios of salt (e.g., Schlichting and Wund, 2014). This finding is a major contribution of the present study because most previous works on multigenerational effects used only one single

clonal lineage (e.g., Chen et al., 2014; Silva et al., 2016). As a recommendation, the response pattern exhibited by one single clonal lineage should only be extrapolated to the population level with much caution.

5.3 Association between tolerance to metals and to NaCl

In the present study, were found no evidences of co-tolerance between metals and increased salinity levels. Crustacean's gills have been reported as very important structures in ionic change and transport (Freire and MacNamara, 1995; Freire et al., 2007; Henry et al., 2012). Besides functionality on hemolymph osmoregulation, gills are also organs where metals can be uptake from the surrounding environment. More precisely, these structures are provided, in their basolateral membranes, with sodium (Na^+/K^+-ATPase) and carbonic anydrase channels (Grosell et al., 2002). The possibility that the mechanism responsible for the tolerance to metals confers some level of protection against salt stress should not be overlooked. Blanchard and Grosell (2006) observed mortality in the killifish *Fundulus heteroclitus*, when exposed to copper under exposures to freshwater and high saltwater concentration (salinity of 28), while at intermediate salinities under copper exposure (5, 11 and 22 ppt, approximately 9.0, 18.6 and 35 $mScm^{-1}$) no mortality was observed, possibly due to competition between ions. Still, in saltwater, no apparent Na^+ uptake deregulation associated with the inhibition of sodium transporters was observed in any of the Cu concentrations (0, 30 and 150 μgL^{-1}). Nevertheless, in the present study, despite correlations being not found, some inversions on clonal sensitivity between SW and Cu and between NaCl and Cu and Co were observed. For instance, the clonal lineage N91 was one of the most tolerant lineages to lethal levels of Cu but one of the most sensitive to SW and to NaCl (feeding behavior); E99 present one of the highest tolerance to NaCl at lethal levels but one of the lowest tolerance to lethal levels of Co. These inversions may be related with the competition of metals (as cations) with Na^+ and/or K^+, interfering with normal physiological functions associated to ionic regulation. Since smaller animals (like cladocerans) are more sensitive due to their higher sodium turnover rates, acute exposure to metals may rapidly cause inhibition of sodium transporters in the basolateral membranes of the respiratory organs (Grosell et al., 2002).

The inversions on the sensitivity by some clonal lineages may increase the susceptibility of the population to future stressors, as many freshwater ecosystems are already subjected to several stress sources. The exposure to one stressor, acting as a directional selective pressure, will lead to the loss of the most sensitive genotypes within a population (Ribeiro and Lopes, 2013). The impoverishment at the genetic level – genetic erosion – can compromise the response of the population to deal with future stressors, as discussed by Ribeiro and Lopes (2013) within the framework of the multiple stressors differential tolerance (working-) hypothesis. For instance, the pre-exposure of population of midges to a high concentration level of tributyltin (TBT; 8.93 µg Sn/kg dw) lead to genetic erosion and significantly increased the population vulnerability to a later stressor (cadmium), when comparing with non-pre-exposed populations (Vogt et al., 2010). Similarly, the clonal lineages here studied that were tolerant to copper and also sensitive to salinity would be wiped out from a population after a sequential exposure to these two chemicals, leading to the occurrence of genetic erosion.

6. Conclusions

This study showed that, overall, NaCl could induce similar or high lethal and sublethal toxicity than SW. Generational exposure to low levels of salinity resulted in diverse responses among the six clonal lineages, which suggests that natural populations of cladocerans will be able to cope with long-term exposure to low levels of salinity. Furthermore, although some clonal lineages exhibited a higher sensitivity to increased salinity after multigenerational exposure, it seems that, at the population level, this may be compensated with the presence of other clonal lineages capable of maintaining or increasing its tolerance under salinization scenarios. Nevertheless, the loss of some genotypes from the population may occur, foreseeing a loss of genetic diversity that may have consequences in the resilience of natural populations when exposed to future environmental perturbations.

Though no correlation was found between lethal or sublethal levels of NaCl or SW and lethal tolerance to metals, some inversions in tolerance to NaCl or SW and metals were found,

suggesting that sequential exposures of cladocerans populations to these stressors may lead to intense genetic erosion events in natural populations. Even so, the effects of salinization on daphnids populations already impacted by other types of stress (e.g., anthropogenic activities) can have repercussion at other trophic levels (e.g., producers and secondary consumers), since this group (daphnids) is a major link on energy transfer in trophic webs.

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Supplementary Information

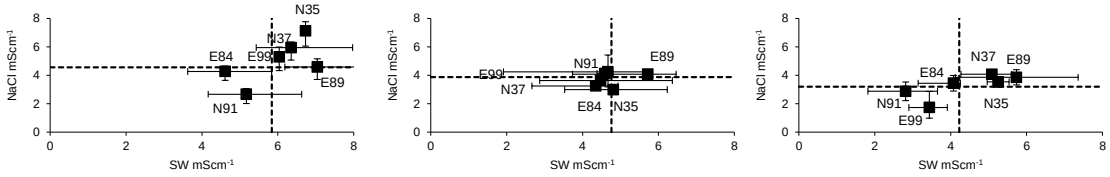
Supplementary Table S1: Range of concentrations ($mScm^{-1}$) and dilution factor (d.l.) tested for each clonal lineage (E84, E89, N37, N35, N91, and E99) within each tested endpoint. NaCl – sodium chloride; SW – natural seawater. FI – feeding inhibition. JGR – Juvenile growth rate. TR – Total reproduction. F0, F1 and F2: generations.

		Range of Concentrations in $mScm^{-1}$ (number of tested dilutions)			
		Lethal (d.l. x1.4)	FI (d.l. x1.2)	JGR (d.l. x1.1)	TR (d.l. x1.2)
NaCl F0	E84	3.04-11.7 (5)	2.30-4.78 (5)	2.08-3.70 (7)	0.53-1.89 (8)
	E89	2.74-14.6 (6)	2.34-4.87 (5)	2.22-4.76 (9)	
	N37	3.80-14.6 (6)	2.31-4.79 (5)	2.54-4.52 (7)	
	N35	3.80-14.6 (5)	2.62-5.42 (5)	2.13-3.79 (7)	
	N91	1.67-9.13 (6)	1.32-5.42 (9)	2.54-4.52 (7)	
	E99	2.13-11.4 (6)	1.90-3.94 (5)	1.96-4.62 (9)	
NaCl F1	E84	2.71-10.4 (5)	2.65-5.51 (5)	2.25-3.97 (7)	0.53-1.89 (8)
	E89	3.80-14.6 (5)	2.11-4.38 (5)	2.75-4.88 (7)	
	N37	3.80-14.6 (5)	2.97-8.47 (7)	2.46-4.30 (7)	
	N35	3.80-14.6 (5)	2.71-5.63 (5)	2.71-3.97 (5)	
	N91	3.35-18.3 (6)	3.13-7.81 (6)	2.71-3.97 (5)	
	E99	3.80-14.6 (5)	3.62-9.01 (6)	2.71-3.97 (5)	
NaCl F2	E84	2.71-10.4 (5)	1.84-3.82 (5)	2.46-3.97 (6)	0.53-1.89 (8)
	E89	3.80-14.6 (5)	1.76-3.65 (5)	2.75-4.88 (7)	
	N37	2.71-10.5 (5)	2.80-5.80 (5)	2.75-3.97 (5)	
	N35	3.80-14.6 (5)	2.33-4.84 (5)	1.87-3.33 (7)	
	N91	3.80-14.6 (5)	2.17-5.42 (6)	2.71-3.97 (5)	
	E99	3.8-14.6 (5)	3.09-6.41 (5)	2.46-4.30 (7)	
SW	E84	2.47-9.50 (5)	1.53-5.49 (8)	2.62-4.64 (7)	n.a.
	E89	4.85-18.6 (5)	3.66-7.66 (6)	3.40-6.02 (7)	
	N37	4.85-18.6 (5)	3.43-8.54 (6)	3.51-6.23 (7)	
	N35	2.47-9.50 (5)	3.18-8.70 (6)	3.65-5.35 (5)	
	N91	2.47-9.50 (5)	1.77-4.41 (6)	3.08-4.95 (6)	
	E99	2.47-9.50 (5)	2.29-4.75 (5)	3.38-4.95 (5)	

n.a. – non applicable. No reproduction tests were performed with SW.

Supplementary Table S2: Range of values for the parameters measured during the assays (highest and lowest pH and conductivity values are presented). No data is presented for dissolved oxygen (DO) as this endpoint was always above 7.4 mg/L (≥ 3 mg/L as stipulated by OECD guideline no 202). Abbreviations: NaCl – sodium chloride; SW – natural seawater. FI – feeding inhibition. JGR – Juvenile growth rate. TR – Total reproduction. F0, F1 and F2: generations.

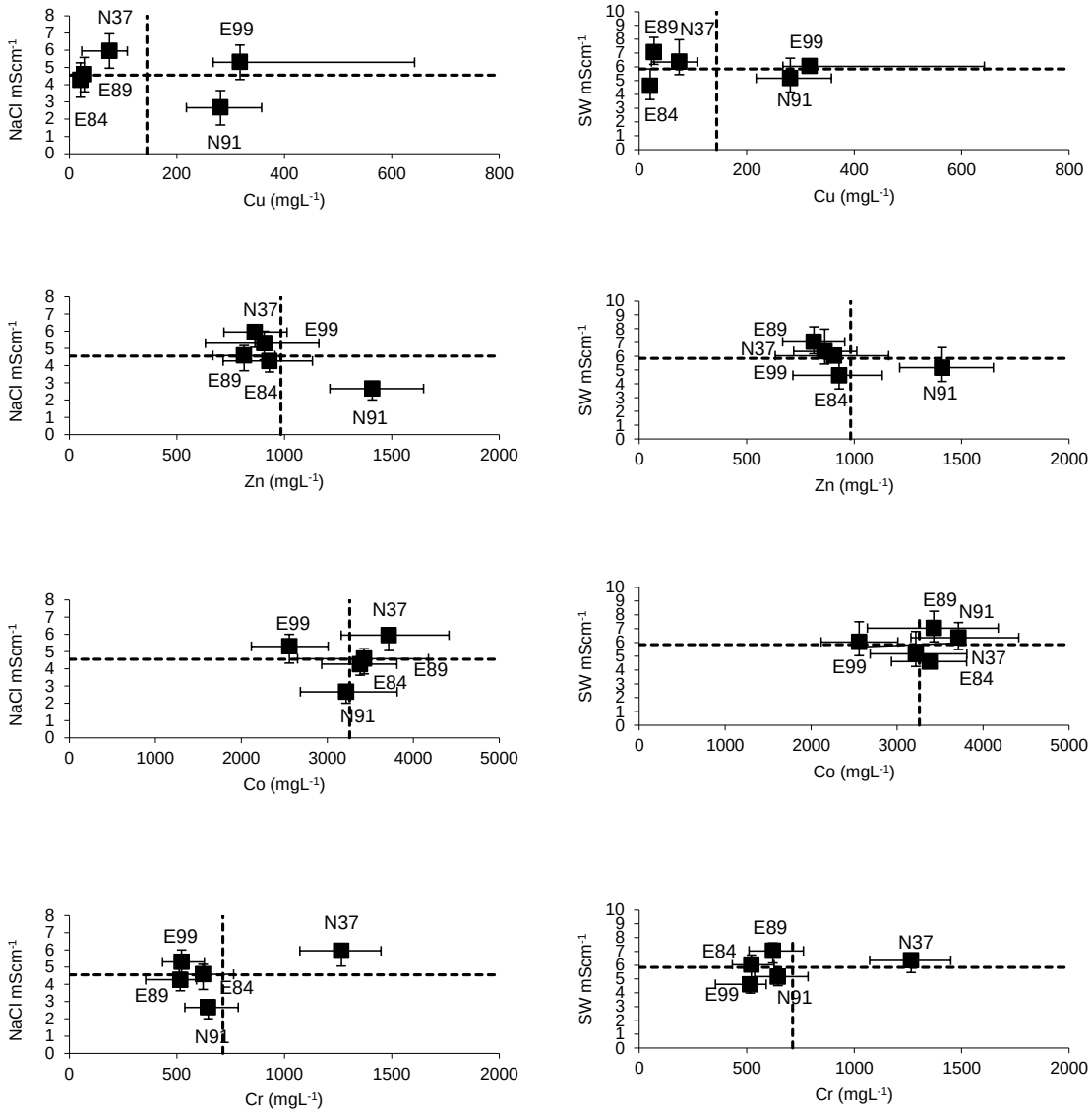
		NaCl			SW
Parameter	**Endpoint**	**F0**	**F1**	**F2**	**F0**
pH	**Lethal**	7.52 – 7.62	7.85 – 7.92	7.68 – 7.77	7.45 – 7.47
	FI	7.71 – 7.75	7.83 – 7.84	8.01 – 8.03	7.45 – 7.53
	JGR	7.74 – 7.85	7.81 – 7.86	7.83 – 7.85	7.41 – 7.54
	TR	7.71 – 7.83	7.68 – 7.74	7.72 – 7.79	-
Conductivity mScm^{-1}	**Lethal**	2.17 – 15.3	3.24 – 14.3	3.26 – 14.4	3.72 – 19.7
	FI	1.87 – 5.57	2.60 – 8.81	2.32 – 6.53	2.09 – 10.03
	JGR	2.47 – 5.01	2.75 – 5.11	2.41 – 5.10	3.76 – 7.57
	TR	0.76 – 2.12	0.76 – 2.10	0.76 – 2.11	-



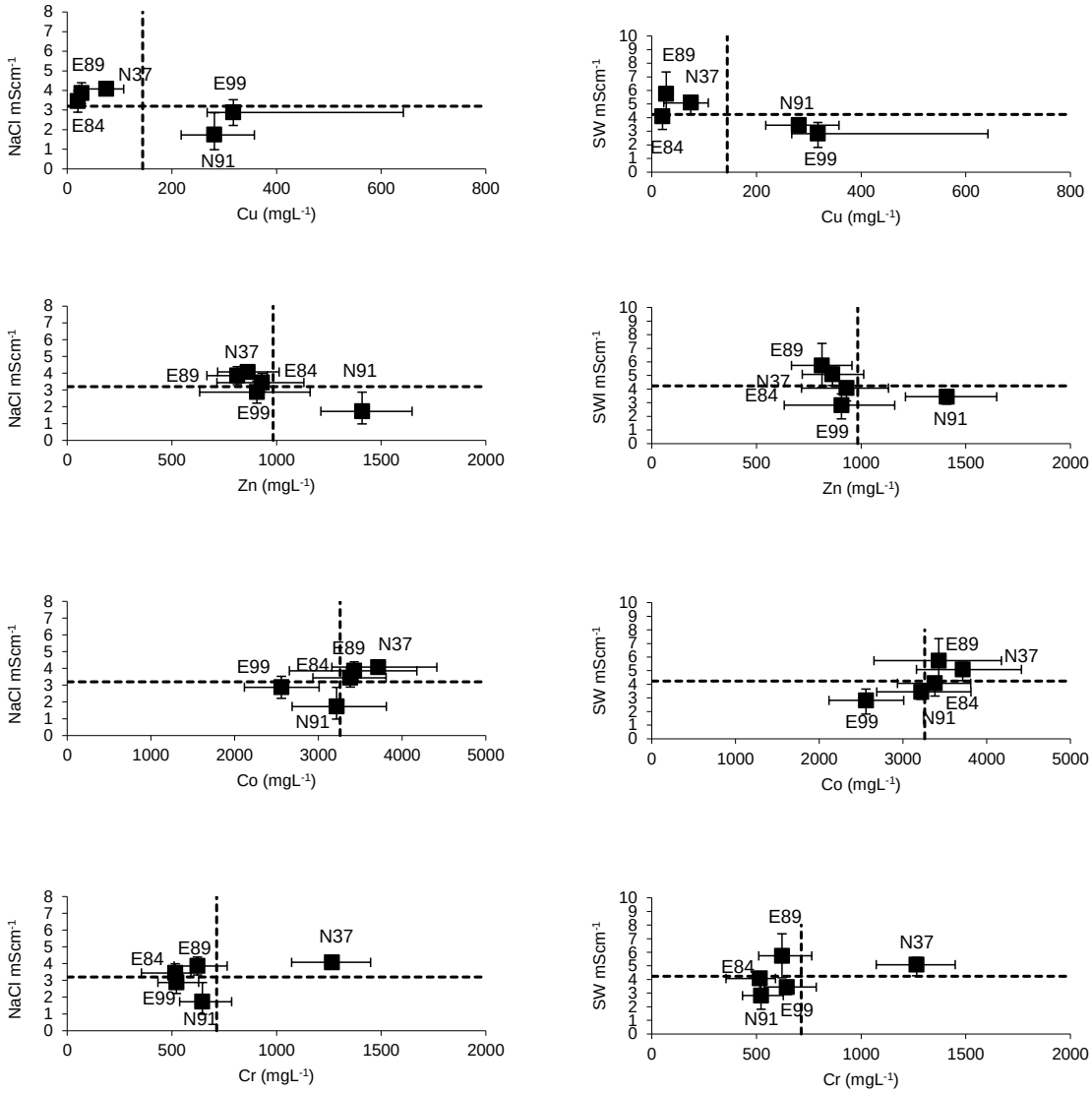
Supplementary Figure S1: Pairwise comparisons between median effective concentrations (LC_{50} or EC_{50}) after exposure to sodium chloride (NaCl) and natural seawater (SW) for the six clonal lineages of *Daphnia longispina*. a) mortality after 24h of exposure; b) somatic growth after 72h of exposure and c) feeding after 24h of exposure. Black squares and error bars indicate the LC_{50} or EC_{50} with the respective 99% confidence limits. Dashed lines indicate the mean of the set of the tested endpoints for the six clonal lineages.

Supplementary Table S3: Pearson's correlations (R) and p values between sodium chloride (NaCl) lethal and sublethal endpoint and lethal tolerance to metals (copper, cobalt, zinc, and chromium) for five *Daphnia longispina* clonal lineages. FI – feeding inhibition. JGR – Juvenile growth rate. TR – Total reproduction.

		NaCl			
		Lethal	**FI**	**JGR**	**TR**
Cu	R	-0.27	-0.82	-0.69	0.61
	p	0.65	0.09	0.19	0.27
Zn	R	0.13	0.6	-0.26	-0.61
	p	0.83	0.28	0.68	0.27
Co	R	-0.86	-0.84	0.41	0.35
	p	0.06	0.08	0.49	0.56
Cr	R	0.52	0.4	-0.08	-0.4
	p	0.36	0.51	0.9	0.5



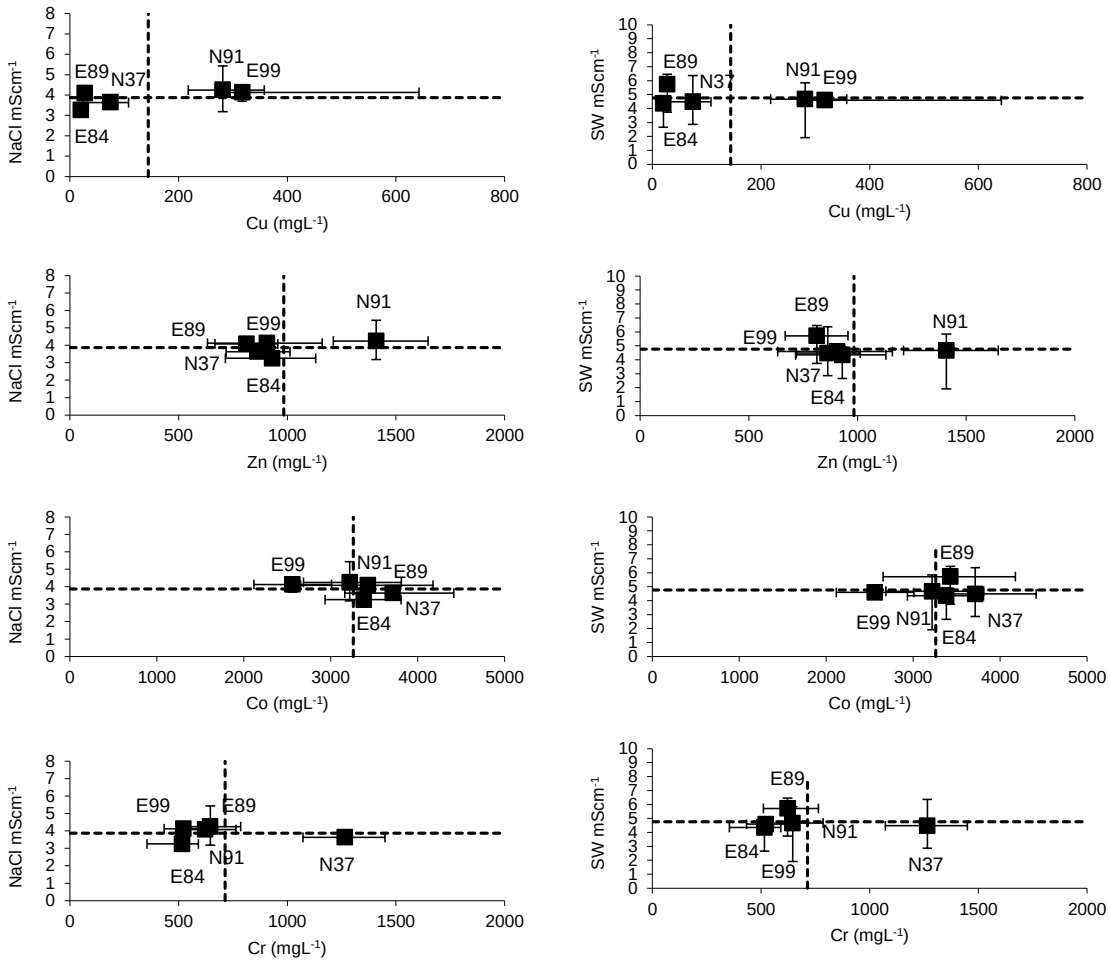
Supplementary Figure S2: Pairwise comparisons between median lethal concentrations (LC_{50}) for the five clonal lineages of *Daphnia longispina* exposed, for 24h, to lethal levels sodium chloride (NaCl – graphics on the left), lethal levels natural seawater (SW – graphics on the right) and lethal levels of four metals (copper, zinc, cobalt, and chromium). Black squares and error bars indicate the LC_{50} with the respective 99% confidence limits and dashed lines indicate the mean of the set of the tested endpoints for the five clonal lineages.



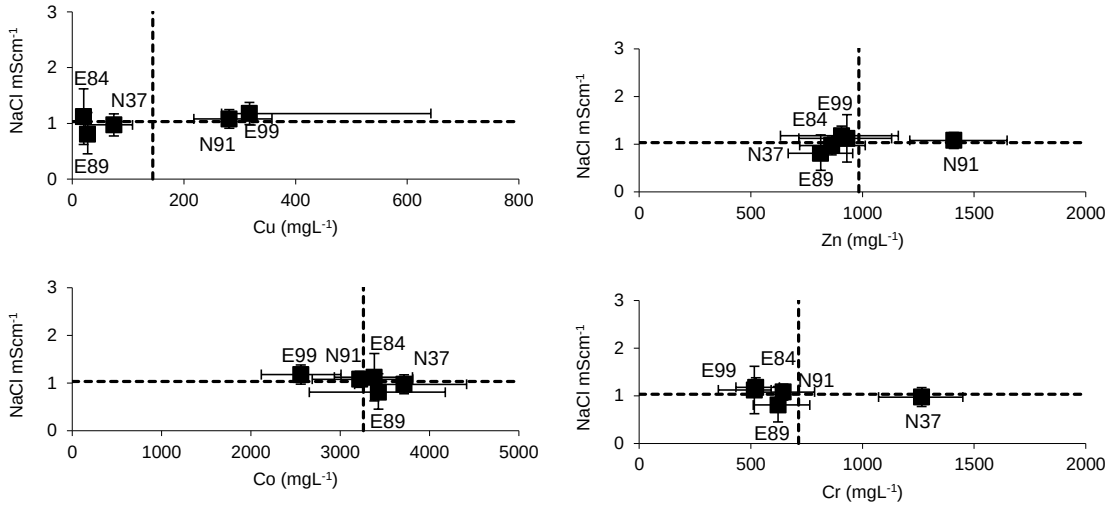
Supplementary Figure S3: Pairwise comparison between median sublethal effective concentrations (EC_{50}), for feeding, of sodium chloride (NaCl) and natural seawater (SW) and lethal levels of four metals, for the five clonal lineages of *Daphnia longispina*. Black squares and error bars indicate the EC_{50} or LC_{50} with the respective 99% confidence limits and dashed lines indicate the mean of the set of the tested endpoints for the five clonal lineages.

Supplementary Table S4: Pearson's correlations (R) and p values between natural seawater (SW) lethal and sublethal endpoints and lethal tolerance to metals (copper, cobalt, zinc, and chromium) for five *Daphnia longispina* clonal lineages. FI – feeding inhibition. JGR – Juvenile growth rate.

		SW		
		Lethal	**FI**	**JGR**
Cu	R	-0.15	-0.83	-0.27
	p	0.81	0.08	0.67
Zn	R	0.14	0.82	0.11
	p	0.83	0.09	0.86
Co	R	-0.54	-0.42	-0.25
	p	0.35	0.48	0.68
Cr	R	0.39	0.51	-0.18
	p	0.52	0.38	0.77



Supplementary Figure S4: Pairwise comparison between median sublethal effective concentrations (EC_{50}), for somatic growth, of sodium chloride (NaCl) and natural seawater (SW) and lethal levels of four metals, for the five clonal lineages of *Daphnia longispina*. Black squares and error bars indicate the EC_{50} or LC_{50} with the respective 99% confidence limits and dashed lines indicate the mean of the set of the tested endpoints for the five clonal lineages.



Supplementary Figure S5: Pairwise comparison between median sublethal effective concentrations (EC_{50}), for total reproduction, of sodium chloride (NaCl) and lethal levels of four metals, for the five clonal lineages of *Daphnia longispina*. Black squares and error bars indicate the EC_{50} or LC_{50} with the respective 99% confidence limits and dashed lines indicate the mean of the set of the tested endpoints for the five clonal lineages.

Chapter V

Does increased salinity influence the competitive outcome of two producer species?

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Does increased salinity influence the competitive outcome of two producer species?

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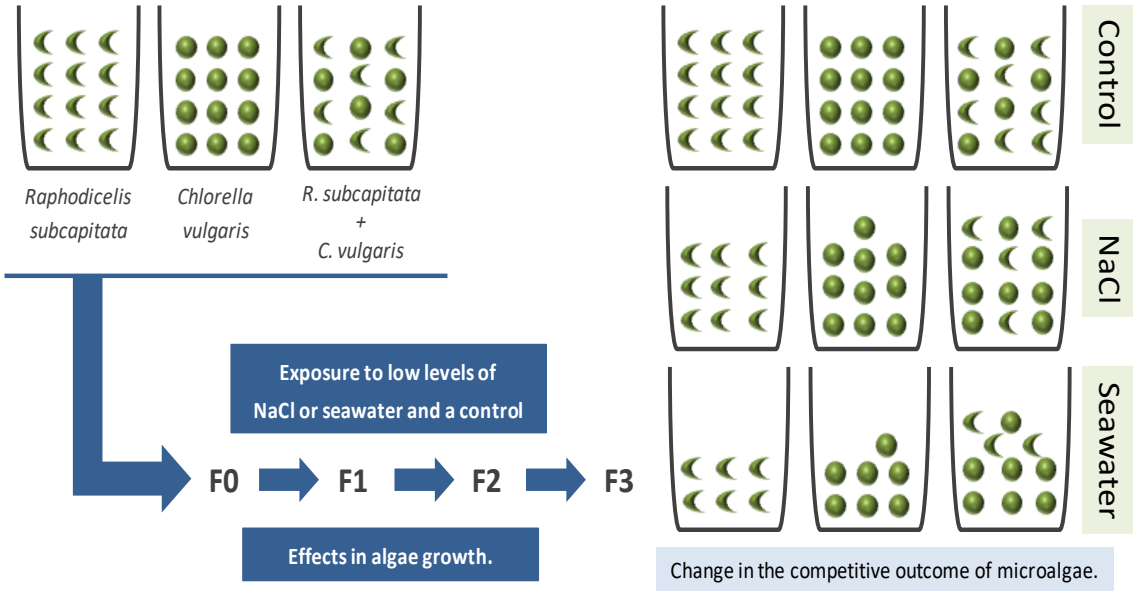
Graphical abstract



Abstract

Within the context of global climate changes, it is expected that low-lying coastal freshwater ecosystems will face seawater intrusion and concomitant increase in salinity. Salinity may provoke disruption of competitive relationships among species. However, species may be capable of acclimating to salinity, which, in turn, may influence the resilience of ecosystems. Accordingly, this work aimed at assessing the effects of multiple-generational exposure to low levels of salinity in the competitive outcome of two species of green microalgae: *Raphidocelis subcapitata* and *Chlorella vulgaris*. To attain this, three specific objectives were delineated: 1) compare the toxicity of NaCl (as a surrogate) and seawater to the two microalgae, 2) determine the capacity of the two microalgae species to acclimate to low salinity levels for seawater and NaCl), and 3) assess the influence of exposure to low salinity levels in the competitive outcome. Results revealed seawater to be slightly less toxic than NaCl for the two microalgae: EC_{25} for growth rate

were 4.63 and 10.3 $mScm^{-1}$ for *R. subcapitata* and 6.94 and 15.4 $mScm^{-1}$ for *C. vulgaris*, respectively for NaCl and seawater. Both algae seemed to be capable of acclimation to low levels of salinity but *C. vulgaris* seemed to acclimate earlier comparatively to *R. subcapitata*. Under competition to control conditions the growth rates of *C. vulgaris* were lower than those of *R. subcapitata*. However, *C. vulgaris* acquired competitive advantage equaling or surpassing the growth rate of *R. subcapitata* with the addition of NaCl or SW, respectively. Multigenerational exposure to low levels of salinity influenced the competitive outcome of the two algae both under control and salinity treatments. Shifts in the algae community structure can be caused by saline stress and, therefore, should not be neglected since algae are an important resource food for many organisms influencing the resilience of higher trophic levels.

Keywords: sodium chloride; natural seawater; competition; microalgae; *Raphidocelis subcapitata*; *Chlorella vulgaris*

1. Introduction

Sea level rise is one of the major driving forces affecting coastal zones (IPCC, 2013). According to the most recent report of the Intergovernmental Panel for Climate Change (IPCC, 2013), this rise is estimated between 26 and 81 centimeters in a worst-case scenario, by the end of this century. In such situation, shorelines are at serious risk of losing territory and, as well, are vulnerable to the action of severe weather conditions. It is expected that this will compromise not only human settlements (Small and Nicholls, 2003; McGranahan et al., 2007), but also a large number of ecosystems located near the coastal line worldwide and the services provided by them (e.g. biodiversity hotspots, economical and recreational activities) (Brito et al., 2012; Roebeling et al., 2013; Liquete et al., 2013).

Salt stress is known to induce changes in life cycle traits (e.g. survival, growth reproduction) in several freshwater organisms (e.g., Gonçalves et al., 2007; Zuanon et al., 2009; Lob and Silver, 2012; Chen and Stillman, 2012). In producers, the toxicity mechanism of sodium (Na^+) and chloride (Cl^-) is mainly related with oxidative stress (production of ROS) and changes in

chlorophyll molecules resulting in the decline of photosynthetic rates (e.g. Kirrolia et al., 2011; Simmons, 2012; Huang et al., 2015). Additionally, salt exposure may lead to changes in DNA structure and induce autophagy of organelles that are distress by salt exposure (Affenzeller et al., 2009).

When in a natural system, abiotic factors (such as salinity) certainly interact with biotic factors (such as predation and competition). This interaction may, therefore, exert a different pressure on organisms and on how they relate, and, for instance, may result in a community structure different from the one that could exist in the absence of increased salinity. This may occur either by the immediate or fast elimination of the less fit species (Sarma et al., 2003) or by shifting the competitive outcome via favoring species that under normal conditions were less competitive (Feniova et al., 2011). For example, Loureiro et al. (2013) demonstrated that, when exposed to low salinity levels (0 and 0.75gL^{-1}NaCl), *Daphnia galeata* was a superior competitor when compared with *Simocephalus vetulus*. But, when exposed at 1.5 gL^{-1} NaCl, a decrease in *D. galeata* population growth relieved the pressure on the less competitive species, allowing an inversion on the competitive outcome (Loureiro et al., 2013).

Concomitant with the fact that organisms do not exist alone in natural environments they can be exposed to a stress factor (like salinity) for long periods of time, especially under the scenarios of sea level rise and coastal flooding. In this case, organisms may develop acclimation processes to the chemicals they are exposed to. The acclimation ability of organisms has been studied for several factors, like metals, temperature, salinity (e. g. Bervoets et al., 1996; Bossuyt and Janssen, 2003; Kwok et al., 2009; Chen and Stillman, 2012; Elmaghrabi et al., 2012; Kearney et al., 2012; Osundeko et al., 2014). For instance, in the study of Johnson et al. (2007), although no significant changes were observed in tolerance to copper, *Chlorella* sp. were able to live in cultured media containing 2 μgL^{-1} Cu, for 100 days. Also, Osundeko et al. (2014) reported that five of six species of freshwater algae were able to increase their growth rates, after an 8-week period exposed to wastewaters. Still, there is a lack of understanding on how freshwater organisms can deal with long-term exposure (i.e., for several generations) under more complex scenarios, like in the presence of competition with other species.

Microalgae are considered key components in water quality assessment, being commonly used in standard risk assessment due to their short life cycles, their widespread distribution and their sensitive metabolism (Wan, 2010). More, require low-cost maintenance since they are easily cultured. As producers, algae are at the bottom of trophic chains contributing with oxygen and organic substances and providing food for other organisms, like fish and invertebrates, (Yang et al., 2008). Therefore, adverse chemical effects on algae can directly affect the structure and function of the entire ecosystem (Satyavani, 2012). So, taking in account the aspects referred above, this work aimed to understand the influence of low levels of salinity, during a long-term exposure on the competitive outcome of two species of freshwater green microalgae (since they compete for light, nutrients and space). For that, three specific objectives were delineated: 1) compare the toxicity of NaCl (as a surrogate) and seawater to the two microalgae, 2) determine the capacity of the two microalgae species to acclimate to low salinity levels for seawater and NaCl), and 3) assess the influence of exposure to low salinity levels in the competitive outcome.

2. Material and Methods

2.1 Test solutions

Increased salinity was simulated through the use of sodium chloride (NaCl, supplied by Merck, St Louis, MO, USA) or natural seawater (SW) (collected at a reference site located in the North Atlantic Ocean; 40°38'33.94"N, 8°44'55.91"W, Aveiro, Portugal).The sampling site for SW was selected because it holds, over the 27 years, the eco-label of blue flag awarded by the Foundation for Environmental Education, being the quality of the water monitored routinely and considered good to excellent according to the Directive 2006/7/CE of the European Union. These two scenarios were studied to address if NaCl could be used as a surrogate of SW for studies on predictive assessment of the adverse effects caused by SW intrusion into freshwater ecosystems. Stock solutions of NaCl (25 gL^{-1} NaCl) were made always fresh by adding directly the salt to the medium used to perform the experiments, Woods Hole (MBL) culture medium (Stein, 1973). Prior to its use, natural SW was filtered through cellulose nitrate membranes of 0.20 µm (ALBET-

Hannemuehle S.L., Barcelona, Spain) and sterilized to remove microorganisms. All tested concentrations were made directly by diluting the stock solution of NaCl and of the natural SW with MBL medium.

2.2 Tested species

Two ubiquitous freshwater species of green microalgae were selected to carry out this study: *Raphidocelis subcapitata* (formerly known as *Pseudokerchneriella subcapitata*) and *Chlorella vulgaris.* Two individual stock cultures of each species were maintained in MBL medium and under laboratorial controlled conditions of light intensity (continuous cool-white fluorescent illumination - 100 µE/m^2/s) and temperature (20±2ºC).

2.3 Growth inhibition assays

Firstly, monospecific growth inhibition assays were performed with each algae species by exposing them individually either to NaCl or SW, in order to understand if NaCl could be used as a surrogate of SW and to establish acclimation concentrations according to each species sensitivity to NaCl and SW. Tests were performed according to the OECD guideline 201 (2006) adapted to 24-well plates under aseptic conditions. For each algae species, it was intended to determine the concentration causing 25% of inhibition in growth after a period of 72h of exposure ($EC_{25,72h}$). This value was chosen due to its proximity to the threshold effect concentration (lowest observed effect concentration - LOEC or EC_{20}). For *R. subcapitata* and *C. vulgaris* the following range of NaCl conductivities were tested, respectively: from 2.61 to 6.94 $mScm^{-1}$, and from 3.97 to 9.18 $mScm^{-1}$ (with a dilution factor of 1.15x). For *R. subcapitata* and *C. vulgaris* the range of natural SW conductivities tested was, respectively: from 8.2 to 16.5 $mScm^{-1}$, and from 10.8 to 21.85 $mScm^{-1}$ (with a dilution factor of 1.15x).

Three replicates per conductivity and control (with MBL only) were made in 24-well plates. Each well contained 900 µl of test solution (prepared in MBL medium) and 100 µL of algal inoculum (initial test cell concentration of 10^4 cells/mL). Test plates were incubated, for 72 h, at 23±1ºC, with continuous cool-white fluorescent illumination (100 µE/m^2/s). To avoid the effects of

shading, algae were resuspended manually twice a day. Absorbance (ABS) measurements, obtained at 440 nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, USA) were converted into cell densities using the equation 1 or 2:

Conc $(cellsmL^{-1}) = -17107.5 + (ABS * 7925350)$ (R^2=0.99), for *R. subcapitata* (equation 1)

Conc $(cellsmL^{-1}) = -155820 + (ABS * 13144324)$ (R^2=0.98), for *C. vulgaris* (equation 2)

Average specific growth rate (μ), for each species, was determined through the following equation (OECD, 2006):

$\mu_{ab} = \frac{\ln D_b - \ln D_a}{t_{b-t_a}} d^{-1}$, (equation 3) where D_b is the cell density at the end of the assay, D_a is the cell density at the beginning of the assay and t_b-t_a is the exposure time interval (72 h).

2.4 Acclimation to low levels of salt stress

Each species was exposed for three generations, under the same conditions as those described for the cultures, to the respective EC_{25} for NaCl or for SW (computed from the monospecific growth inhibition assays of section 2.3). Inoculation from one generation to the next one was made during the exponential growth phase of each microalgae. These acclimated generations were designated as F1, F2 and F3. For all generations the assays followed the same procedure as that described for F0.

2.5 Competition assays

Competition assays were performed under the same light and temperature conditions as for monospecific growth inhibition assays (23±1ºC and continuous cool-white fluorescent illumination - 100 μE/m^2/s), but, for 96 h, and in 150 mL vessels containing 100 mL of solution (EC_{25} of each algae species) or MBL solely (control). Initial cell concentration at each replicate was 10^4 cellsmL^{-1}. Two concentrations of NaCl or SW plus a control (MBL) were established for both species: the $EC_{25,72h}$ computed for *R. subcapitata*, $EC_{25,72h}$ computed for *C. vulgaris*, and control (CTR). Three exposure scenarios were carried out per treatment, consisting of: (i) *R.*

subcapitata solely, (ii) *C. vulgaris* solely, and (iii) *R. subcapitata* plus *C. vulgaris*. For each exposure scenario four replicates were performed.

At the end of the exposure period, all replicates were stirred manually to uniformize the distribution of cells, and ten aliquots (1 mL) from each replicate were collected to eppendorfs tubes and fixed with Lugol. Counting of cells was made under the microscope in a Neubauer chamber. Cell density was used to determine the average specific growth rate (μ) according to equation 3 (please see section 2.3 of Material and Methods) for each microalgae. Competition was evaluated in all generations (F0, F1, F2, and F3).

3. Data analysis

Salinities (measured as conductivity) that caused 25% of growth inhibition (EC_{25}) were computed through non-linear regression analysis, using a logistic model with the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

All data sets were checked for normality through the Kolmogorov-Smirnov test and for homocedasticity through the Levene test.

To evaluate if exposure to salinity treatments affects microalgae growth comparatively to a control condition (no salt addition), within each generation, a one-way Anova was performed followed by Dunnett's test ($p<0.05$). This procedure was applied for results obtained after exposure to NaCl and SW, separately.

To test if microalgae are able to acclimate to low levels of salt during long-term exposure a two-way Anova, followed by Holm-Sidak all pairwise comparison, was performed, individually for NaCl and SW.

To test the if long-term exposure to low levels of salt may significantly influence the competitive outcome between both species, a three-way Anova, followed by Holm-Sidak all pairwise comparison, was performed, with salinity, generation and type of exposure (single or competition) as fixed factors.

4. Results

4.1 Monospecific growth inhibition assays

Monospecific growth inhibition assays fulfilled OECD guideline validity criteria: average growth rates in the controls increased more than 16 times and the coefficients of variation did not exceed 7%, at the end of the 72-h exposure period. *Raphidocelis subcapitata* showed to be slightly more sensitive to NaCl and SW than *C. vulgaris*. The EC_{25} values obtained after exposure to NaCl and SW (95% confidence limits) for this species were 4.6 $mScm^{-1}$ (3.3 – 5.6) and 10.3 $mScm^{-1}$ (9.8 – 10.9), respectively. While for *C. vulgaris* the EC_{25} values (95% confidence limits) were 6.9 $mScm^{-1}$ (6.0 – 7.3) and 15.4 $mScm^{-1}$ (14.2 - 17.5), for NaCl and SW respectively. Both species exhibited a slightly higher sensitivity to NaCl comparatively to SW, as suggested by the lower EC_{25} values computed after exposure to NaCl.

4.2 Exposure to low levels of sodium chloride

When analyzing the results obtained for each species under single exposure (i.e. either *R. subcapitata* or *C. vulgaris* solely), and before being acclimated to the respective EC_{25} (F0), *C. vulgaris* presented lower growth rates than *R. subcapitata*, both when exposed to the control and to the EC_{25} treatments (Fig.1a). Furthermore, still for F0, a significant decrease in growth rates was only observed when *C. vulgaris* was exposed to the respective EC_{25} (7.0 $mScm^{-1}$). While for *R. subcapitata* a significant decrease in daily growth rate was observed at the two tested NaCl concentrations (Fig.1a).

After generational acclimation and under single exposure, growth rates of *R. subcapitata*, though being maintained in the control, decreased from F0 to F1 at the two tested levels of salinity. But, from generation F1 to F2 and F3 growth rates increased under salt exposure, being significantly higher than at F0 ($p<0.05$). Nevertheless, within each generation, significant reductions were always observed between the two NaCl concentrations and the respective control. Significant differences were also detected among the four generations ($p<0.001$). For *C. vulgaris*, growth rates increased significantly from F0 to F1 and to F3, at control and salt concentrations (Fig. 1a).

Significant reduction in growth rate, comparatively to the control, was only observed when exposed to the respective EC_{25} (at F0 and F1) and to the two tested NaCl concentrations (at F2 and F3) ($p<0.05$). Significant differences were detected among generations ($p<0.001$).

Regarding exposures under competition scenarios and before acclimation (F0), as for single exposure, in the control treatment *R. subcapitata* exhibited a higher growth rate than *C. vulgaris*. However, the growth rate of the former species was lower under competition comparatively to single exposure ($p<0.05$; Fig. 1a, 1b). In competition exposure, and at F0, differences in the growth rate of the two species disappeared when exposed to the two concentrations of NaCl ($p<0.05$; Fig. 1a, 1b). Still at F0, significant differences between NaCl concentrations and the respective control were only detected for *R. subcapitata* ($p<0.05$; Fig.1b). After acclimation (> F0), the growth rate values of *C. vulgaris* were always above *R. subcapitata* when exposed to the two salinity levels (Fig. 1b). Exposure to the two levels of NaCl caused a significant decrease in growth rates of *R. subcapitata*, relatively to the respective control, at all generations ($p<0.001$; Fig. 1b). Regarding *C. vulgaris*, when comparing growth rate of F0 exposed under competition versus solely, significant differences were only detected at the lowest NaCl concentration ($p=0.037$; Fig. 1b). Significant differences among generations were detected, growth rates increased from F0 to F3 ($p<0.001$; Fig. 1b).

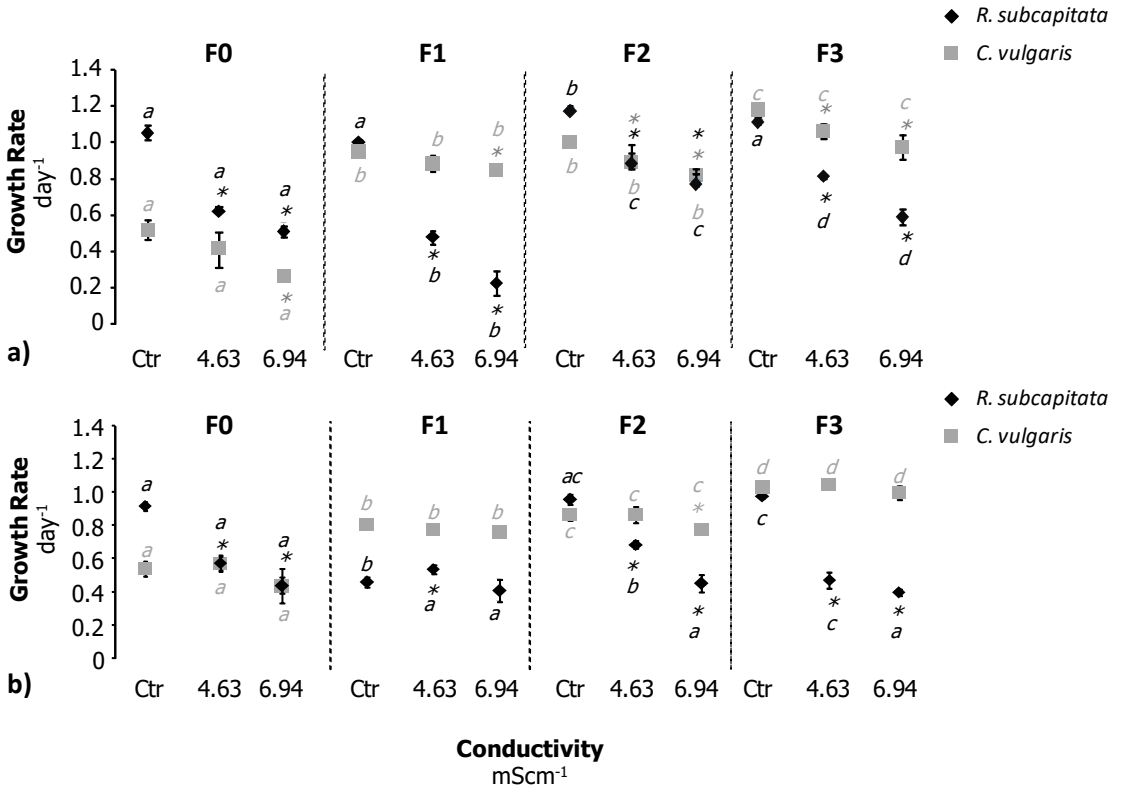


Figure 1. Average growth rates (d^{-1}) of *Raphidocelis subcapitata* and *Chlorella vulgaris*, before and after generational acclimation to low levels (respective $EC_{25,72h}$) of sodium chloride (NaCl) (error bars correspond to the standard deviation), under a) single exposure and b) competition exposure to two levels of NaCl and a control. Letters (a, b, c) indicate homogenous groups between different generations. *represent significant differences ($p \leq 0.05$), within each generation, of each NaCl treatment relatively to the control (black: treatments with *R. subcapitata* relatively to control and grey: treatments with *C. vulgaris* relatively to control).

4.3 Exposure to low levels of natural seawater

When exposed solely to SW and before acclimation (F0) the two species only exhibited significantly different growth rates when exposed to 15.3 $mScm^{-1}$ ($p<0.05$; Fig. 2a). Still in F0, the growth rate of both species were significantly reduced at the two salinity levels comparatively to the respective control ($p<0.05$, Fig. 2a). After generational acclimation, *R. subcapitata* growth rates were similar from F0 to F1 at the three exposure treatments ($p<0.05$; Fig. 2a). But, from F1 to F2 growth rates decreased in control and 10.4 $mScm^{-1}$ (its EC_{25}) ($p<0.05$), increasing again from F2 to F3 ($p<0.05$; Fig. 2a). Significant differences among the four generations were detected ($p<0.001$). For *C. vulgaris*, when exposed solely to low levels of SW, growth rates were similar from F0 to F1 ($p<0.05$), but significantly decreased from F1 to F2, at all treatments ($p<0.05$; Fig. 2a). From F2 to F3, *C. vulgaris* growth rate increased, being more evident at the lowest salt

concentration and control (p<0.05). Significant differences between generations were detected (p<0.001).

Under competition scenarios and before acclimation (F0), significant differences were found between SW dilutions and the respective control for *R. subcapitata*. Growth rate of *R. subcapitata* significantly decrease under control conditions and in the lowest salt treatment under competition comparatively to single exposure (p<0.05; Fig. 2a, 2b). After generational acclimation (>F0), *C. vulgaris* growth rates were higher than *R. subcapitata* at F1 salinity levels and in all treatments at F3, while *R. subcapitata* growth rates surpass *C. vulgaris* growth rates under control conditions of F1 and all treatments of F2. Significant differences between generations, under competition scenarios, were detected for *R. subcapitata* (p<0.001). Regarding *C. vulgaris*, when comparing growth rate of F0 exposed under competition versus solely, smaller growth rates were observed in the latter scenario for all treatments (p<0.05; Fig. 2a, 2b). Still in F0, significant differences were detected between control conditions and the highest salt treatment (p<0.05; Fig. 2b). Significant differences (p<0.001) were detected among the four generations.

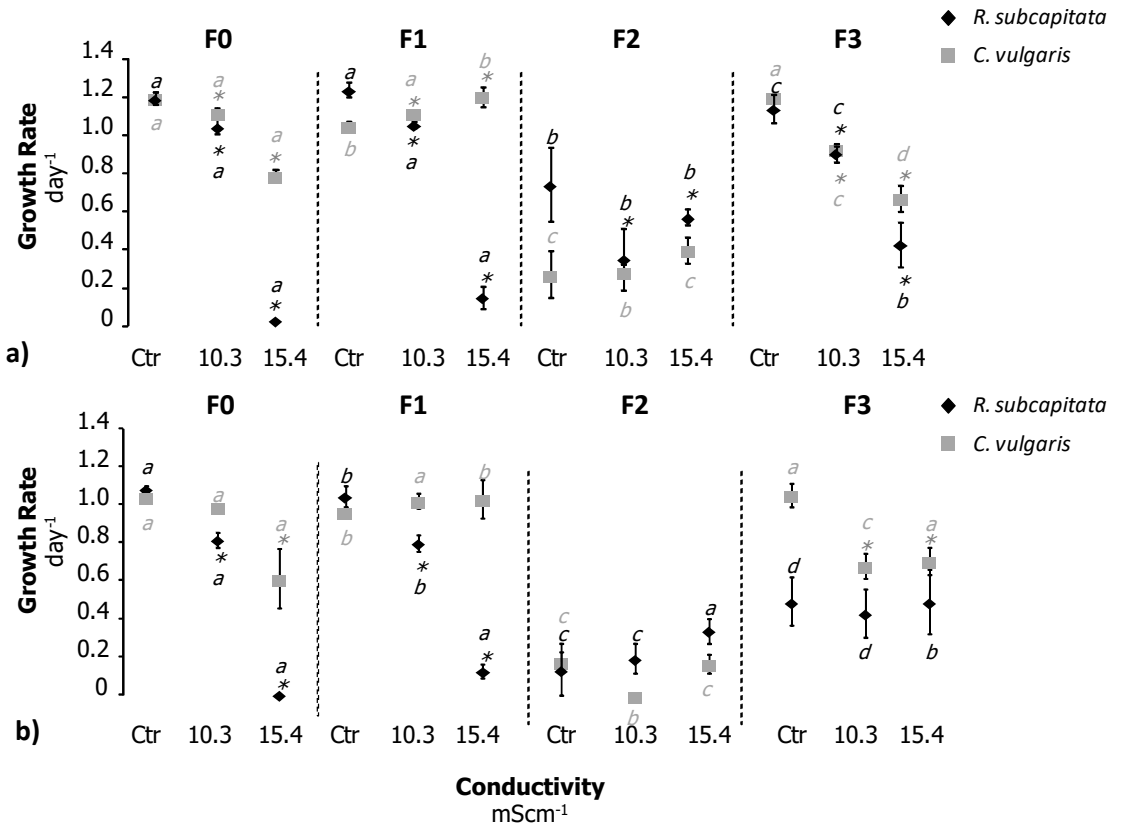


Figure 2. Average growth rates (d^{-1}) of *Raphidocelis subcapitata* and *Chlorella vulgaris*, before and after generational acclimation to low levels (respective $EC_{25,72h}$) of natural seawater (SW) (error bars correspond to the standard deviation), under a) single exposure and b) competition exposure to two levels of NaCl and a control. Letters (a, b, c) indicate homogenous groups between different generations. *represent significant differences ($p \leq 0.05$), within each generation, of each SW treatment relatively to the control (black: treatments with *R. subcapitata* relatively to control and grey: treatments with *C. vulgaris* relatively to control).

5.Discussion

5.1 Monospecific growth inhibition assays

In the present study, *R. subcapitata* presented EC_{25s} (4.6 and 10.3 $mScm^{-1}$) lower than the ones obtained for *C. vulgaris (*6.9 and 15.4 $mScm^{-1}$) both for NaCl and SW. These results are inline with previous works, which also report *C. vulgaris* to be more tolerant to salinity than *R. subcapitata* (Table 1). The observed inter-species differences in sensitivity to salinity are probably related with distinct morphology and physiological aspects inherent to each species (Dunlop et al., 2008). *Chlorella vulgaris* can form colonies as a result of the attachment of daughter-cells during cell division (Watanabe et al., 2006) and may possess the ability to produce a mucilage envelope (Luo et al., 2010; Bock et al., 2011). These two characteristics may function

as a mechanism of protection for the colonies core, insuring that sufficient healthy cells can continue to perform cell division. Secretion of mucilage by *R. subcapitata* has also been observed (Ferguson, 2011), but cells are usually solitary, which may be disadvantageous, because each cell has direct contact with the salt solution. As well, the higher tolerance of *C. vulgaris* to salinity could be related to a more efficient maintenance of internal concentrations of K^+ ions that are known to be involved in Na^+ cellular homeostasis (Rubio et al., 1995; Elmaghrabi et al., 2013). Other mechanisms of tolerance to increased salinity have been reported in microalgae. For example, Arisz and Munnik (2011) observed that in *Chlamydomonas*, the response to increased levels of salinity was related to a phospholipase D pathway combined with the action of a phospholipase C/diacyl-glycerol kinase pathway. In general terms, this is translated in the rapid increase of phosphatidic acid (PA), a secondary messenger lipid, which is involved in the biosynthesis of other compounds that offer protection against salt stress. The accumulation of solutes, like sugars (Kerepesi and Galiba, 2000), aminoacids (Khatkar and Kuhad, 2000; Singh et al., 2002) or fats (Lemoine and Schoefs, 2010) is also a mechanism that help to prevent cell plasmolysis because they act as osmoprotectors. It can be hypothesized that such mechanisms may be triggered differently in *C. vulgaris* than in *R. subcapitata*, determining also their different sensitivities to salt stress. The higher tolerance of *C. vulgaris* to salt stress is further supported by the findings of Talebi et al. (2013). When comparing salt tolerance within several species of *Chlorella*, these authors, found that *C. vulgaris* successfully adapted to high levels of salinity (0.75M NaCl) when compared to *C. salina* (considered a more salt resistant species). Its growth was severely affected at this concentration level but was not totally inhibited. These authors went further and verified that and increased external concentration of Na^+, resulted in an internal decrease of Ca^{2+} (49% reduction). The latter ions are an important element for algae, functioning in photosynthetic activities, and maintenance of thylacoid membrane integrity and glycerol metabolism. This loss was lower for *C. vulgaris* (freshwater) than for *C. salina*, revealing that *C. vulgaris* can grow more than *C. salina* at high salt concentrations. Furthermore, other studies that analyzed cell components, like vacuoles, showed that such compartments are important reservoirs, not only of proteins, aminoacids and sugars, but also of many inorganic ions such as

Na^+, K^+, Ca^{2+}, Cl^-, SO_4^{2-} (Matile, 1978; Ryan and Walker-Simmons, 1983; Martinoia et al., 2000; Etxeberria et al., 2012). Although such analysis was not addressed in this study, differences in the affinity to storage and/or to accumulate the excessive amount of ions may be cell-specific (Wink, 1993) and could also explain the difference in salt tolerance between the two species of microalgae studied in the present work.

The two-tested species of microalgae were more sensitive to NaCl than to SW, exhibiting lower EC_{25s} values when exposed to the latter one. Therefore, these results suggest that the use of NaCl for predictive risk assessment of salinization due to SW is a protective surrogate. These were expected results since earlier studies that compared natural or artificial SW with a surrogate salt (NaCl), observed that the former was less toxic (Ghazy et al., 2009; Kefford et al., 2004). The more complex ionic structure of SW may explain the obtained results. Seawater composition includes, besides sodium (Na^+) and chloride (Cl^-), other ions at lower concentrations, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sulphate (SO_4^{2-}), potassium (K^+) or borate (B^+) (Lee et al., 2007). Therefore, the presence of extra concentrations of these elements in the medium may help producers to increase its tolerance to higher salinity levels. In the present work, algae exposed to NaCl or SW could have gone suffered adverse effects caused by high concentrations of Na^+, but when exposed to SW the presence of other ions, like boron and calcium, could have reduced those adverse effects. Furthermore, the presence of additional Ca^{2+} ions could have decreased the intake of Na^+ ions by algae, since the former competes with the later for the binding sites in proteins located in the cell membrane and cell wall, which could also contribute to the observed lower toxicity of seawater.

Table 1: Ecotoxicological endpoints for *Raphidocelis subcapitata* and *Chlorella vulgaris* exposed to increased salinity. EC_{50} – effect concentration causing 50% of reduction of growth rate. NOEC – no observed effect concentration. LOEC – Lowest observed effect concentration. SW- seawater; NaCl – sodium chloride

	Endpoint	$mScm^{-1}$	Reference
Raphidocelis subcapitata	EC_{50}	0.9 (NaCl)	Santos etal., 2007
	EC_{50}	2.7 (NaCl)	Simmons, 2012
	NOEC	0.5(NaCl)	
	LOEC	5.9 (NaCl)	Leitão, 2013
		9.6 (SW)	
Chlorella vulgaris	2- fold reduction in growth rate	30.5 (NaCl)	Barghbani et al., 2012
	No growth	45.7 (NaCl)	

5.2 Exposure to low levels of salinity

The ability of the microalgae to acclimate to low levels of salinity was also assessed. In the present work, results obtained with generational exposure to low levels of NaCl suggest that the two-studied species were able to cope with low levels of salinity and acclimate to them since, when exposed solely to low levels of salinity, *R. subcapitata* increased its growth rates from F1 to F3. Previous studies reported the ability of *C. vulgaris* to maintain its performance when exposed to 500 mM of NaCl (44.5 $mScm^{-1}$), above which oxygen rates and heating production started to decrease indicating that a possible toxic effect is caused by Na^+ ions on the efficiency of the electron transport chain (Alyabyev et al., 2007). However, for *R. subcapitata*, Dassanayake (2008) found that a five-generational acclimation to salinities of 3 $mScm^{-1}$ were sufficient to reduce growth rates, number of cells divisions and generation time, from the second generation onwards. The earlier acclimation of *C. vulgaris* comparatively to *R. subcapitata* may be related with the mechanisms involved in salinity tolerance described above. For example, stimulation of higher rates of mucous production by *C. vulgaris* after generational exposure to low salinity levels or a faster activation of the phospholipase C/diacyl-glycerol kinase pathway. More, studies performed with *Dunaliella bioculata* (Berube et al., 1999), reported that a long-term contact with

salt, could enhance the metabolism of Golgi apparatus and the endoplasmic reticulum, providing protection against cell plasmolysis, through cellular scaffolding (production of proteins involved in cellular signaling). Although the unicellular algae referred above is a marine organism, this kind of process to deal with salt stress can be, in a smaller scale, a possible mechanism of response in unicellular freshwater species. For *C. vulgaris* results suggest that this species is able to acclimate to low levels of NaCl right after F1 generation, under competition scenarios while for *R. subcapitata* there are no evidences of acclimation under such conditions.

Regarding acclimation to SW different results were observed. A maintenance or increase in salinity tolerance seems to occur from F0 to F1, for *R. subcapitata* and *C. vulgaris*, but a drastic decrease in growth rate of the two algae species follows from F1 to F2, suggesting an increase in sensitivity. Nonetheless, increasing growth rates in F3 generation suggest that both species could afterwards evolve acclimation to SW. Such lower growth rates registered at F2 might be related to the complex chemical composition of SW. It is possible that microelements present in SW (but not in NaCl), along time, accumulated inside the algae cells, constituting a chemical stress after two generations and provoking adverse effects in the cells (e.g. Blakeslee et al., 2013).

This ability to acclimate to low levels of salinity, exhibited by both species, may be an important mechanism to assure the resilience of algae populations, and consequently functions performed by them, and the health of the ecosystem when exposed to such stress. More, by neglecting the integration of such responses in risk assessment we may be overestimating the risks that saline intrusion may pose to freshwater ecosystems (Fischer et al., 2013).

5.3 Competition

In natural environments, intra- or inter-specific competition is a factor that only by itself exerts some kind of pressure on organisms (e.g. Katzmann et al., 2003), since when together, species compete for similar resources (e. g. Bengtsson, 1986) (in the present work case, the two studied algae species can compete, for example, for light, nutrients and space). However, such competitive relationship may be changed due to the presence of other environmental

perturbations, namely chemical substances that may render the most competitive species, under control conditions, the least one in the presence of those substances. In fact, several works have already reported that inter- or intra-specific competition may enhance the toxicity caused by some stressors (Foit et al., 2012; Knillman et al., 2012). On the other hand, competition may be destabilizing (e.g. inter-specific), leading to the shift on the competitive outcome between species or in the worst case, the exclusion of one of the competing species (Hutchinson, 1961). Plus, it was observed that, when exposed under competition, the acclimation pattern to low levels of salinity remained for *C. vulgaris* but not for *R. subcapitata* and, most probably, this resulted in a lower competitive advantage of *R. subcapitata* relatively to *C. vulgaris* when exposed to low levels of NaCl.

In the present work, regarding the influence of competition in the growth rate of the two species, it was observed that under control conditions *R. subcapitata* exhibited a higher growth rate relatively to *C. vulgaris*. However, when exposed to NaCl or SW, it seems that salinity alleviate the competitive pressure over *C. vulgaris*, which gained competitive advantage and was able to match or overcome the growth rate of *R. subcapitata*. These results suggest that small changes in salinity may determine and change the competitive outcome between the microalgae. In addition, the higher salinity tolerance of *C. vulgaris* observed during the course of this experiment certainly favors the outcome of the competition along time. Shifts in competitive outcome have already been seen by Loureiro et al. (2013) experiments between *Daphnia* and *Simocephalus* under NaCl exposure. In the lowest salinity treatments, *Daphnia* presented advantage over *Simocephalus*, but at the highest salt treatment (1.5 gL^{-1}, approximately 2.3 $mScm^{-1}$) was possible to observe an inversion in the competitive outcome. Though, it is important to refer that *Simocephalus* success at the highest salinity treatment was derived from a decreased in the pressure exerted by *Daphnia* population and not by its higher tolerance towards salinity (Loureiro et al., 2013). More, some species are able to activate mechanisms of defense that contribute to their resilience in stressed environments. For instance, findings of Fergola et al. (2007) suggest that *C. vulgaris* competitive advantage is also related to the fact that this species is able to produce an allelopathic substance (chlorellin), i.e. leading to the inhibition of other algae growth. In the present study, we believe

that *C. vulgaris* advantage over *R. subcapitata* is mainly due to its higher tolerance to salinity stress. Chlorellin release is believed to happen only in older cultures (15-days older) (Pratt and Fong, 1940) and the cultures here where never older than 4-days. But, the production of these compounds may affect behavioral features of other species and compromised their performance. For instance, *Daphnia* was shown to present feeding selectivity: it was able to avoid sources that produced toxins (Tillmans et al., 2011). Alike, in a freshwater ecosystem suffering from saline intrusion, if the main food source (e.g. *C. vulgaris*), although present, may not be suitable to sustain other communities that depend on it to feed and survive (e.g. zooplankton). Thus, algae communities have the potential to regulate the dynamics of zooplanktonic communities and, certainly, through chain reactions will also influence fish assemblages (Carpenter and Rounds, 2013; Nicolle et al., 2010). Therefore, since algae communities are the basis of the trophic chain, these changes may result in major alterations in stressed freshwater ecosystems, for example by causing indirect effects at higher trophic levels.

6. Conclusions

Regarding the aspects mentioned above, the use of NaCl as surrogate to assess salt stress should be a careful decision. At the initial growth inhibition assays and at F0, we observed that SW was less toxic than its surrogate. These results previously suggested that NaCl could be used as a safe surrogate, because its higher toxicity could be work as a worst-case scenario. Predictions based in NaCl toxicity would actually be considered as a more protective approach in ecological risk assessment regarding sea level rise. Furthermore, the results obtained in the long-term exposure suggest that SW maybe more toxic than NaCl. So, the use of this salt as a surrogate should be used in a preliminary approach to rapidly understand the effects of saline intrusion, but, long term exposure and more complex scenarios (like the competition scenario here presented) must be studied using more ecologically relevant scenarios and by using, preferably, natural SW. *Raphidocelis subcapitata* dominance in single and competition treatments under controlled conditions was substituted by *C. vulgaris* dominance under salt stress. Also, decreasing growth

rates along generational exposure to low salinity levels reflects the susceptibility of freshwater ecosystems equilibrium and with probable consequences to higher trophic levels.

7.Acknowledgements

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Chapter VI

Mesocosm simulations of seawater intrusion into warm temperate coastal freshwater lagoons and its aftermath dilution

Mesocosm simulations of seawater intrusion into warm temperate coastal freshwater lagoons and its aftermath dilution

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Abstract

Coastal freshwater ecosystems will face seawater intrusions as a consequence of sea level rise, predicted to be of 80 cm by the end of 2100 by the worst scenario projected by the International Panel for Climate Changes. Salinity is an abiotic factor influencing communities' abundance, structure and composition by, for example, influencing relationships among populations that constitute a community. The present work intended to evaluate the long-term effects of a pulse of seawater intrusion in the structure and composition of a freshwater invertebrate community simulated in outdoor mesocosms. For this, mesocosms were pulsed-contaminated with natural seawater, collected at the North Atlantic Ocean (52.7 $mScm^{-1}$), to attain different salinity levels (0, 2.02, 3.34, 5.51, 9.09, and 15.0 $mScm^{-1}$). Four distinct periods of exposure were established: (i) a pre-intrusion undisturbed phase (pre-I phase, 21 days) to allow the communities to reach an equilibrium in the mesocosms, (ii) a saltwater intrusion phase (I phase, 33 days of exposure to the different salinity levels), (iii) a period of sequential dilutions (D phase, 58 days) and (iv) a period of recovery, starting from the end of dilutions in phase D onward (post-D phase, 126 days). Physical (pH, dissolved oxygen, conductivity), chemical (nutrients) and biological parameters (macroinvertebrates, zooplankton and chlorophylls) were monitored in all these phases. The most common macroinvertebrate taxonomic groups identified were Diptera, Ephemeroptera and Mollusca. Diptera showed the highest abundances in the control and at salinity 15 $mScm^{-1}$, during the post-D phase, while Mollusca only appeared in samples at salinities 9.1 and 15 $mScm^{-1}$, during this same post-D phase. Ephemeroptera did not recover from the pulse of seawater intrusion at concentrations of 15 $mScm^{-1}$. At post-D phase a decrease in the species diversity of macroinvertebrates was observed with increasing conductivity values. Regarding zooplankton the most abundant groups were, in decreasing order, Rotifera, Copepoda, and Branchipoda during I

phase. Despite the decrease in organism abundance during this phase, a slight increase in organism abundance was observed at the end of this period, suggesting the beginning of recovery of the community. During the post-D period, Rotifera also showed no capacity of recovery from the salinity pulse at the highest conductivity tested (15 $mScm^{-1}$). Plus, still in post-D, some fluctuations in both abundance and species diversity were observed.

Overall, no abrupt decrease in abundances of the studied invertebrate groups was observed after the seawater pulse and recovery period. However, at the end of the experiments changes in the community structure were observed when comparing control conditions and treatments with highest condutivity values, which may foresee changes in the function of these communities.

Keywords: freshwater; communities; salinization; mesocosms

1. Introduction

Salinization of freshwater coastal ecosystems is a global and increasing threat (e.g., Schallenberg et al., 2003; Pinder et al., 2005). The 5th report of the Intergovernmental Panel on Climate Change (IPCC, 2014), predicted a worst scenario of seawater level rise of 80 cm by the end of 2100 suggesting that, in a near future, seawater intrusions in coastal ecosystems will, most probably, be intensified (IPCC, 2014). Furthermore, such intensification will be aggravated by the expected increase in frequency and intensity of extreme climate events (e.g., Casanueva et al., 2014). The deterioration of these coastal ecosystems may entail not only social and economic problems (as providers of several services for human populations) but also can lead to major biodiversity losses since they serve as shelters/habitats for a large number of species (Bobbink et al., 2006; Delaney et al., 2015; MEA, 2016). Salinity may be an important abiotic factor affecting freshwater organisms ability to osmoregulate. Salt stress is known to induce changes at molecular (Hu et al., 2011; Reddy et al., 2011) and individual levels, namely on plants (Çöçü and Uzun, 2011), cladocerans (Leitão et al., 2013; Struewing et al., 2014), chironomids (Bidwell and Gorrie, 2006; Lob and Silver, 2012), frogs (Kearney et al., 2012; Santos et al., 2013), among others. Accurate predictions of effects at higher levels of biological organization demand for mesocosms studies

and field observations, which have also been pursued (e.g., Schallenberg et al., 2003; Pilkaityté et al., 2004; Silberbush et al., 2005; Cañedo-Argüelles et al., 2012; please see Table 1). For instance, a 72-hours exposure, in mesocosms, to a 5 $mScm^{-1}$ conductivity was found to reduce total abundance, richness and diversity of a benthic macroinvertebrate community along the Llobregat River, Spain (Cañedo-Argüelles et al., 2012). A lower salinity intensity of 2.8 $mScm^{-1}$, reduced macroinvertebrate richness by around 30%, at the Meurthe River (France), although without adverse effects on evenness or total abundance along the entire saline gradient (approximately from 0.4 to 5.2 $mScm^{-1}$) (Piscart et al., 2005).

Natural populations and communities may deal with increased salinity over time through tolerance acquisition. As an example, in a study comprising records of zooplankton (e.g., rotifers, cladocerans, ostracods, copepods) and macroinvertebrates (e.g., diptera, coleoptera) samplings from 230 Australian wetlands with different degrees of salinization (from the year 1997 to the year 2000), Pinder et al. (2005) verified that species richness was not affected by salinities lower than 4.1 gL^{-1} (approximately 8.1 $mScm^{-1}$), but declined dramatically above this salinity value. Nevertheless, when the most salt tolerant species were excluded from analysis, the endpoint started to decline sharply at salinities of only 2.6 gL^{-1} (approximately 5.2 $mScm^{-1}$). The acquisition of tolerance, namely by the spread of tolerant species, may favor communities resilience under freshwater salinization scenarios (Pinder et al., 2005). Furthermore, in the case of zooplankton and aquatic plants, the presence of a viable resting eggs or seeds bank can be determinant for recovery after a salinization event (e.g., Ning et al., 2011; Santangelo et al., 2014). Macroinvertebrates, fish and amphibians lack this life stages, though it is also possible their later recolonization of recovered salinized-ecosystem. In the case of macroinvertebrates, the recolonisation will rely on groups with intermediate to high aerial dispersity capacity in their adult phase (many insects) rather than in groups with an aquatic adult stage and very poor dispersal ability (e.g., Anelida, Mollusca) (Heino, 2013). Long-term large-scale experiments can provide realistic insights on how real freshwater ecosystems will respond to increased salinity levels. But to what concerns temperate freshwater ecosystems and considering what is mentioned above, this information is scarce or inexistent, and it is necessary to quickly fill this gap in knowledge. As

far as we are aware of, only very few studies, if any, addressed seawater intrusion in warm temperate freshwater coastal sytems around the North Atlantic. Accordingly, this study intended to evaluate the long-term effects of a sewater intrusion pulse in the structure and composition of a freshwater invertebrate community representative of freshwater Portuguese ecosystems. In this long-term experiment, simulated in outdoor mesocosms, zooplanktonic and macroinvertebrates communities composition and abundance were followed during exposure to several salinity levels and during the recovery of the systems to their initial state. These groups were studied due to their ecological relevance in nutrient recycling processes and as important food sources (e.g., Graça, 2001; Vanni, 2002; Lacerot et al., 2013).

Table 1: Resume of long-term studies focusing the effects of salinization on freshwater communities.

Location	Salinity Levels	Endpoints	Findings	Duration	Study of recovery	Reference
New England Tablelands and Murray River NSW, Australia	1000 mgL^{-1} (≈ 1.98 $mScm^{-1}$, at 20ºC) 5000 mgL^{-1} (≈ 9.90 $mScm^{-1}$, at 20ºC)	Aquatic plants seed bank	>1000: ↓ richness, abundance, above ground biomass	16 weeks	No	Nielsen et al., 2003
		Zooplanktonic egg bank	>1000: ↓ richness and abundance	18 days		
Lake Waihola South Island New Zealand	Gradient: from 1.2 to 4.7 PSU (≈ranging from 2.0 to 7.5 $mScm^{-1}$)	Zooplankton	↓ richness, abundance along the gradient >1.3 PSU cladocerans absent from community composition	2 non-consecutive years (1997/98; 1999/2000)	No	Schallenberg et al., 2003
Murray-Darling Basin NSW, Australia	Gradient: from 300 to 5000 mgL^{-1}	Aquatic plants seed bank	>1000: ↓ richness, abundance	16 weeks	No	Brock et al., 2005
		Zooplanktonic egg bank	>1000: ↓ richness, abundance	18 days		
Dead Sea Basin Israel	Four salinity levels: 0, 10, 20, and 30 gL^{-1} (≈ corresponding to 0, 19.8, 39.6. and 59.4 $mScm^{-1}$)	Aquatic insect community	0 – 10: no significant differences on evenness or total abundance; highest diversity >20: ↓ richness, abundance	41 days	No	Silberbush et al., 2005

Table 1 (Cont.): Resume of long-term studies focusing the effects of salinization on freshwater communities.

Location	Salinity Levels	Endpoints	Findings	Duration	Study of recovery	Reference
Rambla Salada SE Spain	Gradient: from 3.5 to 76.4 gL^{-1} (≈ ranging from 6.93 to 151.3 $mScm^{-1}$)	Biomass of primary producers	↓ richness along the gradient ≈ abundance and evenness (*Cladophora glomerata*, *Ruppia maritima*)	2 non-consecutive years (from June/03 to October/03 and from June/04 to October/04)	No	Velasco et al., 2006
		Macroinvertebrates	↓ richness along the gradient ≈ abundance and evenness			
Massa Lagoon SE Morocco	Gradient: salinity ranging from 12.01 to 24.6 (≈ ranging from 20.1 to 38.7 $mScm^{-1}$)	Zooplankton	↑ salinity: ≠ composition (substitution for more salt-tolerant species)	6 months (from February/08 to July/08)	No	Badsi et al., 2010
Mossoró River Estuary NE Rio Grande do Norte Brazil	Gradient: from 10 to 30 gL^{-1} (≈ ranging from 19.8 to 59.4 $mScm^{-1}$)	Zooplankton	↑ salinity: ≈ richness and abundance ≠ composition (substitution for more salt-tolerant species)	12 months (from October/06 to September/07)	No	Medeiros et al., 2010
Murray River NSW, Australia	46.8 $mScm^{-1}$ 209 $mScm^{-1}$ (salinity levels corresponding to the lowest and highest conductivity sediment samples, respectively)	Aquatic plants seed bank	↓ richness with ↑ salinity	12 weeks	Yes (recolonization after disturbance)	Ning et al., 2011
		Zooplanktonic egg bank	↓ richness with ↑ salinity			
Llobregat River Barcelona, Spain	Four salinity levels: 0, 1.5, 2.5, and 5 $mScm^{-1}$	Macroinvertebrates	at 5 $mScm^{-1}$: ↓ richness, abundance	72 hours	No	Cañedo-Argüelles et al., 2012

Table 1 (Cont.): Resume of long-term studies focusing the effects of salinization on freshwater communities.

Location	Salinity Levels	Endpoints	Findings	Duration	Study of recovery	Reference
Macquarie Marshes NSW, Australia	Gradient: from freshwater to 13.5 gL^{-1} (≈ ranging from freshwater to 26.7 $mScm^{-1}$)	Zooplanktonic egg bank	↓ richness, abundance along the gradient	12 months	Yes (10 months)	Nielsen et al., 2012
Willalooka, South Australia	Three salinity levels: 300; 5000 and 15000 mgL^{-1} (≈ corresponding to 0.60, 9.9 and 29.7 mScm-1; note: salinity levels tested only on the lowest conductivity sediment, Willalooka)	Zooplanktonic egg bank	At 15000 mgL^{-1}: ↓ richness and abundance (significantly lower compared to other two salinity levels)	21 days	No	Toruan, 2012
Imboassica Lagoon Brazil	Gradient: from <2 to > 30 gL^{-1} (≈ ranging from < 3.95 to > 59.4 $mScm^{-1}$)	Zooplanktonic egg bank	<2 to 8 gL^{-1}: ≈ richness, abundance >16 gL-1: ↓richness, abundance	15 months	Yes (ability to hatch when return to freshwater)	Santangelo et al., 2014
University of Maryland Baltimore, USA	Two chloride levels: 177 and 1067 mgL^{-1} (≈ corresponding to 0.58 and 3.49 mScm-1; note: as a measure of dissociation of ions from road salts)	Zooplankton	↑ $[Cl^-]$: ≠ composition (substitution for more salt-tolerant species)	3 months (from May/09 to July /09)	No	Van Meter and Swan, 2014
Vistula Lagoon Baltic Sea	Gradient: from 1.5 to 9.3 PSU (≈ ranging from 2.5 to 14.5 mScm-1; note: eleven sampling sites)	Zooplankton	↑ salinity: ↓richness, abundance ≠ composition (substitution for more salt-tolerant species)	4 months (from June/07 to September/07; repetition of the same sampling period in the years of 2008, 2009, 2010 and 2011)	No	Paturej and Gutkowska, 2015

2. Materials and methods

2.1 Mesocosms

A long-term experiment was carried out in six model outdoor lentic macrophyte-based ecosystems (mesocosms) at the campus of Escola Superior Agrária de Coimbra (Coimbra, Portugal, 40º12'N, 8º27'W) from November 2013 to June 2014. Containers of black rigid polyvinyl chloride, with a total capacity of 1500 L, 1.5 m of diameter and 1 m of depth, were completely buried in the soil. Each mesocosms was filled with 1000 L of water, of which 600 L were collected from a nearby spring-fed stream (Olhos d'Água, Rio Anços – 39º58'N, 8º24'W) and the remaining 400 L came from a well (with a conductivity of approximately 0.27 $mScm^{-1}$).

The bottom of each mesocosms was fulfilled with 300 L of sediment collected in Lagoa dos Teixoeiros (40º18'N, 8º46'W). Sediment characteristics are described at Table 2. The humidity of sediment samples was quantified by removing sediment moisture by oven-drying technique (temperatures between 105-110ºC, overnight). Total organic matter content (TOM) determination was performed through weight loss after dry soil sample combustion at 450ºC for 5 hours according to Byers et al., 1978). Grain size analysis was performed using a sieve tower with meshes between 63 µm and 4 mm.

Macroinvertebrate and zooplankton collection was made at Olhos d'Água (Rio Anços – 39º58'N, 8º24'W) and at reference lagoons (Lagoa dos Teixoeiros, Lagoa das Braças - 40°14'32''N, 8°48'17''W) and Lagoa da Vela (40°16'N, 8°47'W). Macroinvertebrates were collected using a standard hand-net and carefully transported in plastic vessels and zooplankton trough water filtration with a 50 µm conical shaped hand-mesh, with a sample bucket at the far end. Collections were evenly distributed to colonize the each mesocosms. A total of 16 macrophytes specimens per mesocosms were replanted (*Potamogeton* sp. And *Myriophyllum* sp., eight of each) to provide shelter for the test organisms.

Table 2: Mesocosms sediment characterization: humidity (%), total organic matter (TOM - %) and grain size distribution (expressed in % in relation to the total sediment dry weight).

Sediment Characteristics		
Humidity (%)	22.6	
TOM (%)	1.229	
Granulometry		
Denomination	Size range (metric)	%
>2 mm	Gravel fraction	0.033
1-2 mm	Very coarse sand	0.046
0.5-1 mm	Coarse sand	11.99
0.25-0.5 mm	Medium sand	80.07
125-250 µm	Fine sand	7.67
63-125 µm	Very fine sand	0.12
< 63 µm	Silts and clays	0.06

2.2. Experimental design

Four distinct phases were established during this 217-days long experiment: a 21-days long pre-intrusion undisturbed phase (pre-I phase, up to day 0 of the experiment, considered as the day when seawater intrusion was performed), the seawater intrusion phase (I phase, up to day 33), a 58-days long sequential dilutions phase (D phase, up to day 91) and a 126-days long post-dilution phase (post-D phase, up to day 217).

The I phase started with a pulse of natural seawater (SW, conductivity of 52.7 $mScm^{-1}$), collected in the Atlantic Ocean (Portuguese coast: 40°9'10"N, 8°52'22"W) to each mesocom (Venâncio et al., 2017a, 2017b). The following independent salinity intrusions were simulated: 0 (control), 2.02, 3.34, 5.51, 9.09, and 15.0 $mScm^{-1}$ (using a 1.65-fold geometric progression factor), which correspond to salinities of 1.03, 1.75, 2.97, 5.08, and 8.72. This range of salinity levels was chosen based on previous laboratorial tests that suggested that low to intermediate levels of salinity ($2.5 < x < 5$ $mScm^{-1}$) would already induce adverse effects in some groups (e.g., reproduction of the rotifer *Brachionus calyciflorus* and the cladoceran *Daphnia longispina* or growth rate of the green algae *Chlorella vulgaris*; Chapter II). The upper limit was based on previous works that reported that salinity levels below 10 $mScm^{-1}$ did not induce any strong response on the diversity of the exposed community (e.g., Horrigan et al., 2005). The pulse was performed by removing

previously calculated amounts of freshwater and refilling with corresponding seawater volumes, to attain the desired final conductivity.

The D phase consisted of periodic dilutions of each mesocosms by replacing each time 10% of its water volume. During the D phase, eleven dilutions were performed at days 65,68, 70, 75, 77, 79, 82, 84, 86, 89, and 91 after the seawater intrusion (day 0). All water removed from the mesocosms was filtered (50 µm) and organisms retained were returned to the respective mesocosms. Refilling of the water volume was made through the addition of well-water (conductivity of 0.27 $mScm^{-1}$). Dilutions ended when the mesocosms reached conductivity levels near those reported before the I phase.

In the last phase, post-D, mesocosms were left intact, without removal or addition of medium.

2.3 Biological measurements: macroinvertebrate, zooplankton and chlorophyll sampling

Macroinvertebrate collection was performed using a standard hand-net. At each collection, macrophytes in each mesocosms were gently shaken with the hand-net along three equidistant horizontal axes (Fig. 1a). Collection was made in a similar way in all mesocosms and according to the timeline presented at Fig. 2a. Organisms were carefully collected and stored in 50 mL plastic containers with 70% ethanol. In the laboratory, samples were carefully screened to separate organisms from other materials (such as plants remaining) and identified to the lowest possible taxonomic level, according to Tachet et al. (2010).

Zooplankton sampling was performed with the help of a PVC tube with a screw cap (Fig. 1b). The tube was inserted along the water column, without touching the bottom, and then rapidly capped, thereby making vacuum and allowing collecting the water. The sampling procedure was performed three times, making a total of four liters at each mesocosms (Fig. 1b) and according to the timeline presented at Fig. 2a. All collected water was then filtered through a 50 µm mesh sieve. Samples were conserved in 70% ethanol. In the laboratory, every sample was resuspended and, immediately, 1 mL of each was analyzed. Zooplanktonic organisms were identified to the lowest taxonomic level possible (Streble and Krauter, 1988; Witty, 2004).

Water samples to analyze chlorophyll content were collected from the filtered water used previously to collect zooplankton samples (Fig.1b and timeline at Fig. 2a). Chlorophylls (Chl) *a* and *b* were processed immediately according to the Standard Methods of the American Public Health Association (APHA, 1998) with chlorophyll concentrations estimation made according to the following equations:

Chl *a* (μgL^{-1}) = [11.85 (A_{664}-A_{750}) – 1.54 (A_{647}-A_{750}) – 0.08 (A_{630}-A_{750})] × V_1 /(V_2 ×I)

Chl *b* (μgL^{-1}) = [-5.43 (A_{664}-A_{750}) + 21.03 (A_{647}-A_{750}) – 2.66 (A_{630}-A_{750})] × V_1 /(V_2 ×I)

where A_{630} is absorbance at 630 nm; A_{647} is absorbance at 647 nm; A_{664} absorbance at 664 nm; A_{750} absorbance at 750 nm (turbidity correction factor); V_1 is the volume (mL) of acetone at 90% used for extraction; V_2 is volume (mL) of filtered water sample and I the optical length (1 cm) of the spectrophotometric cell.

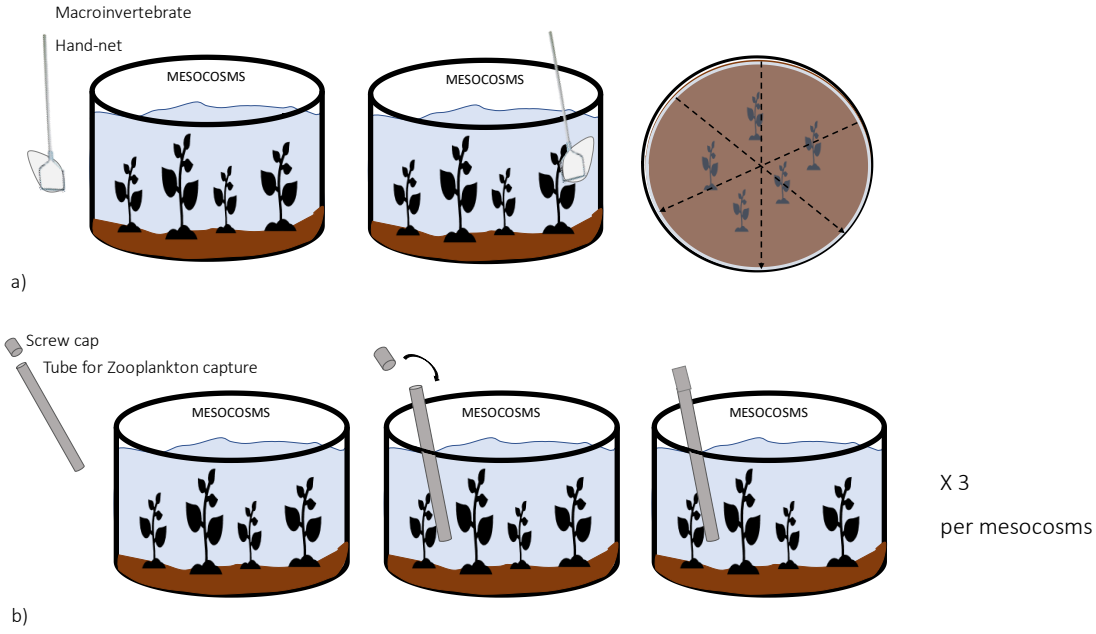

Figure 1: Schematic representation of the collection procedure of a) Macroinvertebrates (where dashed lines represent the equidistant capture trails performed within each mesocosms) and b) Zooplankton.

2.4 Physical and chemical measurements

A sample of the water (approximately 300 mL) was collected from each mesocosms according to the timeline presented at Fig. 2b. Transportation of the samples for chemical analysis to the laboratory was performed in refrigerated boxes (4ºC). A Hach DR/2000 photometer (Hach company, Loveland, CO, USA) was used to quantify the following nutrients concentrations (mgL^{-1}), according to Hach manual: ammonia ($N\text{-}NH_3$; method 8038), nitrates ($N\text{-}NO_3^-$; method 8192), nitrites ($N\text{-}NO_2^-$; method 8507), phosphorous (PO_4^{3-}; method 8048), and sulphates (SO_4^{-2}; method 8051). Three replicates per nutrient were performed, within each water sample.

Conductivity, salinity, pH, and dissolved oxygen were measured in situ with field probes (Wissenschaftlich Technische Werkstätten-WTW conductivity440i, Weilheim, Germany; WTW pH330i and WTW OXI 330i, respectively) whenever macroinvertebrates and zooplankton sampling was performed, according to Fig. 2b. Temperature and light were recorded at 15 min intervals using data loggers (Hobo Pendant Temperature/Light 64K Data-loggers, Bourne, MA, USA). Two data loggers were used per mesocosms: one was left to the surface and the other one was used to measure light and temperature at the sediment surface, in the bottom of the mesocosms (at a depth of approximately of 85 cm and at a place without being shadowed by the macrophytes present at the mesocosms).

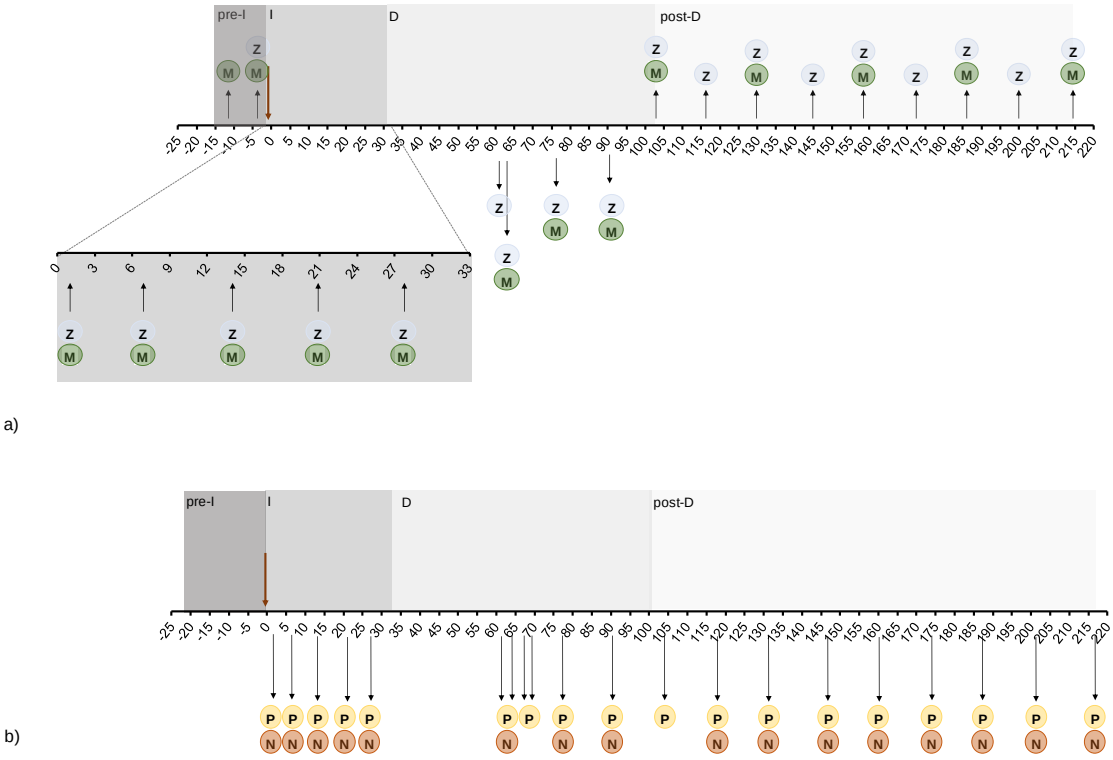

Figure 2: Long-term experiment sampling schedule for a) invertebrates and b) water samples. Abbreviations stand for: Z – Zooplankton; M- Macroinvertebrates; C – Chlorophylls; P – measurement of water parameters (pH, conductivity, dissolved oxygen); N – nutrient analysis of the water samples. Black arrows below the XX axis indicating each sampling time (for invertebrates or water samples). Arrow above the XX axis correspond to the saline intrusion moment (dark orange arrow). The pre-I, I, D and post-D denominations correspond to the pre-intrusion, saline intrusion, dilution and post-dilution phases, respectively.

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3. Statistics and Calculations

Data analysis was performed using the software Primer 6 (Clarke and Gorley, 2006) for macroinvertebrate and zooplankton data separately. Abundance data sets were squared root transformed and Bray-Curtis similarity index was calculated.

Significance of multivariate differences among groups was tested with a one-way analysis of similarities (ANOSIM) (Clark, 1993) – a non-parametric test based on a rank permutation procedure, where *Rho* values (R) were computed at a significance level of 0.001. Significant values close to 1.0 mean dissimilarity between groups, values near 0 indicate even distribution within and between groups and R values below 0 indicate that dissimilarities within groups are greater than between groups.

To compute Spearman correlations coefficients (r) between macroinvertebrate or zooplankton community structures and levels of salinity was applied the BIO-ENV procedure (Clark and Warwick, 2001). Species richness (S) and Shannon-Weaver (H') indexes were also calculated trough Primer 6 software.

4. Results

4.1 Physical, chemical and biological parameters

Salinity levels along the 217-days experiment are shown at Fig. 3. Conductivity increased according to the salinity levels established for each mesocosms (I phase), decreasing after the dilutions carried out during Phase D to a value close to the initial one of each mesocosms (Fig. 3).

Spearman's correlations between conductivity of each mesocosms ($mScm^{-1}$, as a measure of different salinity levels; Fig. 3) and the biological, physical and chemical endpoints have shown that for the I phase, conductivity was negatively correlated with pH (r_s=-0.354, p=0.034), sulphates (r_s=-0.7, p<0.001) and the number of species (r_s=-0.94, p=0.017). For the other parameters, no significant correlations were observed (r_s<-0.83, p>0.06).

For the D period, negative correlations were detected between conductivity and pH (r_s=-0.487, p=0.007) and sulphates (r_s=-0.76, p<0.001), while at the post-D phase negative correlations were only detected between ammonium (r_s=-0.3, p=0.039) and sulphates (r_s=-0.93, p<0.001).

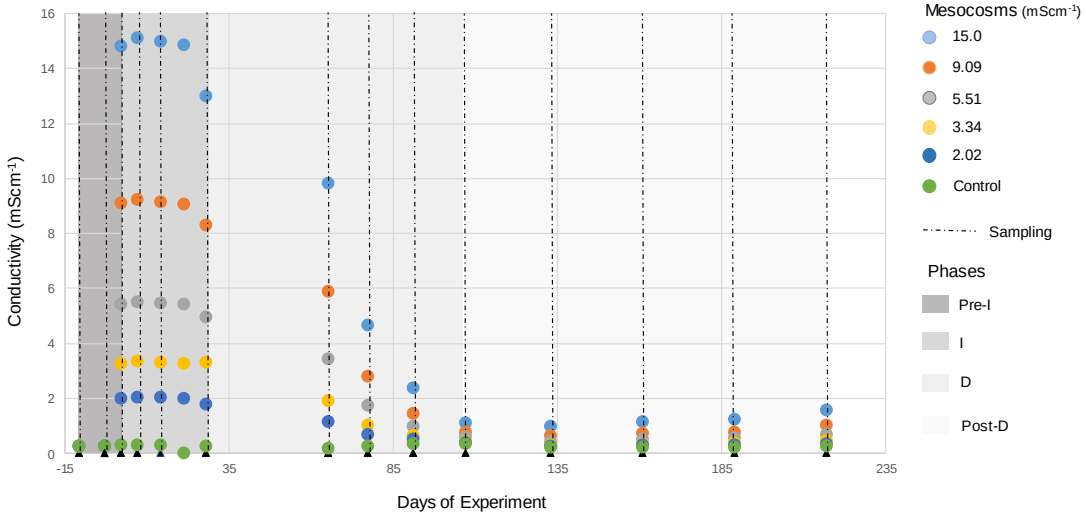

Figure 3: Conductivity ($mScm^{-1}$) values of the water column throughout the outdoor mesocosms experiment within the four established phases: pre-intrusion (pre-I), pulse of saltwater (I phase), periodic dilutions (D phase) and post-dilution (post-D phase).

4.2 Effects of a pulse of seawater intrusion on the invertebrate's community

4.2.1 Macroinvertebrates

Overall ANOSIM analysis between phases, for macroinvertebrates, only revealed significant differences between the phases I and D ($R=0.357$, $p=0.001$) and between the phases I and post-D ($R=0.253$, $p=0.001$). No significant differences were detected between control conditions and salinity levels when considering analysis within each of the established phases ($p \geq 0.008$).

From macroinvertebrate analysis, the most common macroinvertebrate taxonomic groups, identified in the outdoor mesocosms were Diptera, Ephemeroptera and Odonata, which appeared in all phases (Fig. 4). Heteroptera and Coleoptera also appeared during the whole experiment duration but at lower densities (Fig. 4). Diptera showed the highest abundances in the control and at salinity 15 $mScm^{-1}$, in the phase I (Fig. 4 and 5). At the D phase, was noticed an increase in the abundance of the less common groups (in phase I) such as Heteroptera and Coleoptera. Mollusca (composed mainly by snails) presented the highest abundances at the post-D phase. This may have contributed to differentiate the communities of mesocosms previously exposed at different salinity levels (Fig. 5). The Ephemeroptera showed small increments in abundance during the recovery periods (D and post-D phases), but at the highest salinity treatment (15 $mScm^{-1}$) such

recovery was not observed. Diptera and Heteroptera groups increased in abundance during the D and post-D phases. The PCA analysis showed that the post-D phase does not show evidences of segregation between different salinity levels (Fig. 5).

Regarding diversity indexes (Fig. 7a), it was possible to notice an increase in species diversity and evenness with increased salinity in the phase I (Shannon-Wiener index ranging from 0.9 to 1.5 from the lowest to the highest salinity treatment). In the D period, the Shannon-Wiener index ranged between 0.7 – 1.37 and species richness decreased with increasing salinity level (with S=10 in the control mesocosms and S=5 in the highest salinity treatment). In the last experimental phase, the post-D phase, it was noticed an increased in the Shannon-Wiener index at intermediate salinity levels when compared with the previous phase (D phase) and a sharp decrease in this index at the highest salinity treatment when compared to the previous phase (1.4 at the D phase and 0.9 at the post-D phase). Also, the difference in species richness index between control mesocosms and the highest salinity treatments decrease (S= 9, at control mesocosms and S=7 at the highest salinity treatment).

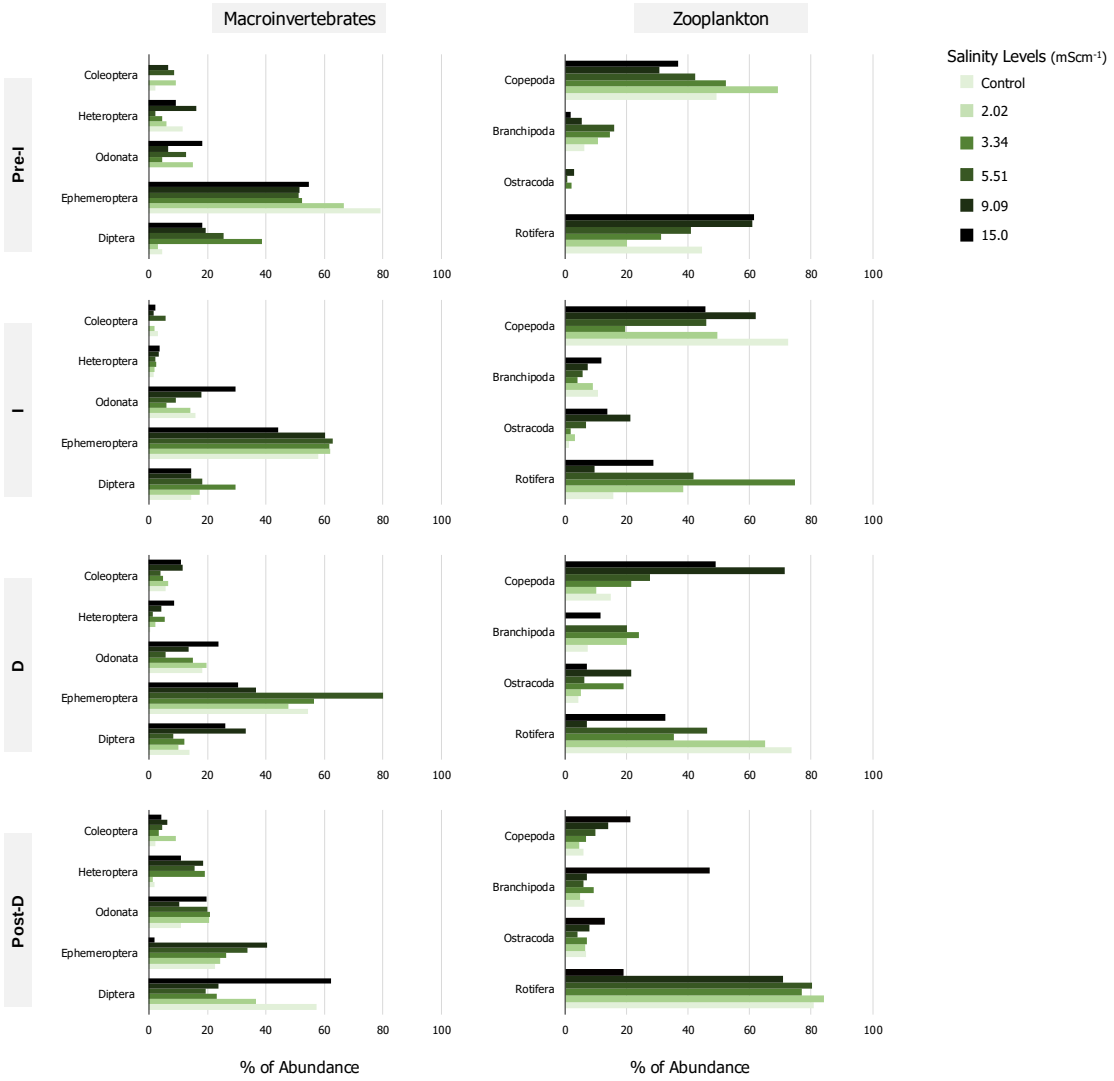

Figure 4: Percentage of abundance of organisms belonging to the five major taxonomic groups of Macroinvertebrates (Coleoptera, Heteroptera, Odonata, Ephemeroptera and Diptera) and the four major taxonomic groups of Zooplankton (Copepoda, Branchiopoda, Ostracoda and Rotifera), sampled in outdoor mesocosms at different salinity levels, for the four periods defined during the experiment: pre-intrusion (pre-I), saline intrusion (I), periodic dilutions (D) and recovery post-dilution (post-D).

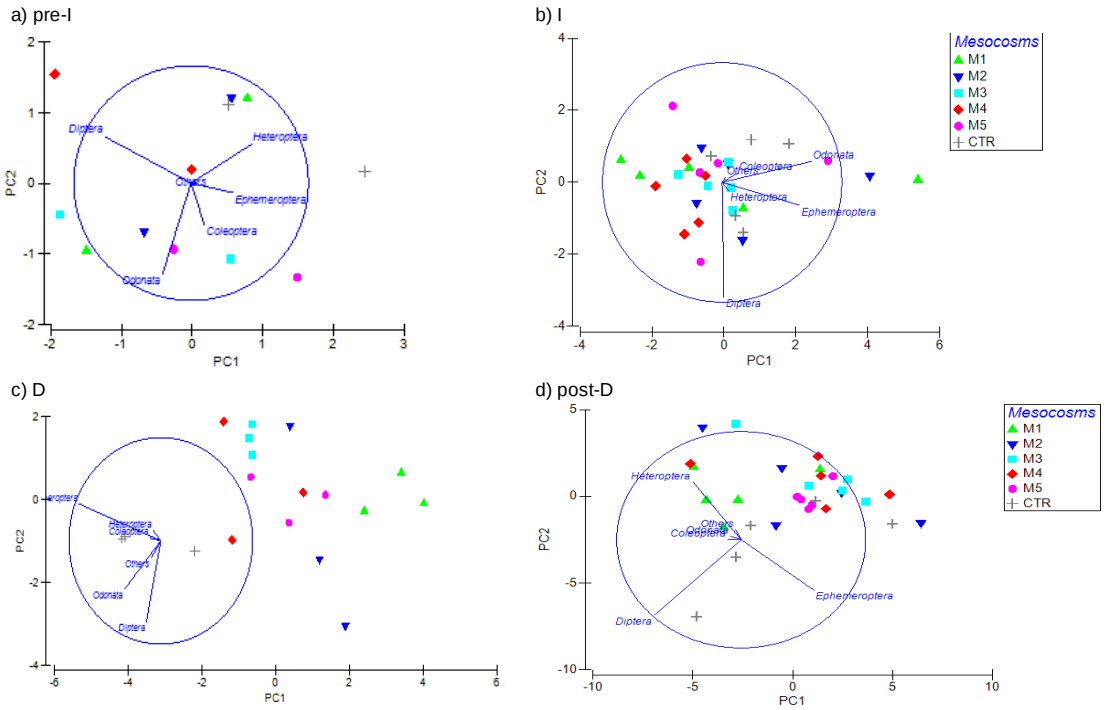

Figure 5: Principal component analysis for the five major taxonomic groups of the macroinvertebrate community in each established phase. pre-I: pre-intrusion phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery. Where CTR- control, M1, M2, M3, M4 and M5 correspond to the following conductivities: 15.0, 9.09. 5.51, 3.34, 2.02 mScm^{-1}, respectively.

4.2.2 Zooplankton

Regarding the zooplanktonic community, ANOSIM comparison between phases showed significant differences between phase I and D (R=0.284, p=0.001), phase I and post-D (R=0.221, p=0.001) and between phase D and post-D (R=0.424, p=0.001). No significant differences were detected between control conditions and salinity levels in any of the established phases (p≥ 0.003). Still regarding zooplankton and during phase I the groups with highest abundances were, in decreasing order of abundances, Copepoda, Rotifera, Branchiopoda and Ostracoda (Fig. 4). As expected, during phase I, a clear decrease in organism's abundance was observed, with stronger effects at the scenario with highest seawater conductivity (15 mScm^{-1}). Still, the most representative groups during this period (I phase) were Rotifera and Copepoda. Furthermore, Rotifera increase in abundance at intermediate salinity levels (3.34 and 5.54 mScm^{-1}) while Copepoda showed to benefit from high salinity levels (9.09 and 15.0 mScm^{-1}), increasing in abundance, also in phase I and contributing to the clear separation of the mesocosms into three distinct groups: high salinity, intermediate salinity levels and low salinity mesocosms including

the control treatment (Fig. 4). Ostracods abundance increased, at phase I at the highest salinity level (15 $mScm^{-1}$). During the recovery periods (D and post-D periods) Rotifera showed the highest recovery, among the four groups, followed by Copepoda. Also, the Branchiopoda showed an increase in abundance. Relatively to the Ostracoda, their presence was constant during the whole experiment and presented their highest abundances at control and lowest salinity levels during the phases I, D and post-D. Still, at the highest salinity levels, during the post-D phase their abundances were above the abundances observed at phases I and D. Overall, in the post-D period, fluctuations in the abundances of both species were observed, that were also observed in control with no clear distinction of groups at the PCA analysis (Fig. 6).

Analysis of diversity indexes showed that, for zooplankton (Fig. 7b), both species richness and Shannon-Wiener indexes were very similar among mesocosms in phase I. After the pulse of salinity (I phase) the values of these indexes decreased: 9 and 4 species were observed in control and at the highest salinity mesocosms, respectively, and the Shannon-Wiener index decreased from 1.4 at the control mesocosms to 0.9 at the highest salinity. During phase D was noticed a very low variability in both indexes, with intermediate salinity levels presenting the highest for both indexes. In post-D phase was noticed a decreased in the number of species with increased salinity, though evenness between groups increased with salinity level.

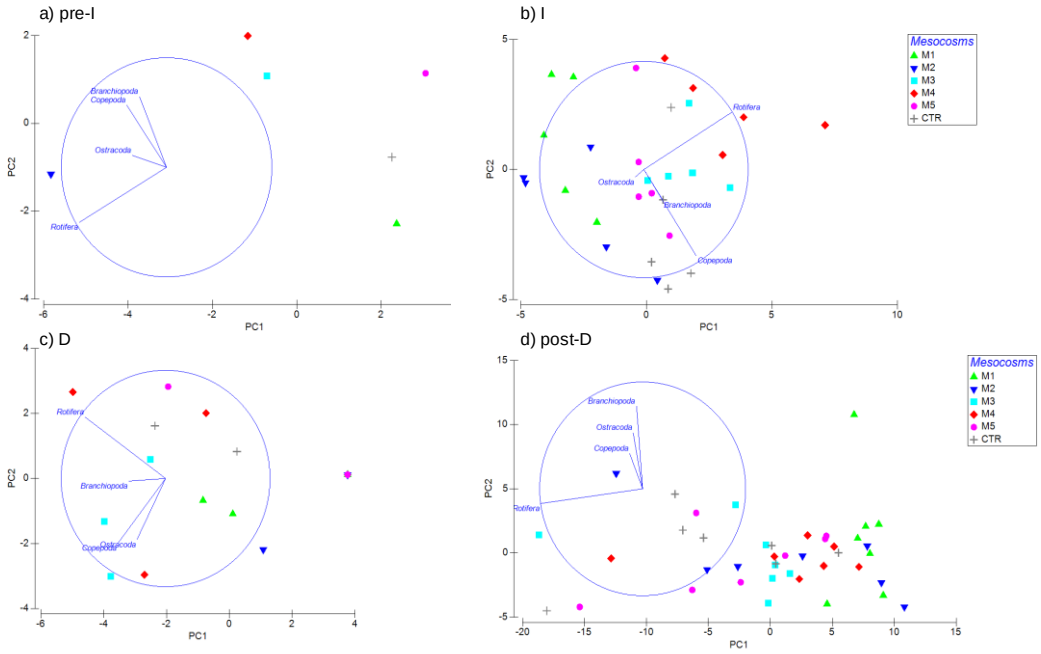

Figure 6: Principal component analysis for the major taxonomic groups of the zooplankton community in each established phase. pre-I: pre-intrusion phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery. Where CTR- control, M1, M2, M3, M4 and M5 correspond to the following conductivities: 15.0, 9.09. 5.51, 3.34, 2.02 $mScm^{-1}$, respectively.

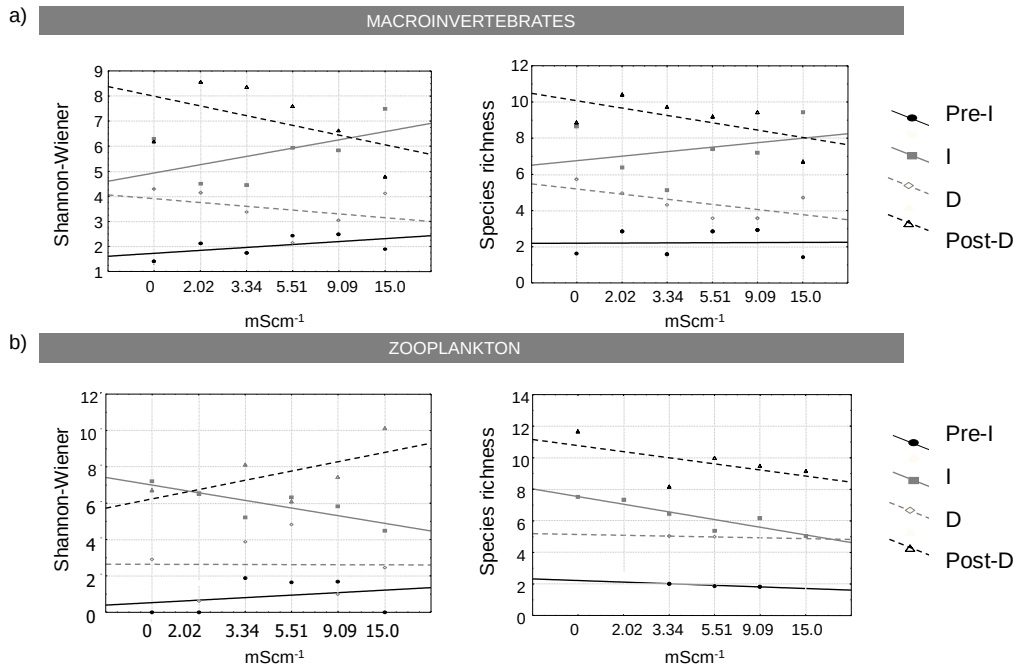

Figure 7: Shannon-Wiener and species richness indexes for a) Macroinvertebrate and b) Zooplanktonic communities exposed to increased salinity levels ($mScm^{-1}$), during mesocosms experiments. Abreviations stand for: pre-I: pre-intrusion phase; I: saltwater intrusion phase; D: sequential dilutions phase; post-D: period of recovery.

5. Discussion

Increased salinity levels may alter the structure or composition of freshwater macroinvertebrate and zooplanktonic communities by reducing species abundance and richness, in agreement with previous works (Kefford et al., 2012; Cañedo-Argüelles et al., 2012; Cañedo-Argüelles et al., 2016). Specifically for macroinvertebrates, it is reported that almost 95% of insect's species have physiological limitations that confine them to freshwater systems (Clements, 1992). However, with a few exceptions, the macroinvertebrate groups that were exposed, in the present work, to salinization were able to cope with it and maintain their abundances after exposure to a pulse of seawater. During the saline intrusion phase (I phase) a decrease of abundances relatively to control was only observed for Ephemeroptera at the highest level of conductivity (15 $mScm^{-1}$) and for Odonata at two intermediate tested conductivities. For Ephemeroptera, such decrease in abundance, at the highest salinity level, was intensified in the following to phases (D and post-D), suggesting this group to be the most sensitive to seawater comparatively to the other four that were here studied, since even after removing the salinity stress (phases D and Post-D) it was not able to recover initial abundances. Actually, these results are in line with data from literature that

has reported Ephemeroptera amongst the least tolerant group to salinity stress (e.g., Szöcs et al., 2012; Kefford et al., 2012; Vidal-Abarca et al., 2013). However, Marshall and Bailey (2004) reported mean control abundances of Ephemeroptera to be 14.5 individuals, while it was on average 9 for treatments exposed to four saline pulses (each pulse of 3.5 gL^{-1} TDS, approximately 6.25 $mScm^{-1}$). But, after the four pulses of salinity, abundances increased to values similar to those reported before the pulses, only within a recovery period of five days (13 individuals) (Marshall and Bailey, 2004). Nevertheless, in the present study, salinity levels that significantly reduced the abundances of Ephemeroptera were higher (15 $mScm^{-1}$) and, though only a saline pulse was performed, it lasted for a longer period of time. Regarding Diptera abundances, the opposite was observed, i.e., abundances increased at the highest tested salinity comparatively to the control. This group of organisms have been reported to have species representatives across the full salinity gradient of inland waters (Tavares et al., 2009), which may be in part related with their ability to induce significant ion transport at the anal papillae to compensate increased levels of salinity (e.g., Donini and O'Donnell, 2007). Furthermore, this group may display strategies of reproduction, like facultative viviparism or ovoviviparism (in both the larvae stages remain partially protected from unfavourable conditions) that may confer them advantages under salinized environments (Meier et al., 1999). Other studies have reported Coleoptera as the most salt tolerant group, in some cases comprising almost 70% of the community, reaching 88% if considered jointly with Heteroptera and Odonata groups (Vidal-Abarca et al., 2013). In the present study, Coleoptera and Heteroptera were the two groups exhibiting the lowest abundances during the phase I (seawater intrusion), but, within each group no effects of salinity were observed in abundances, suggesting their tolerance to increased salinity. Furthermore, during phase D and Post-D the abundances of Heteroptera and Odonata were higher at the seawater than at the control mesocosms, suggesting that their increased tolerance to this stressor may confer them some competitive advantage. Furthermore, though few salinity effects were observed in abundances within each group, changes in the macroinvertebrate structure were observed. For example, in phase post-D, Heteroptera, jointly with Coleoptera, presented the lowest abundances in control conditions. However, in conditions of seawater exposure, its abundances reach those of other

groups like Odonata. These changes on structure of the communities may have impacts in the functioning of the community. For instance, a higher number of predator organisms (Heteroptera) may strongly increase the competitiveness for food resources with other groups (Odonata), leading to further declines in other groups, like Diptera (e.g., Klecka and Boukal, 2012).

For the zooplanktonic community, though significant decreases in abundances relatively to the control, in phase I, were only observed for Copepoda and Branchipoda, overall, a reduction in diversity indexes was observed, following a similar pattern reported in previous studies (e.g., Sarma et al., 2006; Waterkeyn et al., 2010). Furthermore, the abundance of Branchiopoda was significantly higher at the highest conductivity level (15 $mScm^{-1}$) comparatively to the control. However, toxicity data obtained in the previous chapters of this thesis and by other authors [e.g., Jeppesen et al. (1994) defined the maximum optimal salt concentration tolerated by daphnids around 0.05 $mScm^{-1}$] for daphnias would predict that Branchiopoda would exhibit a significant decrease in abundances at such high conductivity levels. Since rotifers abundance was significant lower at 15 $mScm^{-1}$ comparatively to the control, it may be suggested that daphnias abundance increased at this conductivity as they had the opportunity to explore part of the rotifers niche. Actually, some species may benefit from the reduction of abundances of other species. These results are inline with data gathered from literature that point out the ability of rotifers to thrive at high salinity levels while cladocerans and copepods benefit from lower salinity levels (e.g., Sarma et al. 2006; Van Meter et al., 2010; Paturej and Gutkowska, 2015).

Overall, increased salinity levels lead to decreases in abundance and richness of both communities. But decreases in abundance of the most sensitive groups, was sometimes, compensated by the thrive of other groups with similar functions in the ecosystems (for instance, cladocerans abundances decrease while rotifers increase, and both are primary consumers and constitute major food sources for other freshwater organisms) – this redundancy on the functional diversity may assure the resilience of freshwater ecosystem under salinization scenarios (Naeem, 1998). Still, it should be also taken in account that despite the replacement by function-similar species, ecosystems functioning may later be compromised. This may occur because the substitute

species may not perform the function at the same speed or efficiency (Naeem et al, 1994; Naeem, 1998).

Finally, in general, the abundances of organisms, in all treatments, increased from phase pre-I to post-D, his could be a result of increasing spring temperatures and higher number of daylight hours. For instance, small increases in temperature may contribute to organisms grew faster or decrease the mean time between generations in several zooplanktonic species (e.g., Galkovskaja, 1987; Miracle and Serra, 1989; Stelzer, 1998; Bestion et al., 2015).

6.Conclusions

This study comes to highlight that ecosystems may be more resilient to perturbations (salinization) than what was expected. Overall, the results showed that most tested invertebrate groups were able to cope with the pulse of seawater even at conductivities much higher than those reported in previous chapter to induce 50% of mortality to some groups (e.g. rotifers). This, suggest that standard methodologies may lead to overestimation of the risk caused by seawater intrusions in freshwater communities. Furthermore, though few effects were observed in the abundances of the studied groups of invertebrates, exposure to salinity induced changes in the community structure, highlighting the need to perform this type of studies at higher stages of risk assessmnent.

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Chapter VII

The influence of salinization on seed germination and growth under mono and polyculture conditions

The influence of salinization on seed germination and growth under mono and polyculture conditions

Venâncio C, Pereira R, Lopes I

Abstract

Sea level rise is already causing the salinization of soils located in coastal regions worldwide, which may provoke osmotic stress to the producers inhabiting these ecosystems and lead to a decrease of soils productivity. The present work aimed at assessing the effects that increased salinity may provoke in terrestrial plants, using as model species: *Trifolium pratense*, *Lolium perene*, *Festuca arundinacea* and *Vicia sativa*. To attain this main goal, two specific objectives were targeted: i) to determine the sensitivity of the selected plant species to increased salinity (induced by seawater-SW or by NaCl, proposed as a surrogate of SW) and, ii) to assess the influence of culture conditions (mono- or polycultures) on the sensitivity of plants to salinization. To attain the first objective, standard monospecific assays on seed emergence and growth of higher plants were run by exposing, for 14 days, each plant species to a gradient of increasing salinities obtained with SW or with NaCl. The second objective was tackled by exposing, for 14 and 28 days, the four-plant species, under mono- or polyculture conditions (all possible conjugations of pairs of species were tested), to the salinity threshold level (obtained with SW) established to consider a soil salinized (4.0 $mScm^{-1}$). The four-plant species exhibited a higher sensitivity to NaCl than to SW and showed differential tolerances among them to increased salinity. *Trifolium pratense* was the most sensitive species to NaCl ($EC_{50,\text{seed germination}}$ and $EC_{50,\text{growth}}$ of 4.76 and 4.32 $mScm^{-1}$, respectively) while *L. perenne* (only for growth: $EC_{50,\text{growth}}$ of 11.5 $mScm^{-1}$) and *F. arundinacea* were the most tolerant to this salt ($EC_{50,\text{seed germination}}$ and $EC_{50,\text{growth}}$ of 23.1 and 12.2 $mScm^{-1}$, respectively). Soil irrigated with seawater caused no significant adverse effects on seed germination and growth of *L. perenne*. However, SW conductivities of 12.1 $mScm^{-1}$ reduced in 50% the growth of *T. pratense* and conductivities of 22.6 and 23.5 $mScm^{-1}$ caused 50% of inhibition in seed germination of *V. sativa* and *T. pratense*.

Cultures conditions showed some influence on seed germination and growth, but salinization did not influence the pattern of effects caused by culture conditions in these parameters, both when monitoring the effects in aerial and root biomass. However, when considering total productivity, for aerial and root biomass, overall a higher productivity was observed in control comparatively to salinization conditions. Furthermore, under salinization scenarios, polycultures conditions were associated with a higher aerial and root total biomass than monocultures (for instance for *T. pratense* and *F. arundinaceae*). Those results suggest that such salinization effects may be minimized under conditions of polycultures. Thus, this type of cultures should be encouraged in low-lying coastal ecosystems that are predicted to suffer from salinization caused by seawater intrusions.

Keywords: sea level rise; seawater intrusion; terrestrial plants; productivity loss; competition

1. Introduction

Salinization of soils may arise from primary salinization processes (such as weathering of the parental rock or seawater invasion of low-lying systems near coastal areas) or secondary salinization events (e.g., use of poor quality ground water for irrigation/consumption purposes) (European Commission, 2012; IPCC, 2014). This may have major implications in soils quality and fertility (FAO, 2008), since many crops, forage and livestock feeding plants are glycophytes (plants that cannot withstand high salinity conditions). Salt stress exerts immediate effects by changing osmotic pressures decreasing the amount of water that plant roots can uptake from the surrounding environment (e.g., Osakabe et al., 2014). These alterations trigger signalling mechanisms that promote the production of reactive oxygen species (ROS) that can disrupt normal cellular metabolism by causing lipid peroxidation (Hu et al., 2012), protein denaturation or even targeting nucleic acids (Imlay, 2003). Furthermore, with the increase of salt contents in soils, plants are more likely to absorb ions like sodium (Na^+). The extrusion of K^+ to balance the entrance of Na^+ ions impairs many cellular functions that depend on K^+ to prosecute, namely, protein synthesis (e.g., Giri et al., 2007) or stomatal movements (Brugnoli and Bjorkman, 1992),

which when restricted, decrease the availability of CO_2, impairing photosynthetic rates and, consequently plant's growth rates (Parida et al., 2004).

Nevertheless, plants evolved mechanisms that counteract the effects caused by salt stress (Munns and Tester, 2008). For instance, antioxidant mechanisms may be activated to minimize ROS effects (e.g. Hu et al., 2012) or polyamines (to deal with salinity increases and consequent potassium deficiency) may be accumulated to hamper or retard cellular growth helping to maintain the K^+ gradient (Ahmad et al., 2013). In the same line of evidence, other solutes that do not interfere with cellular natural processes (like proline – an antioxidant agent and manitol) help the cells to maintain their primary functions as cell turgor, providing the correct gradient for water uptake by the plant (Ahmad and Prasad, 2011) or can act as scavengers of free ions (Diamant et al., 2001). In addition, plants are supplied with natural substances (like phytohormones) that help seed germination and growth under adverse conditions (e.g., Jamil and Rha, 2007; Jayakannan et al., 2015). These adverse effects that salinization may cause in terrestrial plants may lead to a decrease in the productivity of soils. These effects may be aggravated considering the proliferation of monocultures to respond to human food demands (e.g., FAO, 1999). In this scenario, it is important to understand if effects of soils salinity on terrestrial plants is influenced by the type of culture conditions (mono- or polyculture). Cardona-Olarte et al. (2006) reported the existence of such influences in an experiment performed with two mangrove species. These authors observed that *Laguncularia racemosa* grew faster at a low salinity level (salinity of 10) under a polyculture scenario (cultured with *Rhizopora mangle*) than at the same salinity level in monoculture, contributing two-fold more to the net primary production. However, as salinity increased (to 40) and the immersion period was prolonged, a significant decrease on net primary production of *L. racemosa* was reported.

Following the information mentioned above, this work aimed at assessing the effects of increased soil salinity in seed's germination and in the growth of four terrestrial species of plants: *Trifolium pratense, Festuca arundinacea, Lolium perenne* and *Vicia sativa*. Two specific objectives were tackled: i) to assess seeds emergence and growth of each species, under salinity stress caused by natural seawater (SW) and by sodium chloride (NaCl), to assess the use of this later one as a

possible surrogate at early stages of ecological risk assessment, and, ii) to assess the effects of salinization in seedlings growth under different culture conditions (mono- and polyculture).

2. Material and Methods

2.1 Tested solutions

Natural seawater (SW), from the North Atlantic Ocean, was collected at a reference site (40°38'33"N 8°44'55"W, Aveiro, Portugal), classified by the Foundation for Environmental Education from good to excellent in terms of water quality, as it is frequently monitored according to the Directive 2006/7/CE of the European Union and of the Council of February 15th, transposed to national Portuguese law by Decree-Law no. 135/2009 of June 3rd (Venâncio et al., 2017a, 2017b, 2017c). Prior to its use for phytotoxicity assays, seawater was filtered through cellulose nitrate membranes of 0.20µm (ALBET-Hannemuehle S.L., Barcelona, Spain). This procedure aimed at removing particles in suspension and plankton. All tested SW dilutions were made with distilled water (Gesellschaft für Labortechnik mbH Water Still 2012).

Sodium chloride solution (NaCl; supplied by Merck, St Louis, MO, USA) was tested as a possible surrogate of SW. The solution was prepared with distilled water. All solutions were always made fresh to initiate the phytotoxicity assays.

2.2 Tested species

To conduct the present work, four species of terrestrial plants were chosen: two dicotyledonous species (*Trifolium pratense* and *Vicia sativa*) and two monocotyledonous species (*Lolium perenne* and *Festuca arundinacea*). These species are proposed as test species in terrestrial ecotoxicity standard protocols (ISO, 1995; OECD, 2006) since they are easily found worldwide, being commonly used as cover crops, forage or food for livestock.

2.3 Standard monospecific assays on emergence and growth of higher plants

Standard monospecific assays on seed emergence and growth of higher plants were carried out by exposing each of the four selected plant species to a gradient of increasing salinities generated with SW or with a NaCl stock solution plus to a control. For this, the standard ISO guideline 11269-2 (1995) was followed. For each treatment (SW dilutions, NaCl concentrations or controls) four replicates were carried out, each consisting of a plastic container (11.7 cm diameter, 6.2 cm height) filled with 200g of artificial OECD soil (2006) spiked with the respective SW dilution, NaCl concentration or control water (distilled water). The volume of the test solution added to soil was calculated for adjusting the soil moisture to 45% of the maximum Water Holding Capacity (WHC_{max}) of the soil. To maintain the soil moisture constant over the duration of the assay, each test vessel was introduced in a larger plastic container filled with distilled water. Furthermore, each test vessel was perforated and equipped with a rope that allowed the communication with the inferior plastic container and enabled the ascending of distilled water by capillarity to the test soil. The level of the soil moisture was regularly checked during the assay and water level in inferior plastic containers was kept constant. At each replicate, ten seeds (for *V. sativa*) or twenty seeds (remaining tested species with very small seeds) were added and carefully covered with a thin layer of the spiked soil. All assays were run under controlled conditions of temperature (20±2°C), photoperiod (16^{L}: 8^{D}) and light intensity (25.000 lux). According to the ISO guideline, every day the number of emerged seeds was counted but only the first five to emerge were left to grow, while the remaining were counted and harvested (ISO 11269-2, 1995). At the end of the assay, after 14 days of exposure (counted from the emergence of the last of the five seeds), plants were clipped by the stem right above the soil surface. The biomass above soil (expressed as mg of dry weight) of each sample was assessed after samples were dried in the oven at 60ºC, by weighing in a balance (Kern, EW1500-2M, resolution of 0.01 g).

To irrigate the soil, a total of 12 concentrations/dilutions were set either for NaCl or SW (plus a control), for each of the tested species. For NaCl, tested concentrations ranged from 2.6 to 22.8 $mScm^{-1}$ per kg of soil (with a dilution factor of 1.2x) while for SW the dilutions ranged from 6.5

to 48.0 $mScm^{-1}$ per kg of soil (with a dilution factor of 1.2x). Salinity is expressed for NaCl solution and for SW it is assumed that each individual concentration/dilution corresponds to the worst-case scenario, because the effective salinity in soil will always be lower.

2.4 Polyculture assays

To assess the effects of increased salinity in scenarios of polyculture of terrestrial plants, only exposure to SW was carried out. This decision was based on the results previously obtained with monospecific seed germination and growth assessment assays, which showed that a few of the studied species (e.g., *L. perenne*) could tolerate irrigation water whose conductivity matched the conductivity of natural SW (≈ 52 $mScm^{-1}$). Therefore, it was important to understand how species deal with each other, under long-term exposure to SW.

To assess the influence of increased salinity on a polyculture scenario, each plant species was exposed solely or with any of the other species to a control treatment (distilled water) and to a salinity level of 4.0 $mScm^{-1}$ per kg of soil. This salinity value was chosen because it corresponds to the threshold value above which a soil is considered saline (please see Micheli et al., 2002). A full cross design was carried out to perform these polyculture exposures, i.e., each species was tested against the other three (a total of six plant pairs were tested): (i) *T. pratense* versus *V. sativa*, (ii) *T. pratense* versus *L. perenne*, (iii) *T. pratense* versus *F. arundinacea*, (iv) *V. sativa* versus *L. perenne*, (v) *V. sativa* versus *F. arundinacea*, and (vi) *L. perenne* versus *F. arundinacea*. The methodology used to run the assays was similar to the one used to carry out the monospecific emergence and growth assays (please see section 2.3 of Materials and Methods). For each treatment four replicates were carried out, which consisting of plastic trays (34.5 x 23.5 cm) filled with 1.5 kg of artificial OECD soil spiked with distilled water (Control) or with SW (4.0 $mScm^{-1}$). As reported for the monospecific emergence and growth assays, here each tray was perforated at the bottom, where a rope was introduced, and placed in larger plastic containers (43.5 x 28.5 cm) filled with distilled water to maintain soil moisture constant during the assay. For each treatment six replicates were performed. At each replicate 40 seeds were introduced at the beginning of the assay. For monoculture exposure, the 40 seeds belong to the same species; for

scenarios of polyculture, 20 seeds were from one species and 20 seeds from another plant species. Every day the number of emerged seeds was counted and only the first 30 emerged seeds were left to grow. The test began when all 30 seeds emerged. Similarly, in polyculture scenarios, only the first 15 seeds of each species were left to grow, and the beginning of the test started from the germination of the last seed onwards. All assays took place at controlled conditions of temperature (20±2°C), photoperiod (16^{L}: 8^{D}) and light intensity (25.000 lux).

Two periods of harvesting were performed: at day 14 and day 28 after germination of all 30 seeds. Three of the six replicates were harvested at day 14, while the remaining replicates were harvested at day 28 (end of the assay; to assess prolonged effects of exposure to SW). At the end of each period (14 or 28 days of exposure), aerial biomass (above soil) and root biomass (below soil surface) were assessed for each species. For that, plants were gently removed from the soils and clipped at the stem at the point that separated the plant biomass above soil surface from the part below soil surface. Roots were carefully washed to remove soil particles that could influence the final weight and dried with absorbent paper to remove excessive water. Both biomasses (above and below soil, expressed as mg of dry weight) of each sample were assessed after drying in the oven at 60ºC. Weighing's were performed in a balance (Kern, EW1500-2M, resolution of 0.01 g).

3. Data analysis

Calculation of effect conductivities provoking X% of reduction on seed emergence and growth of higher plants (EC_x) were estimated by fitting the logistic model to the obtained data by using the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA). To infer if NaCl had a similar toxicity as SW, a comparison of EC_x and respective confidence intervals was made for each species.

Regarding polyculture, data analysis was performed, separately for aerial and root dry mass, to identify possible differences between: i) monoculture control *vs* monoculture salt stress, to assess the effects of the salt stress; ii) polyculture control *vs* polyculture salt stress, to assess the influence

of salinity; iii) monoculture control *vs* polyculture control, to understand the effects of the interaction between species solely, and iv) monoculture salt stress *vs* polyculture salt stress treatment, to assess the influence of both salinity and presence of another species factors. For i) and ii) a two-way Anova, followed by Holm-Sidak all pairwise comparison ($p<0.05$) was performed and for iii) and iv) was applied a three-way Anova, followed by Holm-Sidak all pairwise comparison ($p<0.05$).

4. Results

4.1 Standard monospecific assays on seed emergence and growth of higher plants

Regarding exposure to increased levels of salinity caused by NaCl, *T. pratense* presented the lowest $EC_{50,NaCl}$ both for seeds germination and growth parameters: 4.76 and 4.32 $mScm^{-1}$, respectively) (Table 1). *Vicia sativa* and *L. perenne* showed similar sensitivities to NaCl, as their upper and lower confidence intervals, respectively, overlapped. *Festuca arundinacea* was the most tolerant species to increased levels of NaCl with an EC_{50} of 23.1 and 12.2 $mScm^{-1}$ for seeds germination and growth, respectively, and was also the species that presented the lowest difference in sensitivity to NaCl and SW: EC_{50} for NaCl was almost 2-fold lower than that for SW (23.1 and 41.6 $mScm^{-1}$, respectively) (Table 1). *Festuca arundinacea* and *T. pratense* were the species that started to exhibit significant effects on seeds germination at lower salinity levels, with $EC_{20,NaCl}$ of 3.64 and 2.99 $mScm^{-1}$, respectively (Table 1). Regarding growth, *T. pratense* and *V. sativa* were the ones that start to suffer earlier the effects of increased salinity ($EC_{20,NaCl}$ of 2.06 and 3.91 $mScm^{-1}$) (Table 1).

Results obtained for germination and growth with increased salinity levels of SW showed that, among the four-tested species, *L. perenne* was the most tolerant species, as no adverse effects were observed for seed germination and growth even when soil was irrigated with a solution whose conductivity corresponded to natural SW conductivity ($\approx$ 52 $mScm^{-1}$) (Table 1). Additionally, *L. perenne* registered an increment in growth of about 30% at the highest concentration comparatively to the control. The most sensitive species, for both endpoints, were *T. pratense* and *V. sativa* with computed $EC_{50,SW}$ for germination and growth of 23.5 and 12.1

$mScm^{-1}$ and of 22.6 and 16.7 $mScm^{-1}$, respectively. These were also the two species where significant adverse effects started to be observed at lower concentrations ($EC_{20,SW}$ of 12.5 and 13.9 $mScm^{-1}$ for germination and 7.11 and 7.56 $mScm^{-1}$ for growth, for *T. pratense* and *V. sativa*, respectively) (Table 1).

Overall, NaCl solutions caused higher toxicity than SW dilutions to the tested species, both for seed germination and for growth, (with the exceptions of *F. arundinaceae* and *V. sativa*, NaCl and SW induced similar toxicity regarding dry weight). The highest differences, in germination and dry weight, between NaCl and SW EC_{50} were detected for *L. perenne* (more than 4-fold higher for SW) followed by *T. pratense* (3-fold for dry weight rate and 5-fold for seed germination).

Table 1: Conductivities for sodium chloride (NaCl) and natural seawater (SW), provoking 10, 20 and 50% (EC_{10}, EC_{20}, and EC_{50}, respectively) of inhibition on germination and dry weight of four species of terrestrial plants. 95% confidence limits are depicted within brackets. n.d. – could not be determined as no inhibition on germination or growth could be detected at the irrigation water treatment corresponding to 100% SW.

Species	**Conductivity per kg of soil $mScm^{-1}$**				
	Seed Germination			**Growth[a]**	
	EC_{10}	**EC_{20}**	**EC_{50}**	**EC_{20}**	**EC_{50}**
Lolium perenne					
NaCl	3.46 (1.46-5.48)	5.22 (2.96-7.47)	10.5 (8.13-12.8)	5.26 (1.23-9.29)	11.5 (7.08-15.9)
SW	n.d.	n.d.	n.d.	n.d.	29.9%*
Festuca arundinaceae					
NaCl	1.92 (-)	3.64 (0.03-7.26)	23.1 (20.4-25.8)	6.43 (-)	12.2 (8.64-15.8)
SW	23.1 (15.6-30.6)	28.7 (22.2-35.2)	41.6 (36.6-46.5)	12.4 (-)	24.6 (10.1-39.1)
Trifolium pratense					
NaCl	1.85 (-)	2.99 (0.59-5.39)	4.76 (3.04-6.48)	2.06 (0.95-3.17)	4.32 (2.95-5.68)

SW	9.88	12.5	23.5	7.11	12.1
	(5.70-14.1)	(8.5-16.5)	(19.4-27.6)	(5.16-9.06)	(10.3-13.8)
Vicia sativa					
NaCl	2.93	4.14	7.45	3.91	6.94
	(1.66-4.19)	(2.77-5.50)	(6.07-8.86)	(1.99-5.83)	(4.99-8.89)
SW	10.4	13.9	22.6	7.56	16.7
	(4.5-16.3)	(7.9-19.9)	(16.9-28.2)	(-)	(7.51-26.0)

*at the highest tested concentration, a growth stimulation of 29.9% was recorded.

[a]EC_{10} could not be estimated for growth since these values were below the range of concentrations tested.

4.2 Polyculture assays

Increased salinity, under monoculture scenarios, significantly reduced *V. sativa* aerial biomass, comparatively to the control, after 14 or 28 days of exposure ($p<0.001$; Fig. 1b, 1c). For the other tested species, in general, increased salinity did not alter significantly aerial biomass either after 14 or 28 days of exposure (Fig. 1).

The presence of other species (scenarios of polyculture) also affected aerial biomass of the tested plant species (Fig. 1). Aerial biomass significantly decreased for *V. sativa* after 14 days of exposure, when this plant was exposed for 14 days, under control conditions, along with *L. perenne* ($p=0.024$; Fig. 1b). As well, aerial biomass changed significantly for *V. sativa* and *L. perenne* after 28 days of exposure, under control conditions: in the case of *V. sativa* it decreased when cultured with any of the other three plant species ($p<0.001$, Fig. 1b, 1c, 1d) while for *L. perenne* aerial biomass decreased when cultured together with *V. sativa* and *F. arundinacea* ($p<0.001$, Fig. 1b, 1f).

Finally, exposure to salinity influenced the final dry weights of plants (biomass) when in polycultures (Fig. 1). After 14 days of exposure, significant interactions, under salinity stress, were found for *T. pratense* when cultured together with *L. perenne* ($p=0.024$; Fig. 1a) and for *V. sativa* when along with *L. perenne* ($p=0.002$; Fig. 1b): *T. pratense* aerial biomass increased while *V. sativa* aerial biomass decreased. After 28 days of exposure, significant changes in dry weights,

under salinity stress were found for *V. sativa* ($p<0.001$; Fig. 1b, 1c) and *L. perenne* ($p\leq0.007$; Fig. 1b, 1f). *Vicia sativa* aerial biomass decreased when cultured together with *L. perenne* and *T. pratense*; and *L. perenne* aerial biomass decreased when cultured together with *V. sativa* and *F. arundinacea*.

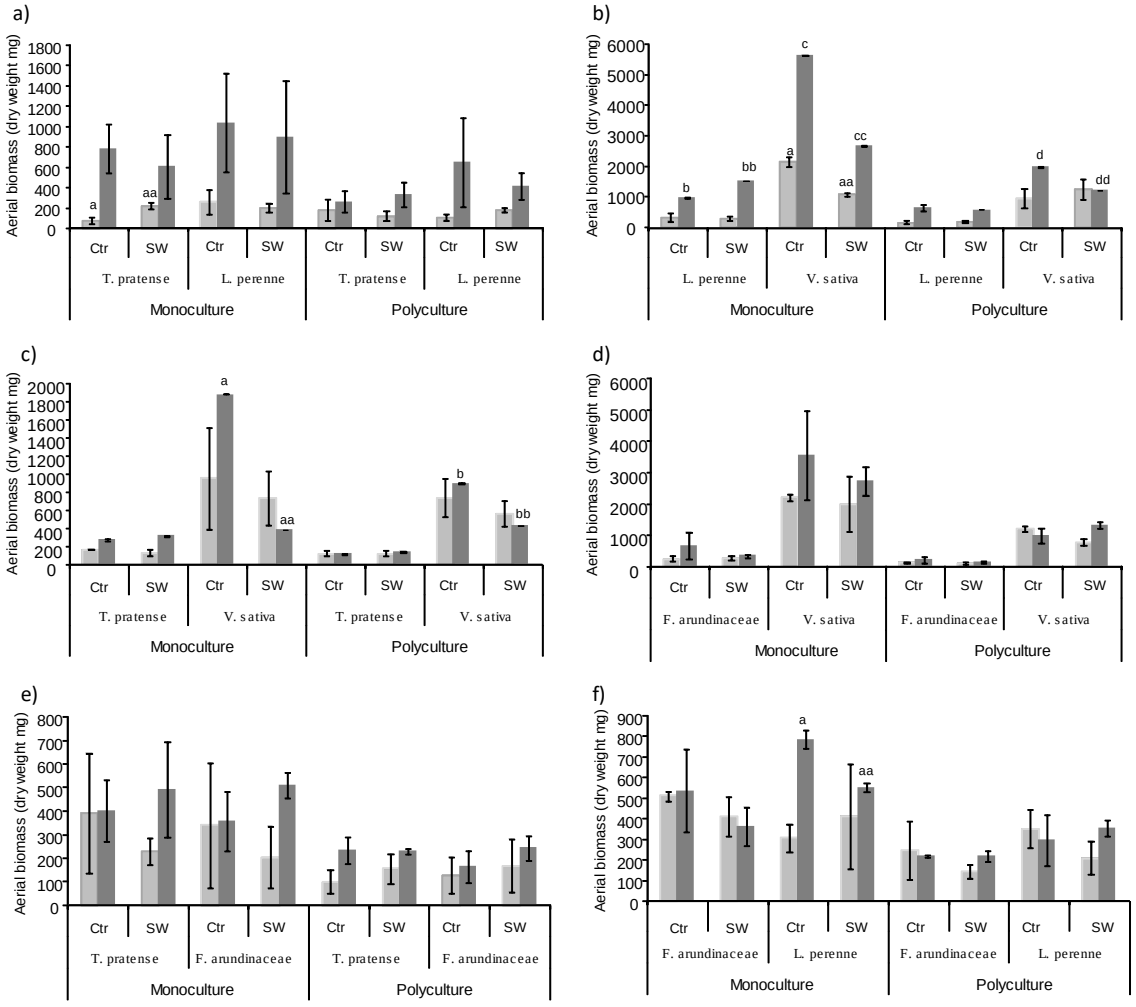

Figure 1 – Average aerial biomass (mg) for each of the six plants pairs exposed for a period of 14 days (light grey) and 28 days (dark grey) to a salinity level of 4 $mScm^{-1}$, either in the absence (Monoculture) or presence of a different species (Polyculture). Ctr corresponds to the control treatment and SW to the salt treatment (4.0 $mScm^{-1}$). Error bars represent standard deviation. Letters (a to d) represent significant differences (Holm-Sidak, $p<0.05$) between paired comparisons of control and SW treatments, of the different exposure periods, within each scenario (Mono- or Polyculture).

Overall, increased salinity, under monoculture scenarios, significantly changed root biomass of *T. pratense*, comparatively to the control, after 28 days of exposure (Fig. 2c), of *L. perenne* after 28 days of exposure ($p \leq 0.001$, Fig. 2a, 2b, 2f) and of *V. sativa* after 28 days of exposure ($p < 0.001$; Fig. 2b, 2c, 2d). In the case of *L. perenne* root biomass increased in two cases while in the other case it decreased, for *V. sativa* root biomass decreased and for *T. pratense* also decreased (Fig. 2c). For the other tested species, increased salinity did not alter significantly root biomass either after 14 or 28 days of exposure.

Regarding the effect of polyculture on root biomass of the tested plants (Fig. 2), significant changes, under control conditions, were observed for *L. perenne* and *F. arundinacea* after 14 days of exposure: *L. perenne* root biomass decreased when cultured with *T. pratense* and *V. sativa* ($p \leq 0.046$; Fig. 2a, 2b) and *F. arundinacea* root biomass increased when cultured with *V. sativa* ($p = 0.038$; Fig. 2d). After 28 days of exposure, significant reductions in root biomass were detected for: *T. pratense* when cultured with *L. perenne* or with *V. sativa* ($p \leq 0.004$; Fig. 2a, 2c); *L. perenne* when cultured with *F. arundinacea* ($p = 0.002$; Fig. 2f); *F. arundinacea* ($p < 0.001$; Fig. 2f) and *V. sativa* when cultured with *T. pratense* or with *L. perenne* ($p < 0.001$; Fig. 2c, 2b).

Increased salinity influenced root biomass when species were in polyculture (Fig. 2). After 14 days of exposure, significant interactions between polyculture scenarios and salinity level, were detected for *L. perenne* ($p \leq 0.001$; Fig. 2a, 2b), *V. sativa* ($p = 0.016$; Fig. 2b) and for *F. arundinacea* ($p = 0.026$; Fig. 2d). After 28 days of exposure, significant changes in polyculture scenarios, under salinity stress were found. In all competition scenarios, root growth decreased for *V. sativa* ($p < 0.001$; Fig. 2b, 2c), *L. perenne* ($p < 0.001$; Fig. 2b) and for *T. pratense* ($p < 0.001$; Fig. 2c).

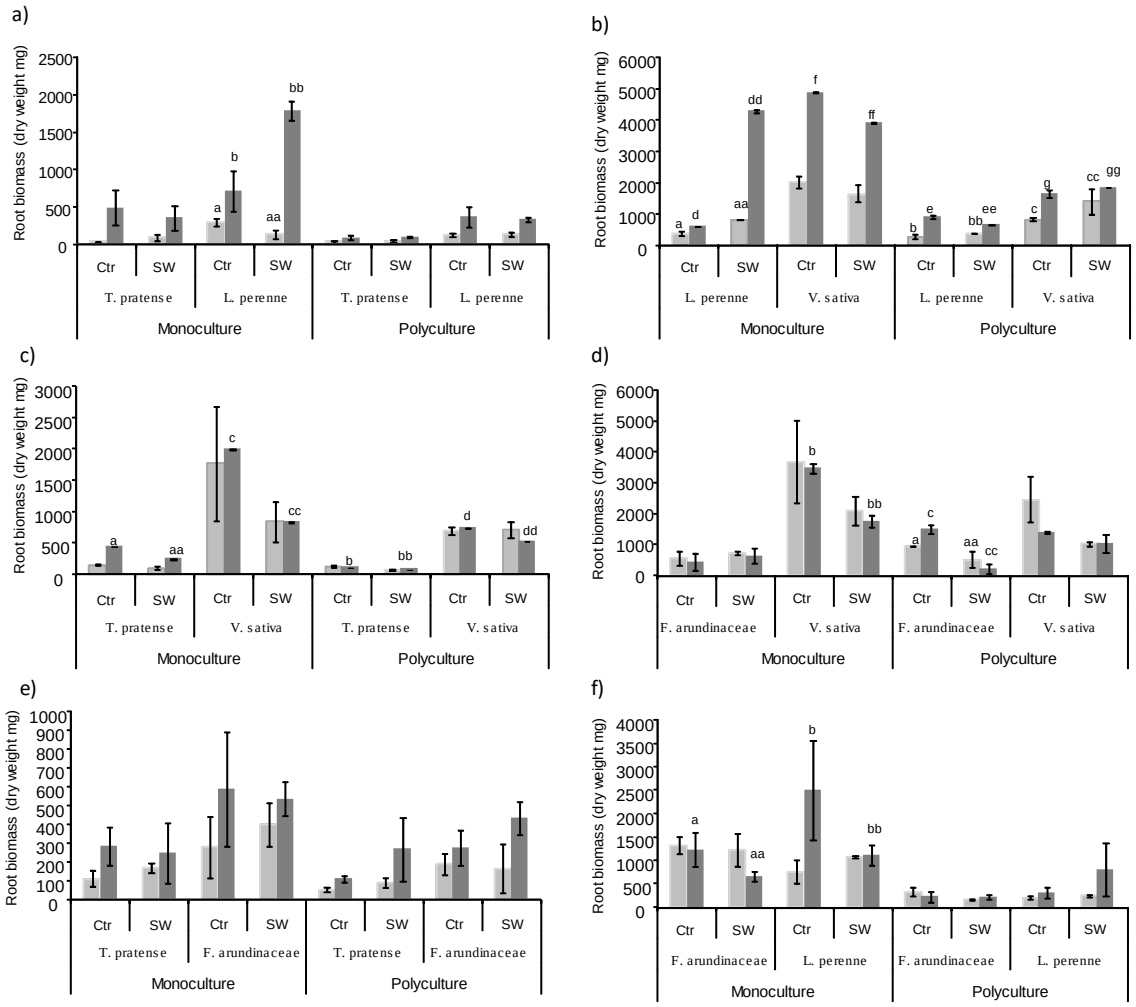

Figure 2– Average root biomass (mg) for each of the six plant pairs exposed for a period of 14 days (light grey) and 28 days (dark grey) to a salinity level of 4.0 $mScm^{-1}$, either in the absence (Monoculture) or presence of a different species (Polyculture). Ctr corresponds to the control treatment and SW to the salt treatment (4.0 $mScm^{-1}$). Error bars represent standard deviation. Letters (a to g) represent significant differences (Holm-Sidak, $p<0.05$) between paired comparisons of control and SW treatments, at the different exposure periods, within each scenario (Mono- or Polyculture).

5. Discussion

5.1 Monospecific assays on seed emergence and growth of plants

The results obtained after exposing the four-plant species to increased salinities revealed that, overall, NaCl exerted higher adverse effects in germination and fresh weight than SW. In some cases, *L. perenne* (germination and fresh weight) and *T. pratense* (germination), the difference in the toxicity exerted by NaCl and SW exceeded 4-fold. Based on the results of previous studies for soil invertebrates and fungi (e.g. Pereira et al., 2015; Venâncio et al., 2017b), NaCl was already expected to be of similar or higher toxicity than SW. The complex mixture of ions in SW might

explain its lower toxicity (Wiesenburg and Little, 1988), since the presence of ions, other than Na^+ and Cl^-, in small dosages may present an advantage for the organisms (e.g., Shaul, 2002; Tuteja and Mahajan, 2007). For instance, the presence of calcium (Ca^{2+}) in SW, may constitute an advantage as this ion intervenes in the abscisic acid cycle (ABA), which plays a major role in membrane integrity and ionic regulation/transport (e.g., Parida et al., 2004). Furthermore, through regulation of ABA levels, Ca^{2+} may also buffer plants against the effects of Cl^-, by decreasing the accumulation of these ions (Cl^-) in the leaves (Gomez-Cadenas et al., 2002).

Plants respond to saline stress in two phases, but the time of response may vary from species to species (Munns and Tester, 2008). The first phase involves response to osmotic stress, which is rapidly induced; the second phase, commonly denominated ion-specific, involves accumulation of ions inside the cells. In the first phase, due to osmotic stress the ability of roots to extract water from the external environment decreases with increasing salt concentration (i.e. osmotic pressure), which leads to a reduction in shoot growth (Munns and Tester, 2008). This osmotic stress may be also determinant during germination, since the growth of the embryo is stimulated through imbibition, i.e., water uptake by the dehydrated seed (Bewley, 1997). If the embryo growth is affected at an early stage, the posterior growth of the plant will also be affected. Zapata et al. (2003) reported 100% germination rates at a salinity level of 150 mM of NaCl (approximately 17.5 $mScm^{-1}$) in seven out of nine species of lettuce cultivars. Yet, these authors also found that, all cultivars, despite the high germination rate, presented higher respiration rates, ethylene production and, later, reduction in fresh weights, suggesting that seeds and embryos went through osmotic stress. In the present work, it was possible to observe that, in their majority, the $EC_{50,SW}$ computed for germination were very close to those computed for fresh weight, in the case of NaCl or higher regarding SW. In both cases, this suggests that effects observed in fresh weight could not only result from the direct action of NaCl or SW in the plant but be a consequence of effects induced during the embryo phase and that accumulated with those induced at the seedling/plant stages. The second phase of reaction to saline stress (ion-specific) occurs mainly through the accumulation of Na^+ ions in the leaves, which has a direct effect on chlorophylls activity. Old leaves are known to be especially sensitive to the accumulation of this ion and rapidly

died, resulting in the reduction of total photosynthetic activity and subsequently in growth (Hu et al., 2014; Qados, 2011). Nevertheless, in the present work, some plant species, were able to cope with salinity levels with a conductivity corresponding approximately to one third of SW, this may be related with the fact that plants can respond to osmotic stress by activating ion pumps that either restrict Na^+ influx into the plant (called SOS1) or compartmentalize the excess of Na^+ into tissues or vacuoles (HKT1 and NHX1 ion channels, respectively) (Munns and Tester, 2008). Furthermore, they may have activated selective transmembrane channels, the aquaporins, that are known to also intervene in salt tolerance response (Glenn et al., 1999; Hill et al., 2004).

The results of monospecific assays also revealed that the two monocotyledonous (*F. arundinacea* and *L. perenne*) species were, in general, more salt-tolerant (considering the EC_{50}) than the dicotyledonous species (*T. pratense* and *V. sativa*). Data already available in the literature support these results. Glenn et al. (1999) stated that, the Na^+ uptake rate is much lower in monocotyledonous species than in dicotyledonous, probably due to its lower cation exchange capacity, which underlies a low cellular Na^+:K^+ ratio and different ion accumulation strategies, during saline stress, between this two groups (Conn and Gilliham, 2010).

Finally, grounded on the monospecific assay results, it seems that NaCl may be used as a protective surrogate for early stages of risk assessment of SW intrusion in low-lying coastal terrestrial ecosystems. Pereira et al. (2015) also made this recommendation after running toxicity assays with soil invertebrates and registering that NaCl seemed to exert a similar or higher toxicity than SW. However, care should be taken in the use of NaCl for these assessments since risk may be highly overestimated, as shown here for *L. perenne* and *T. pratense*.

5.2 Polyculture assays

In this work the effect of culture conditions (mono- or polyculture) on the growth of different plants species, while dealing with the threshold established to consider a soil salinized (4.0 $mScm^{-1}$), was also studied. Regarding the effects of salinity itself (control *versus* SW within monoculture scenario), overall, it did not change the root and aerial biomass of the four plants species. This

was quite an expected result since the $EC_{20,SW}$ after 14 d of exposure to a gradient of salinity were higher than 7.11 $mScm^{-1}$, i.e., above the 4 $mScm^{-1}$ tested in the polyculture scenarios. The lack of significant effects at this salinity level may indicate that the mechanisms associated with salt tolerance, mentioned in the previous section, may reveal efficient to cope with this salinity level. However, it must be highlighted that an exception on this no-effect pattern was observed for *V. sativa* (one of the most sensitive species to SW in the monospecific assays): after 28 d of exposure to soil moistened with 4 $mScm^{-1}$ SW, this species revealed a significant decrease in root and aerial biomass comparatively to the control. This higher sensitivity of *V. sativa* may be related with the fact that this was the species exhibiting, under controlled conditions, the highest weights for root and aerial biomass. It would be expected that a bigger plant needs more water for the maintenance of cell turgor, consequently under osmotic stress could undergo a greater desiccation stress ending up in a reduced growth (Schwinning and Weiner, 1998). Furthermore, plants' response to salinity stress may also vary according to their life cycle. All species here tested are considered perennial, except for *V. sativa*. Munns (2002) referred that perennial species respond to salinity stress within months or years, while annual species the timescale is reduced to days or weeks. Thus, ionic toxicity may be exerted in a faster and shorter period for annual species. This finding comes, also, highlight the importance of long-term studies (e.g., employment of 28 days assays) as a complement to standard guidelines (e.g., 14 days), as these later ones may not be long enough for effects to be detected. Summarising, the results obtained with *V. sativa* alert for two important concerns that must be consider when carrying out risk assessment of SW intrusion on terrestrial ecosystems: (i) the need to assess long-term effects, since a 14 d exposure did not identified significant effects (considered as a no effect concentration; $EC_{10,SW}$ always above 9.88 $mScm^{-1}$) at salinity levels that induce significant effects after a period of 28 d of exposure (4 $mScm^{-1}$); and (ii) the threshold currently accepted to consider a soil saline should be revised since it may induce sublethal effects in some plant species.

When analysing the effects of polyculture solely on the growth of the plants (control conditions: mono *versus* polyculture scenarios), it was observed that, though some effects were observed after a 14-d exposure period, major alterations in aerial and root biomass were observed after 28 d of

exposure, here again denoting the importance of long-term studies. Both for aerial and root biomass, an overall tendency for a decrease in biomass under inter-species polyculture scenarios was observed. These results may be explained, for instance, by the root capacity, of the four plants, to produce exudates, composed of allellopaths, that inhibit the growth of the competitors' roots and consequently of their aerial biomass (Schwinning and Weiner, 1998).

The comparison of aerial biomass between control and SW, under polyculture scenarios, only revealed significant effects for *V. sativa*. This species exhibited a significant decrease in aerial biomass when exposed to SW for 28 d with *L. perenne* and *T. pratense*. These results are most probably directly linked with the highest sensitivity that this species demonstrated to salt stress. When analysing the results obtained for root biomass, significant effects were observed just for a few cases. *Vicia sativa* exhibited lower root biomass when competing with *F. arundinaceae* (14 d) and with *T. pratense* (28 d), which is in line with the results observed for aerial biomass. Furthermore, *L. perenne* and *F. arundinaceae* had their root biomass diminished when exposed for 28 d to SW with *V. sativa*. The fact that only in a few cases significant effects of SW were observed may suggest that under low levels of salinity the effects of inter-species relationship lap up the effects of SW.

Finally, the comparison of biomass of plants exposed to SW, revealed a general increase in root and aerial biomass for each species individually under polyculture conditions (except for *V. sativa*) when compared to monoculture conditions. These results meet the conclusions draw above, i.e., inter-species competition seems to greatly influence the aerial and root biomass of the tested plants.

6. Conclusions

The obtained results showed that the use of NaCl as a surrogate of SW for risk assessment of soil salinization may greatly overestimate the risk and, thus, its use should be cautious. Standard monospecific assays with plants seem to underestimate the risk of soil salinization since they could not detect sub-lethal effects observed after prolonged exposure (28 d) at low levels of salinity, under monoculture conditions. Furthermore, results suggest that the threshold value

above which a soil is considered saline, 4 $mScm^{-1}$, should be revised since at this or lower conductivity values, adverse effects were observed both for aerial and root biomass in the studied plant species, under such scenario. The reduced root biomass in some species, under monoculture conditions, observed at the low level of salinity (4 $mScm^{-1}$), foresees the worsening of costal soil erosion processes namely under the frame of climate changes associated with sea level rise, which may subsequently result in the disruption of the soil structure and communities. However, when assessing effects of salinization under polyculture scenarios, overall the productivity, for aerial and root biomass, increased. Polyculture conditions should be encouraged in coastal terrestrial ecosystems that are predicted to suffer from salinization.

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Chapter VIII

Salinity induced effects on the growth rates and mycelia composition of basidiomycete and zygomycete fungi

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Salinity induced effects on the growth rates and mycelia composition of four species of wood decaying fungi

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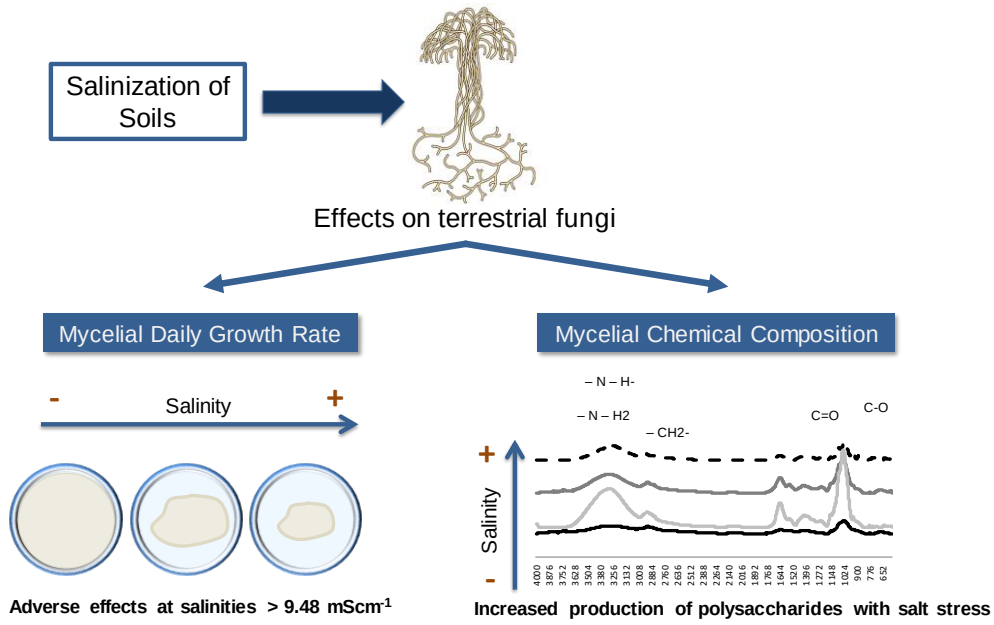
Graphical abstract



Abstract

Soil salinization, as the combination of primary and secondary events, can adversely affect organisms inhabiting this compartment. In the present study, the effects of increased salinity were assessed in four species of terrestrial fungi: *Lentinus sajor caju*, *Phanerochaete chrysosporium*, *Rhizopus oryzae* and *Trametes versicolor*. The mycelial growth and biochemical composition of the four fungi were determined under three exposure scenarios: 1) exposure to serial dilutions of natural seawater (SW), 2) exposure to serial concentrations of NaCl (potential surrogate of SW); and 3) exposure to serial concentrations of NaCl after a period of pre-exposure to low levels of NaCl. The toxicity of NaCl was slightly higher than that of SW, for all fungi species: the conductivities causing 50% of growth inhibition (EC_{50}) were within 14.9 and 22.0 $mScm^{-1}$ for NaCl and within 20.2 and 34.1 $mScm^{-1}$ for SW. *Phanerochaete chrysosporium* showed to be the less sensitive species, both for NaCl and SW. Exposure to NaCl caused changes in the

biochemical composition of fungi, mainly increasing the production of polysaccharides. When fungi were exposed to SW this pattern of biochemical response was not observed. Fungi pre-exposed to low levels of salinity presented higher EC_{50} than fungi non-pre-exposed, though 95% confidence limits overlapped, with the exception of *P. chrysosporium*. Pre-exposure to low levels of NaCl also induced changes in the biochemical composition of the mycelia of *L. sajor caju* and *R. oryzae*, relatively to the respective control. These results suggest that some terrestrial fungi may acquire an increased tolerance to NaCl after being pre-exposed to low levels of this salt, thus, suggesting their capacity to persist in environments that will undergo salinization. Furthermore, NaCl could be used as a protective surrogate of SW to derive safe salinity levels for soils, since it induced toxicity similar or higher than that of SW.

Keywords: fungi; salinity; seawater; pre-exposure; FTIR

1. Introduction

Salinization of soils may occur as a natural process due to the accumulation of salts resulting from the degradation of the soil parent rock. However, considering the most recent reports on climate change, increasing sea level, and severe and frequent weather events can also act as main drivers for salt intrusion in coastal regions (IPCC, 2013). Furthermore, these same reports point to a global increase in mean temperatures and scarce rainfall events. All these factors will eventually promote salinization of coastal soils, which may be exacerbated by other factors, such as uncontrolled water consumption by society in general and inappropriate irrigation practices (European Soil Portal, 2012). The excessive amount of salts in the soils may change water-holding capacity and promote ions imbalance, thus impacting life cycle traits of the groups of organisms that inhabit it (e.g. Pereira et al., 2015). Within this framework of soil salinization, it is pertinent to understand its effects on fungi, since these organisms play a pivotal role in the functioning of soil communities. Namely they contribute to the availability of water and nutrients to plants and other edaphic organisms (Genre and Bonfante, 2012), to soil's aggregation (Ritz and Young, 2004; Genre and Bonfante, 2012) due to a complex net of hyphae that are able to spread and cover a large soil area, to carbon and nutrient recycling (Dighton, 2007; Baldrian and Valášková, 2008)

or even contribute as a food source (e.g., rhizosphere bacteria) (Ballhausen and de Boer, 2016). Furthermore, their almost unique capacity to degrade lignin (only shared with bacteria) makes them relevant players in the degradation of plant material (Schwarze et al., 2013). Following this context, an increasing attention has been given to this group of organisms within the research field of ecological risk assessment studies. Within the context of salinization scenarios, some works have already reported the effects of increased salinity in soil fungi. Castillo and Demoulin (1997) observed that the growth of *Microporus xanthopus* was inhibited at salinities between 35-40 gL^{-1} NaCl (corresponding to 69.3 and 79.2 $mScm^{-1}$) and that the growth of *Schizophyllum commune* never decreased below 50% even when it was exposed to salinities of 35 gL^{-1} (salinity of natural seawater). Aksu and Balibek (2010) reported that the growth rates of Rhizopus arrhizus only declined by 20-25%, even after being exposed to 50 gL^{-1} NaCl (conductivity of 98.9 $mScm^{-1}$). These published results suggest a high tolerance of terrestrial fungi to salinity. Such high tolerance may be related with their capacity to produce many extracellular compounds that helps to control the excessive uptake of chemical compounds present in the surrounding environment, either by immobilizing them externally or by metabolizing them (Fink-Boots et al., 1999; Nesci et al., 2004). For instance, Fink-Boots et al. (1999) observed an increased production of extracellular enzymes and consequent degradation of metabolites (phenol compounds and superoxide anion radicals) in basidiomycete species exposed to temperature stress. Furthermore, in four food spoilage fungi strains (*Aspergillus* section Flavi) exposed to NaCl (osmotic potential of -3.0, -7.0, -10.0 MPa), the total content of polyols (mainly glycerol) and sugars (mainly glucose) increased with increasing salt concentration (Nesci et al., 2004). These fungi stress-induced responses have been associated with its high tolerance to salinity.

Though some knowledge already exists on the sensitivity and mechanisms of response of fungi to salt stress, little is known regarding the effects that prolonged exposure to low salinity levels may exert on fungi. One of the few published works focusing on long-term effects of salinity in fungi was done by Langenfeld-Heyser et al. (2007). These authors monitored the growth and stress responses of *Paxillus involutus* when exposed to NaCl. They found that a liquid culture of this basidiomycete was able to growth at NaCl concentrations as high as 100 mM (approximately

11.56 $mScm^{-1}$). Furthermore, although the growth of P. involutus started to be affected at 200 mM NaCl (23.13 $mScm^{-1}$), it could tolerate up to 500 mM NaCl (57.84 $mScm^{-1}$) after being exposed for three weeks to NaCl.

Regarding salinity tolerance observed in wood decay fungi, information available is scarce if not inexistent. Accordingly, the present work aimed to assess the effects of increased salinity on the mycelial growth and biochemical composition of four species of terrestrial fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Rhizopus oryzae* and *Lentinus sajor caju*). For this, two specific objectives were delineated: (i) to assess if NaCl could be used as a surrogate of natural seawater for risk assessment purposes and (ii) to evaluate the effects of prolonged exposure to low salinity levels on the growth and mycelial composition of fungi.

2. Materials and Methods

2.1 Test solutions

Increasing levels of salinity were set up by using a gradient of sodium chloride (NaCl) and of natural seawater (SW) conductivities. Conductivity values (expressed in $mScm^{-1}$) were employed as a measure of salinity. The salt NaCl was supplied by Merck (St Louis, MO, USA) and the SW was collected at a site located in the East coastal region of the North Atlantic Ocean (40°38'33"N, 8°44'55"W: Aveiro, Portugal), monitored under the European Union regulation (Directive 2006/7/CE) for its water quality. For each NaCl concentration, culture medium powder [3.9% Potato Dextrose Agar (PDA), Merck, Darmstadt, Germany] and the previously calculated quantities of NaCl (to reach the desired final conductivity) were weighed separately and added to the adequate amount of ultrapure water (1 L). Before being used for ecotoxicological assays, SW was filtered through cellulose nitrate membranes with a pore size of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain) to remove associated natural biota. Each SW dilution (in which the culture medium powder was also directly dissolved) was prepared by adding ultrapure water to seawater. All concentrations/dilutions were sterilized prior to its use in an autoclave for 20 minutes, at 121 ºC and 1 Bar (Uniclave 88, AJC).

2.2 Test species

Four terrestrial fungi species, including three saprophytic basidiomycetes [*Phanerochaete chrysosporium* (Burdsall 38388), *Lentinus sajor caju* (Fries) Fries and *Trametes versicolor* (Pilát 38412)] and a zygomycete (*Rhizopus oryzae*, Went & Prins. Geerlings, 31002) were used in this study. *Phanerochaete chrysosporium*, *T. versicolor* and *R. oryzae* were obtained from the BCCMTM/MUCL Culture Collection (Belgium) whereas *L. sajor caju* was obtained from UNESP (São Paulo State University, Brazil). These species were selected because their physiology and ecology is very well known and they have been used in several ecotoxicological studies as model species representative of wood decaying fungi (e.g. Purkayastha et al., 1994; Baldrian and Gabriel, 1997; Fahil, 1997; Guillén and Machuca, 2008; Galindo et al. 2013; Uyar and Uyar, 2016, Xie et al., 2016).

Individual cultures of each species were preserved in the laboratory, in PDA, in the dark, at 4ºC. Prior to the beginning of the tests, new individual cultures were obtained. Each fungi species was cultured in PDA at 28 ºC in the dark for 3-5 days depending of the species.

2.3 Growth inhibition assays

Growth inhibition assays were carried out by exposing each of the four fungi species to gradients of salinity constituted by seven to eight conductivities of NaCl and SW, in 90 mm Ø Petri dishes. Detailed descriptions of the conductivities ranges ($mScm^{-1}$) tested for each species and time (days) of exposure to NaCl and SW are shown in Table 1. Negative controls (only PDA medium) were also performed for each assay.

Table 1 – Description of the conductivities range ($mScm^{-1}$) tested and the duration of the assays (days) for each fungus species. NPE: fungi non-pre-exposed to low levels of salinity; PE: fungi pre-exposed to low salinity levels; SW-natural seawater. The dilution factor used for all serial dilutions was 1.2x.

	Time of exposure (days)		**Ranges of conductivity ($mScm^{-1}$)**	
Fungi	**NPE-NaCl/SW**	**PE-NaCl**	**NPE_NaCl/PE-NaCl**	**SW**
Lentinus sajor caju	8	11	10.2 – 30.5	10.2 – 36.6
Trametes versicolor	8	8	10.2 – 30.5	14.7 – 52.7
Rhizopus oryzae	8	9	10.2 – 36.6	14.7 – 52.7
Phanerochaete chrysosporium	3	3	14.7 – 52.7	14.7 – 52.7

For each fungal species, the assay was started by placing, at each replicate, a single circular 7 mm Ø agar disk collected from the edge of an active growing culture. Four replicates were performed per conductivity and control. Time of exposure was dependent on the growth rate of fungi, thus, an assay ended when the control Petri dishes were totally covered by the respective mycelium. According to this, exposure occurred during three (for *P. chrysosporium*) or eight (*L. sajor caju*, *T. versicolor* and *R. oryzae*) days, at 28ºC and in the dark (Table 1; NPE). The average diameter of the disks was measured at the beginning and at the end of the assays, by measuring the minimum, maximum and diagonal diameters, as highlighted in Figure 1. Daily growth rate (*DGR*; $mmday^{-1}$), for each species was determined by using Equation 1:

$$DGR_{ab} = \frac{D_b - D_a}{t_b - t_a}, \text{ mmday}^{-1}, \quad \text{(Eq. 1)}$$

where D_b is the average of the three measured diameters at the end of the assay, D_a is the diameter of the disk at the beginning of the assay (7 mm) and t_b-t_a is the exposure time interval (in days). At the end of the assays, the mycelium of each fungus was carefully collected from the surface of the solid medium, avoiding pulling out pieces of the solid medium, using a sterile cell scraper. Mycelium from the four replicates was pooled and samples were stored and frozen in sterile tubes at -80ºC until further analysis. Afterwards, these samples were lyophilized (at -85ºC, 0.08 mBar for 24 hours; Telstar, LyoQuest-55) and reduced to powder and homogenized to obtain a uniform distribution of the sample. Then, samples were analyzed by Attenuated Total Reflectance Fourier

Transform Infrared Spectroscopy (FTIR-ATR) [Perkin Elmer (USA) Spectrum BX FTIR instrument] to assess any molecular changes in the fungal mycelium.

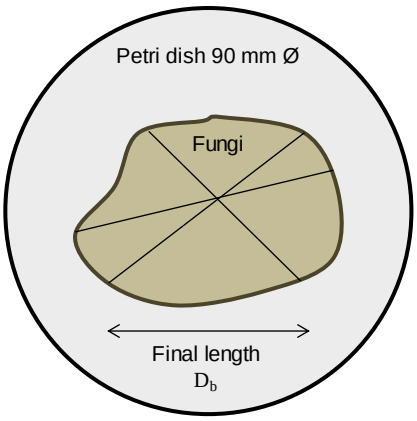


Figure 1: Schematic representation of the measurements made of the minimum, maximum and diagonal diameters of the mycelium of fungus.

2.4 Period of pre-exposure to low levels of NaCl (PE-NaCl)

To evaluate the effects of prolonged exposure of the fungi to low levels of salinity, individual cultures of each species were obtained and maintained under the same conditions as previously described (section 2.3) in PDA medium spiked with NaCl concentrations corresponding to the conductivity that caused an inhibition of 25% in the daily growth rate (EC_{25}) of each fungi species. This conductivity was selected because it is close to the threshold value for significant effects (lowest observed effect concentration or concentration causing 20% of effect). To perform these assays, only NaCl was tested since previous studies (please see section 2.3 of Material and Methods and also Results section) suggested NaCl as exerting a higher or similar toxicity comparatively to SW. The period of pre-exposure (PE) to the respective EC_{25} NaCl concentration corresponded to the period need to re-culture each fungus three times, therefore, this period of pre-exposure to low levels of salinity depended on the fungi growth rate (Table 1). Every time the mycelium covered the whole surface of the culture medium in the Petri dish (90 mm Ø) it was re-cultured by introducing a 7 mm Ø disk (of each fungus mycelium, collected into a new Petri dish) in new Petri dishes (90 mm Ø) with freshly prepared culture media spiked with the respective EC_{25} NaCl concentration. This procedure was repeated three times, leading to the following exposure periods: 33, 24, 27 and 9 days for *L. sajor caju*, *T. versicolor*, *R. oryzae* and

P. chrysosporium. After the period of pre-exposure to the EC_{25} NaCl concentration (PE-NaCl) the daily growth rate of each fungi species was reassessed (Fig. 1), by performing the same assays as described in section 2.3 and by testing the same range of NaCl conductivities (Table 1). At the end of each assay, mycelium was again collected carefully from the surface of the solid medium (avoiding collection of solid medium) with the help of sterile cell scrapers. Mycelium obtained from each of four replicates was pooled and stored in sterile tubes (-80ºC) and lyophilized (-85ºC, 0.08 mBar for 24 hours). Lyophilized samples were reduced to powder and homogenized before analysis through FTIR-ATR spectroscopy.

2.5 Fourier Transform Spectroscopy (FTIR-ATR)

The FTIR analyses were carried out for fungi exposed to NaCl (with no pre-exposure to low levels of salinity - NPE-NaCl; and with pre-exposure to low levels of salinity - PE-NaCl) and to SW (Table 1). Three replicates were made for each sample. For each replicate were performed 64 readings. Final spectra profiles corresponded to the average values of those 64 readings. Spectra profiles obtained correspond to the total amount of compounds at the cellular wall and cytoplasmatic compartment of the cells.

The infrared (IR) spectra of lyophilized mycelia were obtained at a 4 cm^{-1} resolution within the 4000 – 550 nm range. The spectra were obtained by comparison with the room spectra, by using air as the background spectrum. Two corrections were made, the baseline correction and the smooth correction by using the software Spectrum 8.

The FTIR-ATR data obtained was evaluated in accordance to the peak attribution and molecular vibrations of specific chemical bonds as described by Naumann (2009) (Table 2).

Table 2: Correspondence between profiles absorbance peaks obtained with FTIR-ATR and macromolecules present in fungi mycelia.

Peak/Wavenumber (cm^{-1})	Functional groups/Association with macromolecules
3650 - 3000	O-H bond vibrations from carboxyl, hydroxyl and phenol groups Amides N-H vibrations
3000 - 2765	CH_2 symmetrical and asymmetrical stretching
1750 - 1500	Proteins with functional groups, amides I and II Proteins and lipids with functional groups, CH_2 and CH_3 Phosphate compounds with functional group P-O
1495 - 1200	Symmetric C-H deformation from groups C-H_2 and O-H deformations and C-H elongation from phenolic groups (within 1462 – 1454 cm^{-1}) C-O elongations and O-H deformations (around 1230 cm^{-1})
1200 - 1180	Polysaccharides with functional groups C-O-C and C-O-P
1150	C-O stretching and CH_2 bending
1070	C-O stretching.

3. Data analysis

Conductivities (as a surrogate measure of salinity) provoking X% of effect (EC_x) for growth inhibition were computed through non-linear regression analysis by fitting a logistic model with the program Statistica for Windows 4.3 (StatSoft, Aurora, CO, USA).

The highest tested conductivity with no statistical significant effect comparatively to the control (NOEC) and the lowest tested conductivity presenting a statistical effect comparatively to the respective control (LOEC), computed for each fungi species under the three exposure scenarios (NPE-NaCl, SW and PE-NaCl), were determined through a one-way Anova, followed by the multicomparison Dunnett's test ($p \leq 0.05$) (data presented as supplementary material). To test the hypothesis that NaCl and SW exert similar toxicity to the fungi, the overlapping of confidence limits (95%) was analysed and the NOEC and LOEC values were compared. A two-way ANOVA analysis could not be used for this comparison because the range of conductivities tested was not the same for NaCl and SW.

To test if these species of fungi were able to cope with low levels of salinity after pre-exposure to low levels of NaCl, a two-way ANOVA was performed followed by the Holm-Sidak

multicomparison test ($p \leq 0.05$), to compare growth rates at different concentrations for non-pre-exposed (NPE-NaCl) and pre-exposed fungi (PE-NaCl).

Additionally, a principal component analysis (PCA) and cluster analysis were performed using the FTIR-ATR areas determined for the specific regions described in Section 2.4 (obtained for control and the three highest salt treatments for each tested scenario: NPE-NaCl, SW and PE-NaCl), in order to assess possible relationships between the biochemical composition of the here studied fungi and the salinity levels to which they were exposed to. Multivariate analysis was performed with external peak areas (Supplementary Table S1) by using the software in Primer 6 Software Package.

4. Results

4.1 Effects of NaCl *versus* Seawater on the growth of fungi

The analysis of results obtained for mycelial growth rate suggest *P. chrysosporium* to be the most tolerant fungi species to NaCl (EC_{50} of 32.1 $mScm^{-1}$). The other three tested species exhibited similar sensitivities to NaCl (with confidence limits overlapping or with very close upper and lower limits): *T. versicolor*, *L. sajor caju* and *R. oryzae* with EC_{50} of 14.9, 18.7 and 22.0 $mScm^{-1}$, respectively (Table 3). Additionally, *P. chrysosporium* showed to be the fungi that started to undergo adverse effects at higher salinity levels of NaCl, with EC_{20} of 17.8 $mScm^{-1}$, respectively. The other three species of fungi started to experience effects on growth at lower salinity levels with EC_{20} of 9.48, 11.1 and 11.9 $mScm^{-1}$, for *T. versicolor*, *L. sajor caju* and *R. oryzae*, respectively (Table 3).

Table 3. Conductivities ($mScm^{-1}$), with corresponding 95% confidence limits depicted within brackets, causing 10, 20, 25 and 50% (EC_x) of growth inhibition in fungi exposed to sodium chloride [NaCl, for non-pre-exposed (NPE-NaCl) and pre-exposed (PE-NaCl) fungi)] and natural seawater (SW). * indicates differences between EC_x computed for NPE-NaCl and EC_x computed for SW (no overlap of confidence limits at 95%). Letters (a,b) indicate differences between EC_x computed for NPE-NaCl and EC_x computed for PE-NaCl (no overlap of confidence limits at 95%).

Fungi	**Conductivity ($mScm^{-1}$)**			
	EC_{10}	**EC_{20}**	**EC_{25}**	**EC_{50}**
Lentinus sajor caju				
NPE-NaCl	8.89[a]	11.1	13.7[a]	18.7*
	(6.75-11.0)	(9.64-13.2)	(11.7-15.8)	(16.9-20.4)
SW	8.47	12.0	14.1	23.7*
	(6.61-10.3)	(10.0-14.0)	(11.8-16.3)	(21.5-26.2)
PE-NaCl	4.35[b]	8.09	9.30[b]	22.8
	(2.54-6.16)	(5.83-10.4)	(8.80 – 9.40)	(19.8 – 25.9)
Trametes versicolor				
NPE-NaCl	7.06*[a]	9.48*	10.7*	14.9*[a]
	(4.97-9.13)	(7.36-11.6)	(8.50-12.9)	(12.8-16.9)
SW	18.6*	22.8*	24.9*	34.1*
	(17.0-20.3)	(21.3-24.4)	(23.1-26.6)	(32.6-35.6)
PE-NaCl	9.69[b]	12.4	13.9	20.2[b]
	(8.51-10.9)	(11.3-13.6)	(12.8-14.9)	(19.3-21.2)
Rhizopus oryzae				
NPE-NaCl	8.66	11.9*	14.0*	22.0*
	(6.15-11.2)	(9.31-14.5)	(11.6-16.5)	(19.7-24.3)
SW	13.1	17.4*	19.7*	28.5*
	(10.8-15.3)	(15.2-19.7)	(17.7-21.8)	(26.5-30.6)
PE-NaCl	5.08	8.78	11.4	25.3
	(1.87-8.31)	(4.85-12.7)	(7.01-15.8)	(19.6-31.1)
Phanerochaete chrysosporium				
NPE-NaCl	12.8[a]	17.8 [a]	18.7 [a]	32.1 [a]
	(9.77-15.8)	(14.7-21.0)	(15.8-21.5)	(28.6-35.6)
SW	13.2	19.3	20.2	37.4
	(11.3-15.1)	(17.3-21.3)	(18.2-22.3)	(35.4-39.8)
PE-NaCl	37.0[b]	42.3 [b]	47.4 [b]	57.2 [b]
	(33.8-40.1)	(40.0-44.7)	(45.2-49.5)	(54.1-60.3)

Regarding the results of mycelial growth rate after exposure to increased levels of SW, *T. versicolor*, *R. oryzae* and *P. chrysosporium* showed to be the most tolerant species, with EC_{50} of 34.1, 28.5 and 37.4 $mScm^{-1}$, respectively. *Lentinus sajor caju* was the most sensitive fungus, with

an EC_{50} of 23.7 $mScm^{-1}$ (Table 3). Furthermore, *L. sajor caju* was, as well, the species that started to experience effects on growth at lower salinity levels with an EC_{20} of 12.0 $mScm^{-1}$. The other three species began to experience effects on growth at higher conductivity values (EC_{20}>17.4 $mScm^{-1}$) (Table 3).

Comparing the effects of NaCl and SW on daily growth rate of fungi, the latter exerted a similar or lower toxicity to that of NaCl. Among the four-tested species, *T. versicolor* showed the highest difference in sensitivity to NaCl and SW: this species was 2-fold more tolerant to SW than to NaCl (Table 3; Supplementary Fig. S1).

4.2 Pre-exposure to low levels of NaCl (PE-NaCl)

Though, in general, fungi NPE-NaCl and PE-NaCl exhibited a similar tolerance to NaCl, expressed by similar EC_x and overlapping 95% confidence limits (Table 3), some exceptions were observed. The EC_{50} computed for PE-NaCl *T. versicolor* and *P. chrysosporium* were higher than those for the NPE-NaCl fungi. For the PE-NaCl *P. chrysosporium*, tolerance to NaCl increased 1.8 to 2.9-fold, as expressed by the computed EC_x (Table 3): the EC_{20} from 17.8 to 42.3 $mScm^{-1}$, the EC_{25} from 18.7 to 47.4 $mScm^{-1}$ and the EC_{50} from 32.1 to 57.2 $mScm^{-1}$ (Table 3). Additionally, *P. chrysosporium* growth significantly decreased relatively to the control at a conductivity value of 14.7 $mScm^{-1}$ for NPE-NaCl fungi while for PE-NaCl, this salt only significantly affected the growth of *P. chrysosporium* at a conductivity value of 44.0 $mScm^{-1}$ (Supplementary Fig. 1S). For the other three fungi species, though some differences in the computed EC_x were detected, in most cases the 95% confidence limits overlapped or were very close to each other, suggesting a similar tolerance to increased salinity before and after pre-exposure to low salinity levels (Table 3). Still regarding the results obtained for PE-NaCl fungi, *P. chrysosporium* was again the most tolerant fungus exhibiting the highest EC_{50} (57.2 $mScm^{-1}$). The PE-NaCl *T. versicolor*, *L. sajor caju* and *R. oryzae* showed similar sensitivities to NaCl, with EC_{50} of 20.2, 22.8 and 25.3 $mScm^{-1}$ (Table 3): there were no shifts in the ranking of saline tolerance between NPE-NaCl and PE-NaCl for these three fungi species.

4.3 FTIR-ATR analysis

Overall, analyses of the mycelium composition of the four terrestrial fungi species showed five distinct peaks of absorbance after exposure to saline stress, corresponding to carboxyl, hydroxyl, phenol or amides with O-H and N-H functional groups (within the range 3650–3000 cm^{-1}) (Klaus et al., 2015), lipids with functional groups CH, proteins (1750–1500 cm^{-1}) (Zhao et al., 2007), protein and lipids with functional groups CH_2 and CH_3 and phosphate compounds and polysaccharides with functional groups C-O-C and C-O-P (1200–805 cm^{-1}) (Gonzaga et al., 2005), respectively.

In general, when the four fungi species were exposed to increased concentrations of NaCl exhibited an increment, comparatively to the respective control, in three out of the five identified peaks, namely the peaks corresponding to carboxyl, hydroxyl, phenol, amines, proteins and polysaccharides (Fig. 2a, 2d, 2g, 2j; Supplementary Table S1). However, this pattern was not observed when the fungi were exposed to SW dilutions (Fig. 2b, 2e, 2h, 2k). Actually, only slight changes in the intensity of the peak corresponding to polysaccharides were observed under exposure to SW for *L. sajor caju* (at 24.4 $mScm^{-1}$), *T. versicolor* (at 36.6 and 44.7 $mScm^{-1}$) and *R. oryzae* (at 36.6 $mScm^{-1}$) (Fig. 2b, 2e, 2h). Looking at the effects of salinity, comparatively to the respective control, in fungi pre-exposed to low levels of NaCl (PE-NaCl), a decrease in the intensity of the three peaks was observed for *L. sajor caju*, an increase for *R. oryzae* and the inexistence or only slightly changes in the cases of *T. versicolor* and *P. chrysosporium* (Fig. 2c, 2f, 2i, 2l). When comparing the intensity of the peaks of the bands under control conditions before and after pre-exposure to low salinity levels, some changes were observed: for *R. oryzae*, the intensity of the polysaccharide peak decreased after pre-exposure to NaCl (i.e. NPE-NaCl *versus* PE-NaCl under control conditions; Fig. 2g, 2i), while for *L. sajor caju* and *P. chrysosporium*, an increase in the peaks of the controls after pre-exposure to NaCl was observed (Fig. 2a, 2c for *L. sajor caju*; Fig. 2j, 2k for *P. chrysosporium*). For *L. sajor caju*, *T. versicolor* and *P. chrysosporium*, non-pre-exposed to NaCl, the cluster analysis showed that the control formed an isolated group from the remaining tested treatments, while after pre-exposure to NaCl, the control grouped with the lowest and/or highest salinity treatment (Supplementary Fig. S2, S4, S5). For *R.*

oryzae the control grouped, both before and after pre-exposure to NaCl, with the 30.5 $mScm^{-1}$ (the second highest tested conductivity) (Supplementary Fig. S3).

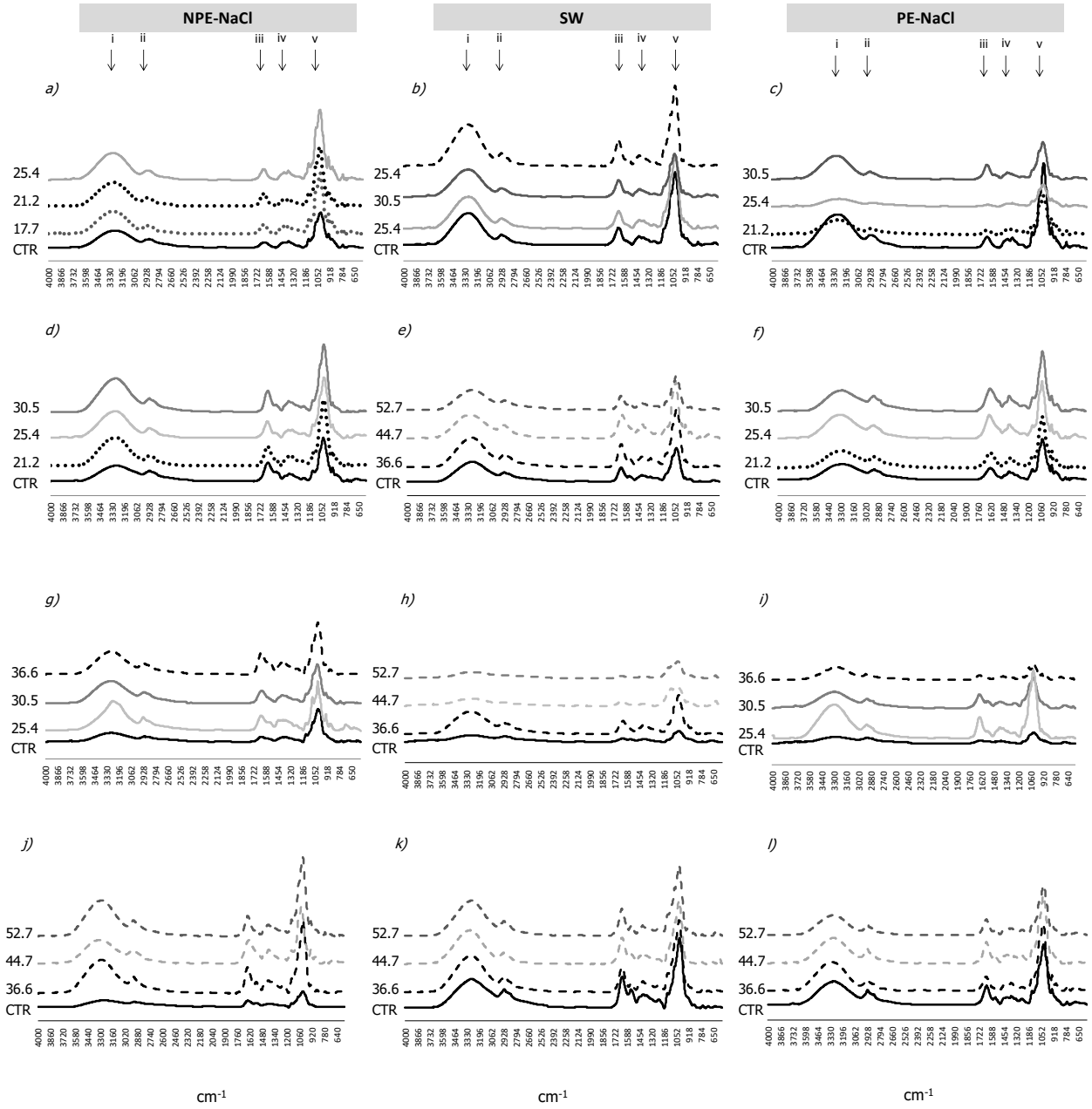


Figure 2: FTIR-spectra of the mycelia of *the studied terrestrial fungi*: *Lentinus sajor caju* exposed to a) sodium chloride (NPE-NaCl), b) natural seawater (SW) and c) pre-exposure to low levels of NaCl (PE-NaCl), *Trametes versicolor* exposed to same conditions (d, e and f), as well as *Rhizopus oryzae* (g, h and i) and *Phanerochaete chrysosporium* (j, K and l), respectively. Values at the left of each curve correspond to the salinity level tested (in $mScm^{-1}$). Indentions on the spectra correspond to: i) peak in 3650–3000 cm^{-1} range for O-H bond vibrations from carboxyl, hydroxyl or phenol groups, and from amides N-H vibrations; ii) peak in 3000-2765 cm^{-1} range for lipids with functional groups CH; iii) peak in 1750-1500 cm^{-1} range for proteins with functional groups, amides I and II; iv) peak in 1495–1200 cm^{-1} range for proteins and lipids with functional groups, CH_2 and CH_3 and phosphate compounds with functional group P=O; and v) peak in 1200–805 cm^{-1} range for polysaccharides with functional groups C-O-C and C-O-P.

5. Discussion

5.1 NaCl *versus* SW

One of the specific objectives of this study was to assess the possible use of NaCl as a surrogate of SW for ecological risk assessment of seawater intrusions scenarios in coastal terrestrial ecosystems. The data obtained for mycelium daily growth rate revealed that NaCl exhibited a similar or higher toxicity comparatively to SW for the four tested fungi, depending on the fungi species, the intensity of the response (EC_x) and exposure ($mScm^{-1}$). Therefore, it is suggested that the EC_{10} computed for NaCl (alternative for the non-predicted effect concentration - NOEC) could be used for the derivation of safety salinity levels in first screening phases of ecological risk assessment (ERA) for seawater intrusions in coastal terrestrial ecosystems.

This highest toxicity of NaCl comparatively to SW is inline with results reported by Pereira et al. (2015). These authors observed a similar or higher toxicity of NaCl comparatively to SW in terrestrial invertebrate species, namely enchytraeids and collembolans. The higher complexity of ions in SW may explain its lower toxicity to biota. Some ions that are present at low concentrations in SW are known to be required in several metabolic functions of organisms. In fungi, for example, magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions may be co-factors of enzymes and contribute to the hyphal extension (e.g. Chardonnet et al., 1999; Kim and Yun, 2005). Furthermore, though sodium (Na^+) and chloride (Cl^-) are the most representative ions in SW, it is expected that for the same level of conductivity they will be present at higher concentrations in NaCl solutions than in SW. These ions have been reported to be the main responsible for the high toxicity of increased salinity to freshwater or terrestrial organisms, and thus, it is suggested to be responsible for the highest effects of NaCl on the growth of the here studied fungi (e.g. Lee and Ray, 2007). In a similar way to what can occur in other organisms, in fungi, the increased extracellular amounts of ions (like Na^+ and Cl^-) increases the difference between the membrane potential of the extracellular and intracellular compartments. In turn, the excitation of the membranes causes the opening of the potassium (K^+) channels, leading to the diffusion of K^+ out of the cell and the entrance of Na^+ and Cl^- ions. This way, the excessive amount of Na^+ ions may

outcompete with K^+ ions for the same binding sites in various cellular functions (activation of enzymes, production of solutes). However, since Na^+ cannot replace K^+, vital cellular functions may be impaired when fungi are submitted to osmotic stress and result in decreased growth rates as shown in the present study (Giri et al., 2007; Evelin et al., 2009). In addition, the adverse effects caused by Cl^- ions, may be induced at lower concentrations than those of Na^+ (e.g., Hirrel, 1981; Luo et al., 2005). This higher toxicity of Cl^- may be related with the fact that Na^+ can form stronger bonds with lipids present in cell membranes while Cl^- remains free in solution having potential to induce toxicity at a higher number of different sites inside the cells (Knetch and Klasczyk, 2013). Overall, the presence of a high content of salts in the medium causes osmotic stress and may inhibit species growth *or induce biomass decline* (*Langenfeld-Heyser et al., 2007; Tang et al., 2009)*. Yet, all fungi species here tested have shown a high tolerance towards salinity. For example, *L. sajor caju*, the most sensitive species, was able to tolerate a third of the salinity (measured in conductivity) of natural seawater and *P. chrysosporium*, the most tolerant tested fungi, was able to tolerate salinities equivalent to 60% of natural seawater. These results are inline with previous works already published which evidences that salt tolerance is species dependent. Castillo and Demoulin (1997) reported that the growth of the white rot fungi *Pycnoporus sanguineus* and *Schizophyllum commune* (GC 41 and AN 131) was only inhibited at concentrations of 60 and 35 g L^{-1} NaCl (corresponding approximately to 97 and 60 $mScm^{-1}$). Ayodele and Ojoghoro (2007) observed that only levels as high as 15 and 20 grams of NaCl (corresponding approximately to 25 and 33 $mScm^{-1}$, respectively) provoked a significant growth inhibition in *Pleurotus tuberregium*.

The tolerance displayed by the four fungi studied in this work may be related both with their ability to accumulate high levels of solutes (e.g. glycerol, manitol) in the organelles, this way maintaining the osmotic balance (e.g. Davis et al., 2000; Managbanag and Torzilli, 2002) and/or, with mechanisms that are activated to reduce the uptake of excess ions (e.g. production of extracellular compounds) (Kogej et al., 2007). For instance, in a study comprising five strains of *Debaryomyces hansenni* and *D. fabryi* (halotolerant yeasts) it was shown that the most salt-sensitive (*D. hansenni* CB5767) and the most salt-tolerant (*D. fabryi* CBS1793) strains presented

similar cellular contents of Na^+ (Michán et al., 2013). Though both strains were able to tolerate similar Na^+ concentration levels, the mechanisms involved in such tolerance were different involving the membrane structure: the most tolerant strain exhibited a permeable membrane but a more efficient mechanism for excretion of excessive ions while the most sensitive strain possessed a more rigid membrane that is believed to prevent the entrance of ions.

Regarding biochemical analysis of fungi mycelia, the highest differences in peaks intensity (between control and salt treatments) were observed after exposure to NaCl while for SW only slightly changes were recorded (for *L. sajor caju* at 24.4 $mScm^{-1}$, *T. versicolor* at 36.6 and 44.7 $mScm^{-1}$ and *R. oryzae* at 36.6 $mScm^{-1}$). Overall, the four fungi exhibited an increase in the production of metabolites associated with groups of carboxyl, hydroxyl phenol, and amides and as well of polysaccharides and proteins after exposure to NaCl. However, after exposure to SW, only slight changes in the production of polysaccharides were observed for *R. oryzae*. These results are in agreement with the low toxicity caused by SW, as confirmed by their effects in growth rates and may be related, as mentioned above, with the complex composition of SW. The presence of other ions in SW may buffer against the effects that Na^+ and Cl^- may cause when solely, for instance, in a solution of NaCl, not only because other ions have central roles in fungi growth (e.g., calcium; Jackson and Heath, 1993) but also because in SW, competition between similar charged ions is also possible diminishing their toxicity towards the organism (e.g., Mount et al., 1997).

The changes in the mycelial biochemical composition (increased content/production of extracellular compounds as carboxyl, hydroxyl, phenol or amides compounds, proteins and polysaccharides) may be part of the responses of fungi to salt stress. They could be probably related with the accumulation of solutes within the cells or with the external mucilage that they produced because both are common mechanisms used by these organisms to cope with salt stress (Vesentini et al., 2005; Vesentini et al., 2006). For instance, the observed increased levels of hydroxyl groups after exposure to NaCl may be related with the production of osmoprotectors enabling the formation of a hydration shield around the hyphae, as described by Galinsky and Truper (1994), which prevents metabolic inactivation of the fungus during osmotic stress.

Additionally, the increased production of polysaccharides may be related with the extracellular immobilization of ions with subsequent reduced internalization, as already reported for other chemicals, namely metals (Baldrian, 2003, Vesentini et al., 2005; Vesentini et al., 2006) or herbicides (e.g. Jaszek et al., 2006). As an example, the addition of 25 µM of paraquat to the liquid culture medium of *T. versicolor* did not changed significantly the growth rates of this particular fungi when compared to a non-exposed culture, indicating that it was able to cope with this concentration of the herbicide (Jaszek et al., 2006). It is suggested that growth was probably maintained at the expense of the production of extracellular compounds. Actually, the investigators have found that at this paraquat concentration, *T. versicolor* extracellular activity of laccase, superoxide dismutase and catalase increased as a defense mechanism to prevent intracellular dysfunction (Jaszek et al., 2006). In the present study, even though growth rates decreased at high salinity levels, growth was not totally inhibited, this may be due to the presence of mechanisms that allow the fungi to counteract the osmotic pressure, for example the release of compounds. Other mechanisms have, as well, been identified in fungi and are associated with their capacity to survive under hypersaline environments, namely alterations at the cellular membrane level, like alterations in membrane phospholipids ratio or the presence of a melanin layer (e.g., Cantrell et al., 2006; Kejžar et al., 2013) and alterations at the genome level, for instance, increased production of proteins with high content on acidic aminoacid residues (e.g., Kis-Papo et al., 2014).

5.2 Effect of pre-exposure to low levels of NaCl in fungi

Among the four-tested species of fungi, *P. chrysoporium* was the only one clearly showing a higher tolerance to NaCl after pre-exposure to the respective EC_{25} NaCl concentration. Thus, corroborating the differential sensitivities and mechanisms of response of fungi to salt stress. This capacity of *P. chrysosporium* to acquire an increased tolerance to salt stress over time, may be associated with its higher capacity for the production and/or retention of solutes. Interestingly, this fungus, after being pre-exposed to low levels of salinity, increased its basal level (under control conditions) of several metabolites (e.g. compounds with carboxyl, lipids, proteins,

polysaccharides), as confirmed by the increase in external peaks areas obtained by infra-red spectra (increments of 53, 10, 9 and 43% for carboxyl, lipids, proteins and polysaccharides, respectively). Furthermore, while differences in the intensity of peaks were observed between the control and NaCl concentrations for NPE-NaCl such differences were not observed for PE-NaCl. These results may be indicative of its capacity to acclimate to osmotic stress after a prolonged exposure. Actually, the ability of fungi to acclimate to chemical stress is well known and it has been widely used to enhance some characteristics of these organisms. For instance, gradual acclimation of fungi to wastewaters has been used by industries to promote the treatment of these waters before their release to the environment (Aissam et al., 2007). Acclimation of fungi (particularly arbuscular mycorrhizal fungi) is commonly employed in agriculture: the tolerance of fungi to high salinity levels helps to improve crop's plants to develop in saline soils (e.g. Sharifi et al., 2007). The production of extracellular compounds has been reported as a common physiological response that leads to acclimation processes (Baldrian, 2003; Huang et al., 2010). This has already been discussed for freshwater microalgae and fungi isolated from saline soils (e.g. Salama et al., 2013; Smolyanyuk et al., 2013). Using a halotolerant mycelial fungus (*Fusarium* sp.), Smolyanyuk et al. (2013) confirmed the retention of cellular compatible solutes as a way to tolerate saline stress: for instance, exposure to hypersaline conditions of 1 and 1.5 M (approximately 116 and 174 $mScm^{-1}$, respectively) resulted in an intracellular increase of arabitol (sugar) by 44% and a two-fold increase on total carbohydrate content in this particular fungus.

Regarding the other three species of fungi that were studied (*L. sajor caju*, *T. versicolor*, *R. oryzae*) effect conductivities computed for growth after pre-exposure to low levels of salinity were very similar as the ones obtained before pre-exposure, indicating that these fungi were not capable of acquiring increased tolerance to salinity. However, *T. versicolor* and *R. oryzae* could maintain their growth rate, after pre-exposure to salinity, suggesting that though they were not capable of acquiring an increased tolerance to the salt they were capable to acclimate to it. *Lentinus sajor caju*, the species exhibiting the highest sensitivity to NaCl, was also the species that, after a pre-exposure to this salt, could not maintain its growth rate, being observed a significant decrease when exposed again in medium without salt comparatively to the non-pre-

exposed fungus. This indicates that, for some fungi, long periods of exposure to low levels of salinity may compromise mycelial growth, diminishing their ability to search for dead wood (foraging), which in turn may compromise the decomposition process in terrestrial ecosystems and consequently soil nutrient's cycling. This is of major concern since fungi must first degrade the most resistant forms of carbon (C) into labile C (the carbon pool from plant residues or particulate that is the most accessible and quickly degraded) so bacteria can convert this form of C. Furthermore, fungi function in nutrient's recycling has been reviewed and it has been suggested that fungi can convert labile C as much and fast as bacteria (de Vries and Caruso, 2016). Additionally, fungal hyphae are known to create a very specific environment (referred to as mycosphere) that is suitable for a very restrict bacterial community, that may also contribute to the rotting/decomposition process (Folman et al., 2008; Hervé et al., 2016). Moreover, salinity stress exerted over wood decay fungi may lead to indirect effects that can be reflected in other organisms: for instance, the role of other decomposer organisms (bacteria) may be compromised since they rely on the growth of fungi hyphae to be transported (hyphae act as carriers/transporters of some bacteria) (e.g. Johnston et al., 2016) or the interaction and behaviour between and within plants and soil's invertebrates may be altered as many of the extracellular compounds produced by fungi can act as chemical cues for these organisms (e.g. Fäldt et al., 1999; Lee et al., 2016).

Salinization of soils may, therefore, interfere directly with decomposition rates and the normal ecosystem nutrient recycling rates through the reduction of growth rates or interfering in the production of extracellular compounds of wood decay fungi. But, wood decay fungi have an intervention that goes further than the direct role of decomposition itself, as above mentioned. Therefore, effects of saline stress over wood decay fungi activity may also lead to a series of indirect effects on other soils organism's life traits.

6. Conclusions

In summary, following the similar to higher toxicity of NaCl, comparatively to that exerted by SW, it is suggested that this salt reveals an adequate and protective surrogate of SW to be used in early stages of ecological risk of salinization. This way, toxicity data already available in the

literature for NaCl may be used for a first risk screening of coastal ecosystems salinization due to seawater intrusion, thus, minimizing the need to use further animals on ecotoxicity assays.

Overall, the four fungi species revealed to be able to cope with low levels of salinity (EC_{25}) for prolonged exposure periods, probably through the regulation of the production of some metabolites, namely polysaccharides. Actually, one of the fungi species was even able to increase its fitness after a prolonged exposure to low levels of salinity, which indicates that some species of this group of organisms may be able to persist in salinized environments. Nevertheless, for two fungi species pre-exposed to NaCl, mycelial growth started to be impaired at salinity levels of 8.09 $mScm^{-1}$, which may be considered a low salinity level since it corresponds to approximately 16% of that of natural SW. Salinity levels higher than this value may easily occur in situations of extreme events where surface seawater intrusions may occur in coastal ecosystems. In such a situation, the decomposition rates and normal soil nutrient recycling may then be compromised since these wood decay fungi will have their growth affected. Furthermore, the EC_{10} values (proposed as a substitute of NOEC in ecological risk assessment) computed for two of the studied fungi (*L. sajor caju* and *R. oryzae*) were very close to the current threshold value for a soil to be considered saline (4,000 μScm^{-1}). Actually, the lower limits of the 95% confidence limits were well below 4,000 μScm^{-1}. Considering these results, it is suggested that the threshold value for the classification of soil salinization should be revised.

Finally, the results here obtained also highlight that indirect effects on soil biota may occur due to fungi exposure to low levels of salinity, since under such scenarios the studied fungi produced larger quantities of external compounds that, though increasing the tolerance of fungi to salinity, may adversely influence the fitness of other wood or soil biota.

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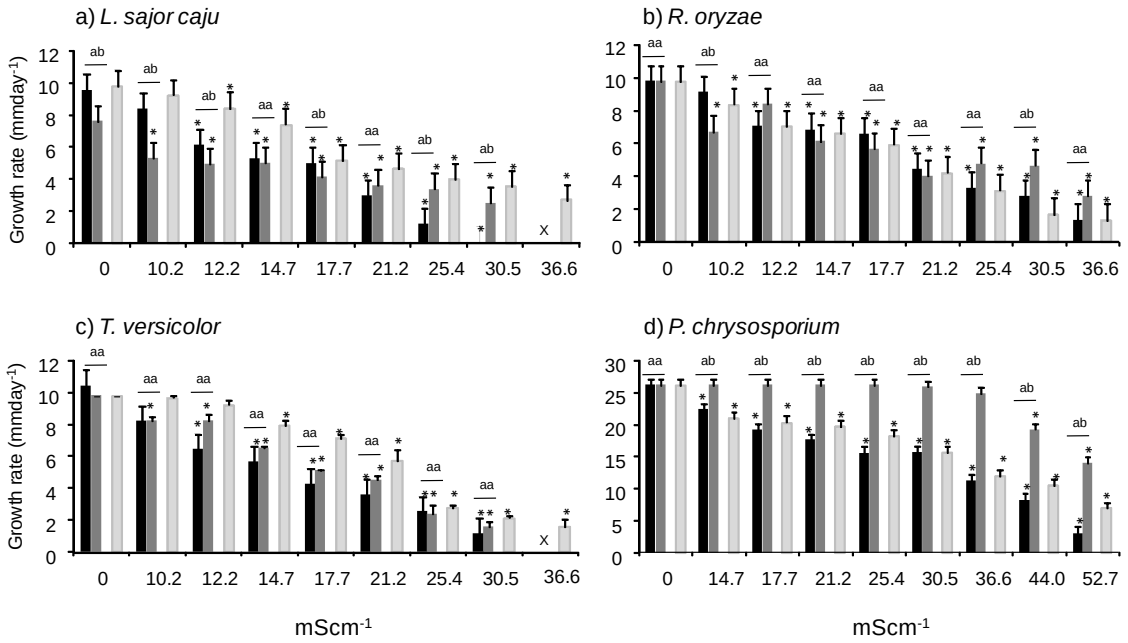
Supplementary Information



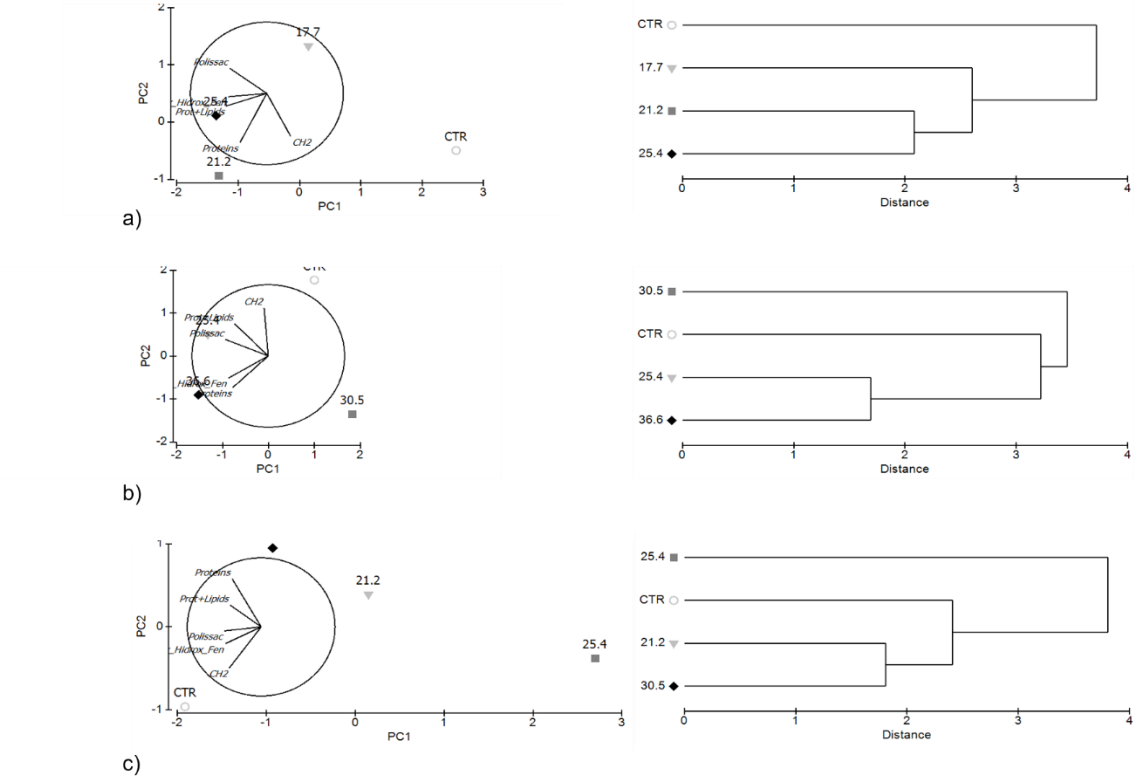
Supplementary Figure S1. Mycelial growth rate ($mmday^{-1}$) of the four species of terrestrial fungi after exposure to increased levels of sodium chloride (NPE-NaCl) (black bars), after pre-exposure to low levels of NaCl (PE-NaCl) (dark-grey bars) and to natural seawater (SW) (light-grey bars) (error bars correspond to the standard deviation). *denote significant differences (Dunnett's, $p \leq 0.05$), for each species, between treatments and the respective control group. X – not tested conductivities. letters^{a,b} – treatments sharing the same letters means no differences in mycelial growth rates before (NPE-NaCl) and after pre-exposure to low levels of NaCl (PE-NaCl).

Supplementary Table S1: External peaks areas of the infra-red spectra (FTIR) of lyophilized mycelia for the four terrestrial fungi species exposed to sodium chloride (NPE-NaCl), natural seawater (SW) and after pre-exposure to low levels of salinity (PE-NaCl). Analyses were performed for control conditions (CTR) and the three higher salinity levels ($mScm^{-1}$) for each exposure condition. Peak areas per interval correspond to: i) 3650–3000 cm^{-1} for O-H bond vibrations from carboxyl, hydroxyl or phenol groups, and from amides N-H vibrations; ii) 3000-2765 cm^{-1} corresponding to CH_2 symmetrical and asymmetrical stretching, respectively; iii) 1750–1500 cm^{-1} for proteins with functional groups, amides I and II (peak around 1540 cm^{-1}); iv) 1495–1200 cm^{-1} for proteins and lipids with functional groups, CH_2 and CH_3 as well as phosphate compounds with functional group P-O and where the small indention between 1462 and 1454 cm^{-1} is corresponding to symmetric C-H deformation from groups C-H_2 and O-H deformations and C-H elongation from phenolic groups; v) 1200–805 cm^{-1} for polysaccharides with functional groups C-O-C and C-O-P, where the peak around 1230 cm^{-1} corresponds to C-O elongations and O-H deformations.

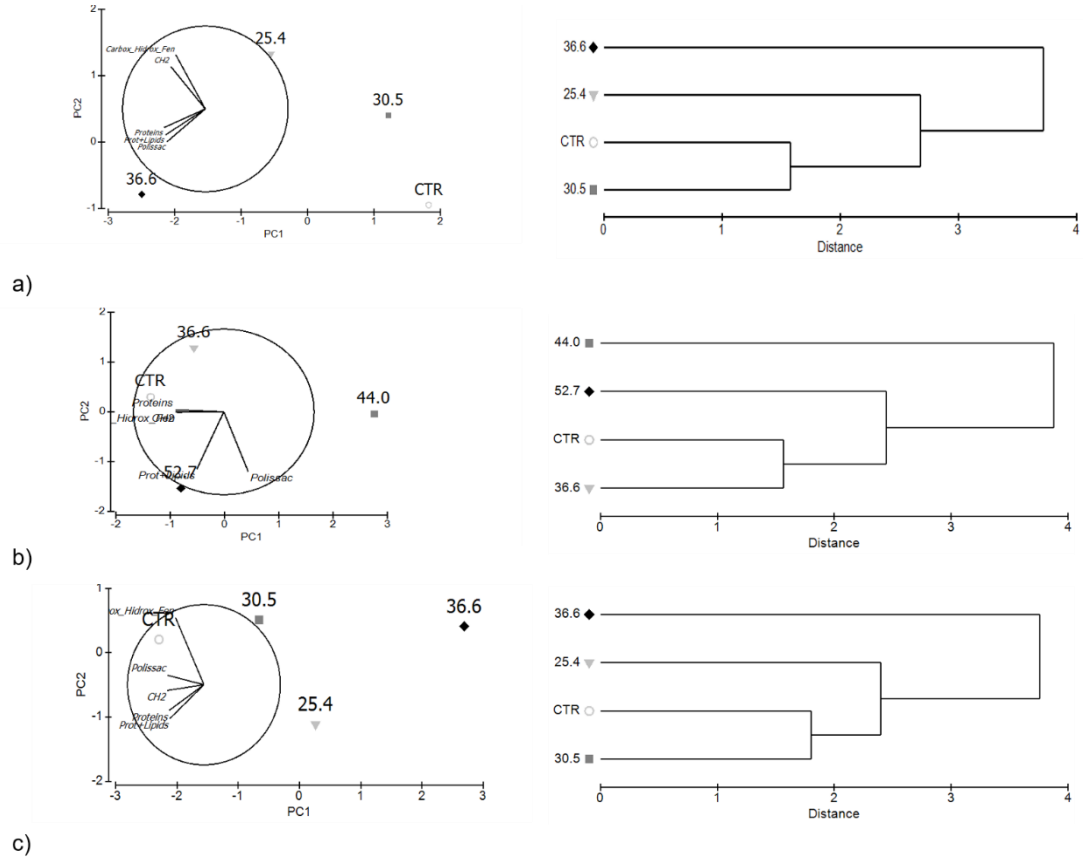
			Peak area per peak interval (cm^{-1})				
	[$mScm^{-1}$]		3650-3000	3000-2765	1750-1500	1495-1200	1200-805
R. oryzae	NaCl	CTR	40.5	12.46	10.67	13.16	39.96
		25.4	77.96	17.89	17.43	19.61	45.99
		30.5	63.67	13.93	10.47	9.95	37.21
		36.6	69.7	16.76	31.96	43.15	103.2

	SW	CTR	21.26	7.61	3.72	3.14	11.56
		36.6	21.94	5.8	3.42	1.81	11.21
		44.0	13.72	1.82	0.533	1.97	20.58
		52.7	22.06	6.26	3.45	3.86	22.18
	PE-NaCl	CTR	52.85	14.18	14.51	14.95	39.51
		25.4	33.51	11.17	12.67	12.12	26.04
		30.5	49.63	11.94	13.34	10.45	31.6
		36.6	36.99	7.92	5.35	6.43	17.07
T. versicolor	NaCl	CTR	61.85	18.32	22.99	15.88	50.25
		21.2	86.62	14.41	18.92	15.85	62.63
		25.4	81.79	13.78	15.69	15.01	59.93
		30.5	92.55	13.89	15.25	13.43	55.88
	SW	CTR	60.84	14.7	14.27	14.63	51.47
		36.6	83.27	14.21	18.66	16.05	54.29
		44.0	83.33	20,00	28.87	22.39	55.22
		52.7	65.52	12.98	14.46	13.85	36.02
	PE-NaCl	CTR	60.03	17.02	15.34	14.05	47,00
		21.2	62.77	17.57	18.91	16.21	55.15
		25.4	78.41	22.19	28.6	23.13	56.93
		30.5	70.8	21.29	25.86	21.28	58.33
P. chrysosporium	NaCl	CTR	29.33	8.68	8.6	8.85	18.75
		36.6	91.72	15.98	14.4	13.73	48.44
		44.0	84.6	14.34	17.2	13.2	53.37
		52.7	97.18	16.03	13.4	14.98	65.28
	SW	CTR	83.93	19.95	27.46	19.93	61.45
		36.6	96.83	14.37	16.77	13.02	60.83
		44.0	100.7	15.37	25.67	18.32	57.45
		52.7	93.39	14.13	18.93	13.93	61.17
	PE-NaCl	CTR	76.2	19.1	18.23	16.6	62.24
		36.6	90.17	14.37	14.41	16.04	62.99
		44.0	70.92	14.35	16.22	14.38	59.69
		52.7	76.52	18.14	20.06	21.01	59.36
L. sajor caju	NaCl	CTR	66.84	17.22	10.34	14.18	47.99
		17.7	73.97	13.51	10.18	14.82	65.3
		21.2	80.7	14.71	14.05	15.58	63.47
		25.4	87.79	15.63	11.38	15.41	74.99
	SW	CTR	67.81	18.01	11.21	14.04	52.31
		25.4	87.85	14.53	16.47	15.24	66.09
		30.5	73.43	11.19	14.44	11.59	39.83
		36.6	103.9	13.66	19.21	13.78	60.95
	PE-NaCl	CTR	109.4	19.97	11.41	15.52	79.16
		21.2	60.83	14.63	10.93	15.99	54.86
		25.4	40.57	11.94	8.55	12.48	35.09

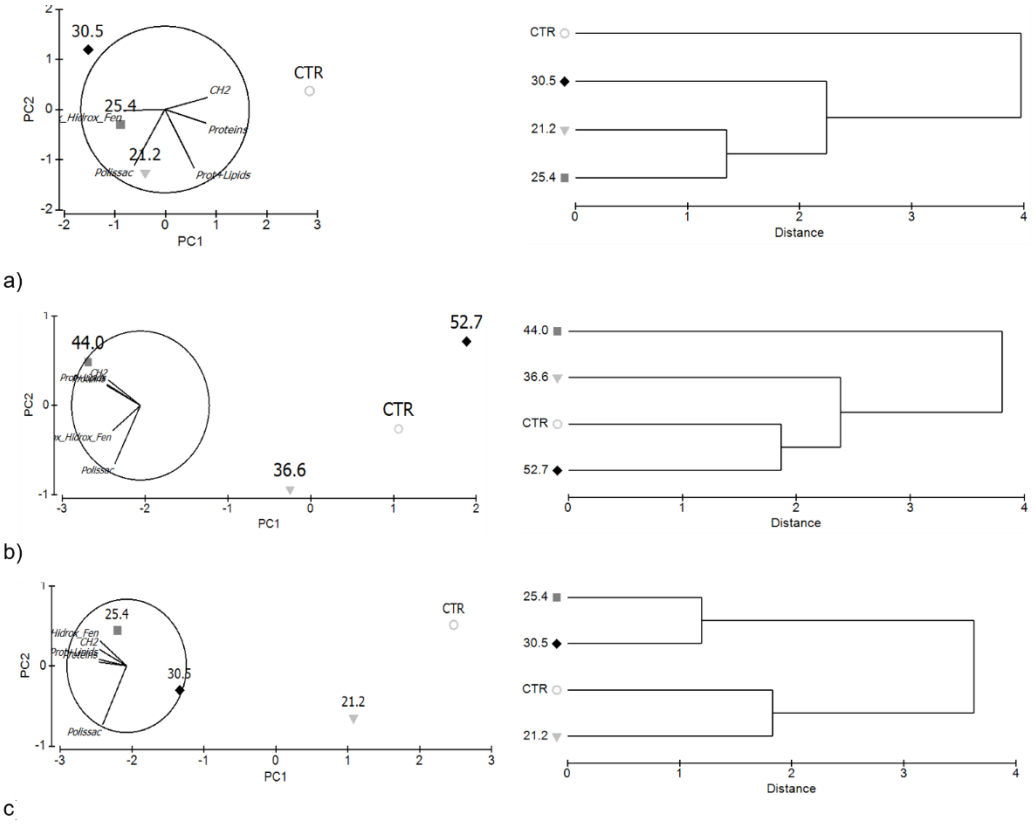
	30.5	85.85	14.47	13.81	15.23	69.11



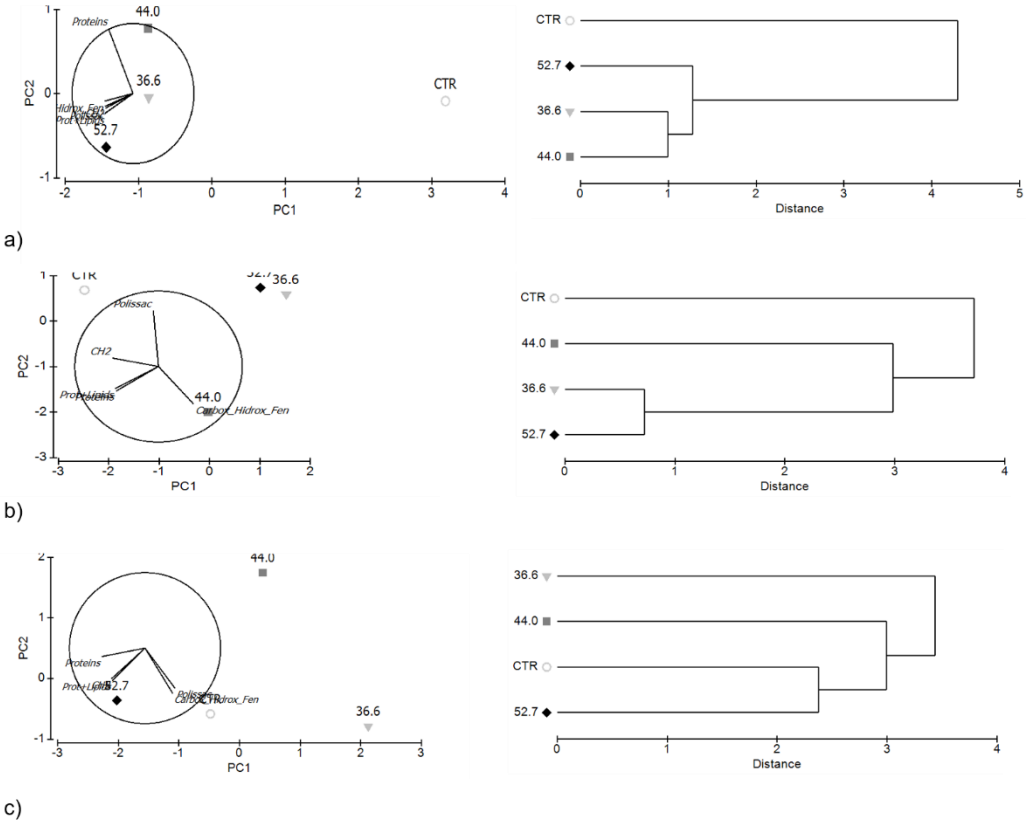
Supplementary Figure S2: Principal component analysis (PCA) and cluster analysis based on FTIR spectra analysis obtained for *Lentinus sajor caju* when exposed to a) sodium chloride; b) natural seawater and c) after pre-exposure to low levels of salinity. CTR stands for control; remaining values are conductivity values of the salinity treatments ($mScm^{-1}$).



Supplementary Figure S3: Principal component analysis (PCA) and cluster analysis based on FTIR spectra analysis obtained for *Rhizopus oryzae* when exposed to a) sodium chloride; b) natural seawater and c) after pre-exposure to low levels of salinity. CTR stands for control; remaining values are conductivity values of the salinity treatments ($mScm^{-1}$).



Supplementary Figure S4: Principal component analysis (PCA) and cluster analysis based on FTIR spectra analysis obtained for *Trametes versicolor* when exposed to a) sodium chloride; b) natural seawater and c) after pre-exposure to low levels of salinity. CTR stands for control; remaining values are conductivity values of the salinity treatments ($mScm^{-1}$).



Supplementary Figure S5: Principal component analysis (PCA) and cluster analysis based on FTIR spectra analysis obtained for *Phanerochaete chrysosporium* when exposed to a) sodium chloride; b) natural seawater and c) after pre-exposure to low levels of salinity. CTR stands for control; remaining values are conductivity values of the salinity treatments ($mScm^{-1}$).

Chapter IX

General Discussion and Conclusions

Considering the actual framework on climate change (IPCC, 2014), it is expected a continued increment on average global temperatures that will lead to an increase in the average sea level rise due to water thermal expansion and to the melting of ice caps and glaciers. Parallel to this, those same projections also point to a high degree of uncertainty and severity of several natural phenomena (e.g., giant waves, sudden storms or droughts periods) for the upcoming years. Such scenarios bring special concern for low-lying coastal areas (either soils and freshwater ecosystems), as their low elevation and high proximity to the sea increases the risk of seawater intrusion and to its salinization, either through surface flooding events (e.g., due to extreme weather phenomena) or through seawater intrusion in groundwater supplies (e.g., increased drought periods may lead to saltwater/freshwater interface to retreat further inland in coastal aquifers). So, to the uncertainty associated with sea level rise and possible salinization scenarios derived from climate change global patterns, along with some persisting knowledge gaps, it is rather difficult to predict what salinity levels might be achieved in these coastal ecosystems and what effects (lethal or sublethal) they might provoke and for how long. To help fill in these gaps, the present work intended to explore the salinization effects that might be caused in coastal terrestrial and freshwater ecosystems, by i) determining if sodium chloride (NaCl) may be used as a surrogate of SW at preliminary risk assessment frameworks of salinization effects; (ii) identifying the most sensitive freshwater and terrestrial ecological receptors to salinization; (iii) assessing if biota is capable of acquire an increased tolerance to low levels of salinization through mechanisms of acclimation or phenotypic plasticity; (iv) assessing the effects of increased salinity on interspecies relationships; and (v) identifying the effects of salinization for freshwater and soil communities under realistic exposure scenarios.

Furthermore, understanding the effects that salinization may provoke to terrestrial and freshwater biota can be helpful to establish threshold values or legislation in order to safeguard vulnerable areas, or implement management or mitigation strategies were seawater intrusion is already a reality.

In this context, the research developed within the present work contributed with the following new knowledge:

Sodium chloride may be used as surrogate for natural seawater in early stages of ecological risk assessment

Sodium chloride (NaCl) proved to be of similar or higher toxicity than that caused by natural seawater (SW) for the majority of the freshwater and terrestrial tested species (both for soil and freshwater biota; Chapter II, III, IV, V, VII and VIII), which suggests that it could be used as a first screening approach in risk assessment frameworks. These results are inline with the few data already available in the scientific literature (Kefford et al., 2004; Ghazy et al., 2009; Pereira et al., 2015). The higher or similar toxicity of NaCl may be considered as a worst-case scenario and decisions based on its toxicity can be understood as a conservative/protective approach, for both compartments, soils and freshwater. The use of toxicity data of NaCl, at early stages of risk assessment of salinization scenarios provoked by seawater intrusion is advantageous, since many toxicity data already exist for this salt, mainly derived from studies related with the impacts of its use as a road deicer (Dougherty and Smith, 2006; Denoël et al., 2010; Lob and Silver, 2012; Van Meter et al., 2012; Coldsnow et al., 2017). The fact that this toxicity data set is already available may avoid performing unnecessary animal experimentation. However, the use of NaCl as a surrogate of SW at early stages of risk assessment must be cautious since SW shown to be more toxic than NaCl to some freshwater species (e.g., the green microalgae *Chlorella vulgaris*, the rotifer *Brachionus calyciflorus* and the chironomid *Chironomus riparius*) and, data from competition experiments in Chapter V, revealed a higher effect on the growth rate of two microalgae species (*Raphidocelis subcapitata* and *C. vulgaris*) when exposed to SW comparatively to when exposed to NaCl.

For the terrestrial species that were tested in this study (plants and wood decay fungi) this tendency was not observed, but, Pereira et al. (2015) observed a higher sensitivity of the microarthropod *Folsomia candida* to SW comparatively to NaCl. Therefore, when running

toxicity assays with NaCl to assess risk of SW intrusion in coastal regions, data collected from literature for several species should be integrated, rather than the use of data for a single species, to extrapolate with more accurately possible effects at higher levels of biological organization.

Short-term or multigenerational exposure to low levels of salinization may cause an increased tolerance of biota to this stressor

Organisms when challenged by chemical alterations in the environment may be able to increase its tolerance to that particular chemical through phenotypic plasticity, aiming at persisting in the new environmental conditions (e.g., Coldsnow et al., 2017). These mechanisms may involve physiological changes that are activated and deactivated within the life cycle of organisms (acclimation) or functional changes that persist through the lifetime of the individual, eventually being transmitted to the descendants (e.g. developmental conversion, epigenetics) (e.g., Berger et al., 2009; Crean et al., 2013). These types of responses have been reported for some species (Lopes et al., 2005; Kim et al., 2015; Coldsnow et al., 2017) and were further studied in the present work both for freshwater and soil species exposed to increased salinity. Regarding exposure to low levels of salinity for a short period of time and within the life cycle of an organism, it was observed that tadpoles of *P. perezi* were able to increase their tolerance to this stressor (Chapter III), being hypothesized that such increased tolerance could be triggered through developmental conversion processes during the embryonic development and/or through acclimation. For multigenerational exposure of freshwater species to low levels of NaCl, results revealed that some species were able to increase their tolerance to this salt. *Daphnia longispina* and *Cylindrospermopsis raciborskii* showed an increased lethal tolerance to NaCl after their exposure to the LC_{50}/8 and EC_{50}/3, respectively, for two-generations (Chapter II). Also, *C. vulgaris* and *R. subcapitata* were able to increase their tolerance to salinity after exposure for three generations to the respective EC_{25} for NaCl or SW (Chapter V). Interestingly, these two algae species did not show an increased tolerance to NaCl (*C. vulgaris* showed a tendency for increased tolerance but was not significant) after being exposed for two generations to the corresponding EC_{50}/4 (lower

than the EC_{25} used in chapter V). These results may point to a dependency on the intensity of salinity to trigger mechanisms related with phenotypic plasticity. This lead us to hypothesize that the fact that no increased tolerance was observed for some freshwater species tested in Chapter II may be due to the low levels of salinity they were exposed to for two generations, rather than a lack of capacity to trigger plasticity mechanisms to increase their tolerance to NaCl. Adding to this, time also revealed to be an important factor to allow organisms acquiring and increased tolerance to salinity. Though an increase in tolerance was observed for some species, it was also observed that some species decreased their tolerance to salinity after multigenerational exposure to low levels of NaCl (*R. subcapitata* and *Lemna minor*, Chapter II). Taking into consideration the results obtained in chapters IV and V, in that a diversity of tolerance responses (e.g., in some cases organisms acquired an increase tolerance from generation F0 to F1 and then from F1 to F2 decreased greatly their tolerance) was observed over generations it is hypothesized that if *R. subcapitata* and *L. minor* were exposed for further generations to NaCl could have acquired and increased tolerance to this salt. Actually, this inconsistent pattern on tolerance acquisition over generations has already been reported in the literature regarding multigenerational exposure to other chemicals (e.g., Silva et al., 2017).

Regarding soils, results from multigenerational exposure performed with wood decay fungi (Chapter VIII) followed a similar pattern as that described for freshwater biota, i.e., some species were able to increase their tolerance towards salinity almost two-fold after prolonged exposure to low levels of NaCl (e.g., *Phanerochaete chrysosporium*). However, it was found that the acquisition of an increased tolerance to NaCl was made at the expenses of the production of polysaccharides compounds. The existence of costs associated with an increased tolerance to chemicals is widely documented in the scientific literature (e.g., Jaszek et al., 2006; Kogej et al., 2007), which is mainly related with the diversion of energy to the detoxification mechanisms. Similarly to what was reported to freshwater organisms, some soil species have been shown to exhibit a higher sensitivity to NaCl after a long-term exposure to low levels of NaCl. Pereira (2014) reported an increased sensitivity to NaCl for the springtail *F. candida* after an exposure

period of 26-week to a salinity level corresponding to the EC_{20} for reproduction, with only 5% of the animals alive.

These findings highlight the need to perform tests with a higher ecological relevancy at latter stages of ecological risk assessment. Standard toxicity assays do not take into consideration the possibility of organisms becoming more or less tolerant to salinity over a succession of generations exposed to such stressor, which may lead to situations of under- or overestimation of risk.

Differential tolerance to increased salinity levels may allow natural populations to cope with long-term exposure to such stressor but the absence of co-tolerance with other chemicals may enhance the possibility of populations being wiped out under future scenarios of contamination by different chemicals.

Genetic diversity allows populations to adapt to environmental perturbations (Maltby et al., 2001). However, chemical contamination may cause a decrease in the genetic diversity of populations by eliminating the most sensitive genotypes (e.g., Lopes et al., 2009). Although, the occurrence of such genetic erosion, the population will be able to persist in the contaminated site but will most probably be mainly constituted by the tolerant individuals to that particular type of contamination. However, if future exposure to different contaminants occurs, then such genetically eroded populations may be at a higher risk of extinction, specially if an inversion exists in the tolerance to the first and second chemical they are exposed to (Leitão et al., 2013; Venâncio et al., 2016). The experiments carried out in Chapter IV tackled this issue by comparing the sensitivity of six different clonal lineages of *D. longispina* to salinity and to four metals. Any positive or negative correlations were observed between the sensitivity of clonal lineages to the two types of chemicals, however, for a few clonal lineages and inversion in the sensitivity to NaCl and metals was observed (N37, E89, E99). These are relevant results within the framework of salinization of coastal regions, since many of these ecosystems are already impacted with chemical contamination originated by several anthropogenic activities (industrial and agricultural

activities). It is expected that populations already impacted by such chemical contamination will have undergone genetic erosion, if such inversion in tolerance to salinity occurs then part of the genotypes that persisted in these populations may be eliminated within scenarios of future seawater intrusions.

Exposure to low salinity levels influenced inter-species relationships

Adding to the effects that salinization may pose at the individual level (e.g., in survival, growth, fertility), that are assessed by the conventional standard toxicity assays, it can also cause effects at higher levels of biological organization. By influencing the interspecies relationship, salinity may directly cause changes in the structure of biological communities. The results obtained in Chapter V of the present work indicated that the competitive outcome of two species of microalgae under NaCl or SW stress was different when compared to that in the absence of this salt: under controlled conditions (no salt), *R. subcapitata* exhibited competitive advantage relatively to *C. vulgaris*, while the opposite occurred under a scenario of salinization. These results suggest that, in addition to the direct effects on the structure of phytoplankton communities, interfering with primary production rates (carbon storage and oxygen production), this change in the competitive output between microalgae species may as well have repercussions in primary consumers since different algae species holds different chemical composition that determines its quality as a food source. For example, several works have already shown the influence of different microalgae species, as food source, in the longevity, fertility and growth of cladocerans (e.g. Martinéz-Jeronimo et al., 1994; Choi et al., 2009).

Regarding soil producers (Chapter VII), long-term exposure (14 or 28 days) to a salinity threshold already established for soils (4.0 $mScm^{-1}$) did not changed the relationship between plant species. It seemed that culture conditions (mono or polyculture) was the main factor to which plants responded to, rather than the salinity level tested. Despite this, it should not be disregarded that higher conductivity levels can change the composition of the terrestrial community of higher plants. Later, this can influence nitrogen and carbon retention rates in soils and, consequently, interfere with soil productivity.

Zooplankton was identified as the most sensitive ecological receptor for freshwaters biota while microinvertebrates were the most sensitive ecological receptors within the terrestrial compartment.

The identification of the most sensitive ecological receptors to increased salinity, can be made by using Species Sensitive Distribution curves (SSD) analyses (e.g., Fig.1 to Fig.6). Species are distributed along a curve according to their sensibility to a determined stressor: at the bottom of the curve lies the most sensitive species while at the top of the curve are found the most tolerant ones. The results obtained in the present work, for lethal and sublethal toxicity of salinization, were merged with that found in literature for temperate species in order to construct SSDs and determine the most sensitive ecological receptors to salinization, at freshwater and terrestrial compartments. In freshwater, in general, cladocerans and rotifers showed to be the most sensitive ecological receptors to lethal and sublethal levels of salinity, either caused by NaCl or SW (Fig.1 to Fig.4). Perturbations at the population level of these groups may impair several processes, especially in energy transfer within trophic webs (D'Alelio et al., 2016). For instance, through grazing, large cladocerans (as daphnids) control algae growth, preventing blooms and eutrophication, while constituting a food source for many fish. In a general way, the SSD-SW curves showed that primary consumers (as daphnids and rotifers) are among the most sensitive ecological receptors, with no organisms from this group (primary consumers) found among the most tolerant species. The lack of functional redundancy between the most sensitive and the most tolerance groups may enhance the effects caused by salinity. On the other hand, fish, macrophytes and water molds were among the most tolerant species to salinity (over five times more tolerant to salinity when compared to zooplankton). This is of major concern, since the species, of these three groups (fish, macrophytes and water molds) are alien species (some considered invasive) in Europe [e.g., *L. minor* (Hussner, 2012) and *L. gibbosus* (Almeida et al., 2014)], foreseeing their higher competitive advantage under salinization scenarios, which may potentiate their invasive capacity in salinity impacted coastal ecosystems.

Regarding the soil compartment, soils microinvertebrates were the most sensitive group of organisms to increased salinity (Fig. 5 and Fig. 6). Furthermore, there exists a great difference between stress values that might induce 50% of effect in these soil microorganisms and between salinity stress values that might induce 50% of effect on other terrestrial species, such as some species of wood decay fungi. The results observed in graphs of figures 5 and 6 shown that before effects started to be observed in the most tolerant groups, the group of soil microinvertebrates may decline abruptly or even disappeared. Soil microinvertebrates play important roles in decomposition and nutrient mobilization functions in the soils (at a different speed scale), which is crucial in poor soils (e.g., Curry, 1989). Therefore, since salinized soil are expected to become poor soil (with low fertility and production; e.g., Heneghan and Bolger, 1998; Bagyaraj et al., 2016), this will be potentiated by the fact that the first group of animals to suffer from salinization is the one that could contribute to minimize the effects of salinity on decomposition and nutrient mobilization (microinvertebrates). Though the decomposition functions may be assured by other groups (like wood decay fungi that presented to be much more tolerance to salt stress), they are still necessary due to their relationship with other soil inhabitants, such as higher plants (e.g., Johnson et al., 2011).

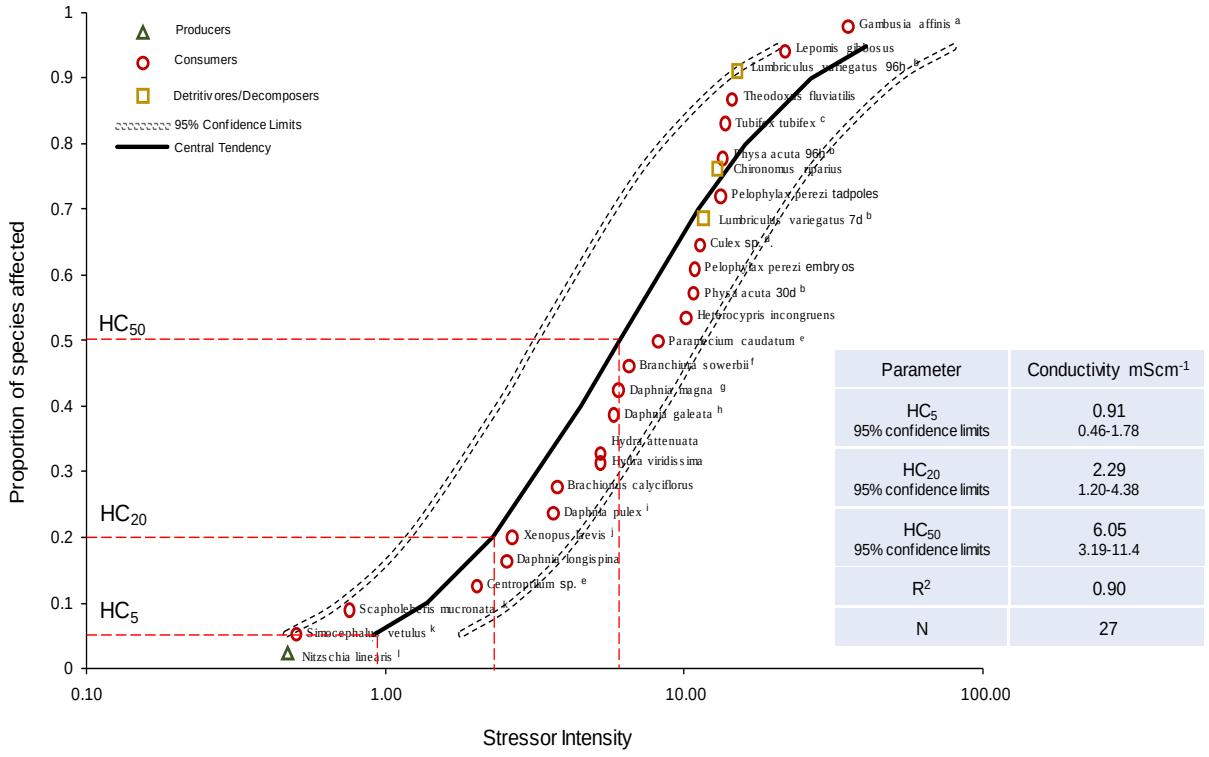
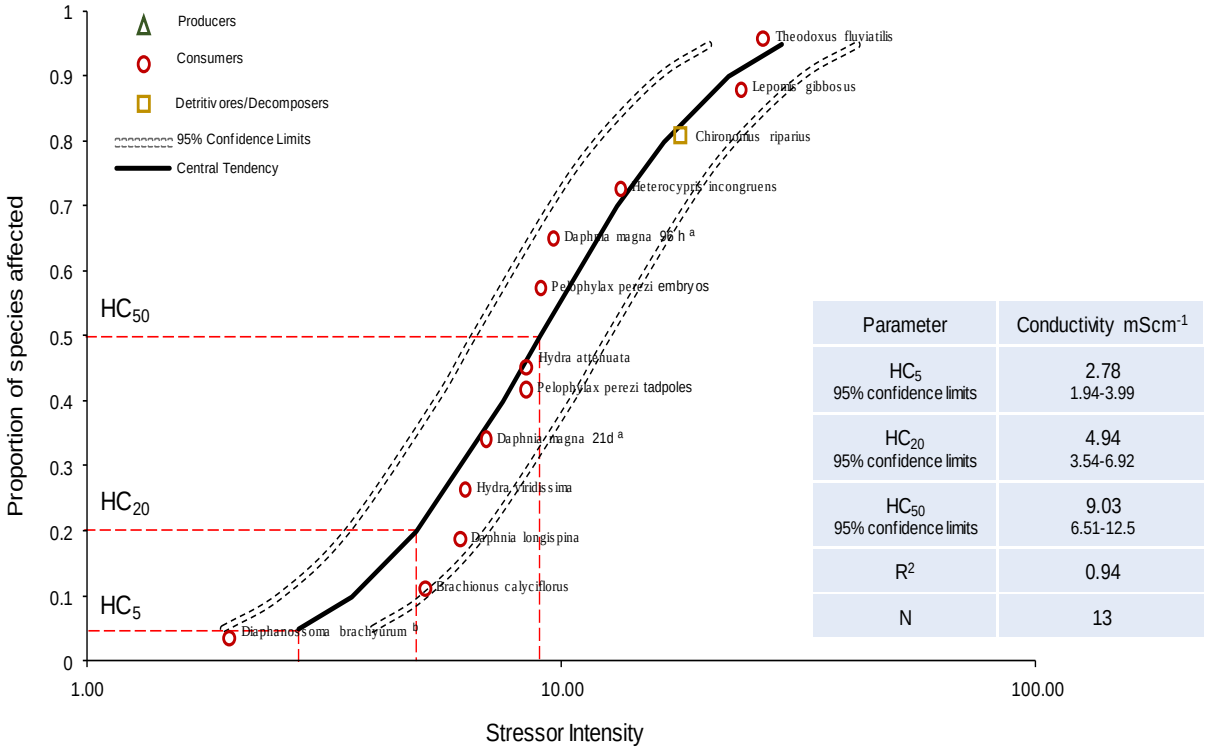
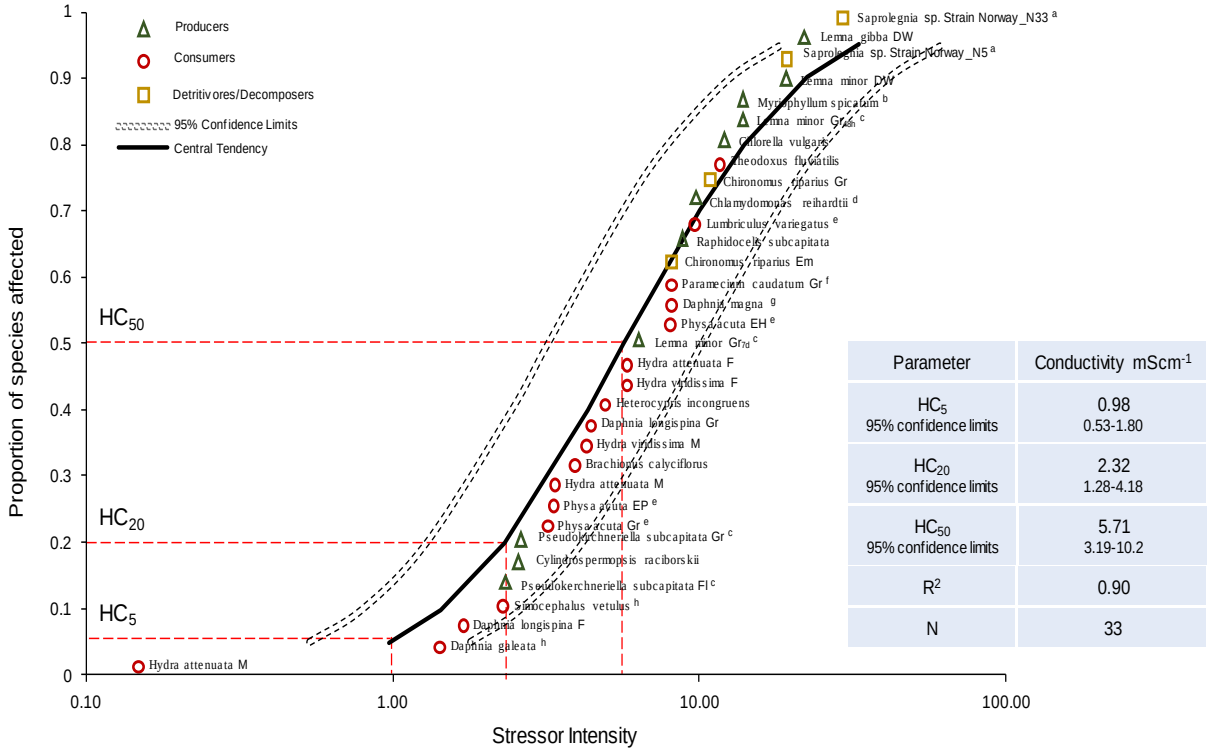


Figure 1: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to lethal levels of sodium chloride (NaCl). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 1 for further details). Superscripts are as follows: [a]Wallen et al., 1957; [b]Paradise, 2009; [c]USEPA, 2008; [d]Thamer and Abdulsamad, 2005; [e]Zalizniak et al., 2006; [f]Kefford et al., 2006; [g]Ghazy et al., 2009; [h]Loureiro et al., 2013; [i]Gardner and Royer, 2010; [j]Dougherty and Smith, 2006; [k]Gökçe and Turhan, 2014; [l]Patrick et al., 1968. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points.



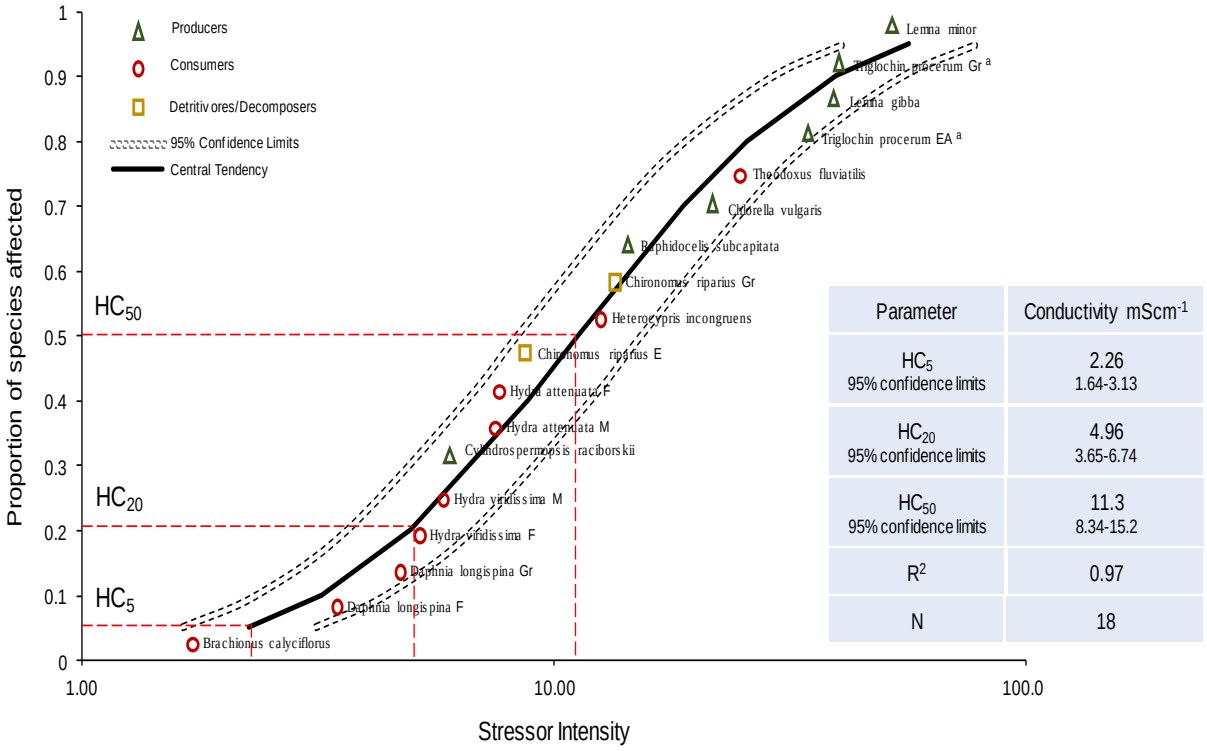
Parameter	Conductivity mScm^{-1}
HC_5 95% confidence limits	2.78 1.94-3.99
HC_{20} 95% confidence limits	4.94 3.54-6.92
HC_{50} 95% confidence limits	9.03 6.51-12.5
R^2	0.94
N	13

Figure 2: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to lethal levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 2 for further details). Superscripts are as follows: [a]Ghazy et al., 2009; [b]Mohammed and Agard, 2007. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2- coefficient of determination of the curve; N-number of data points.



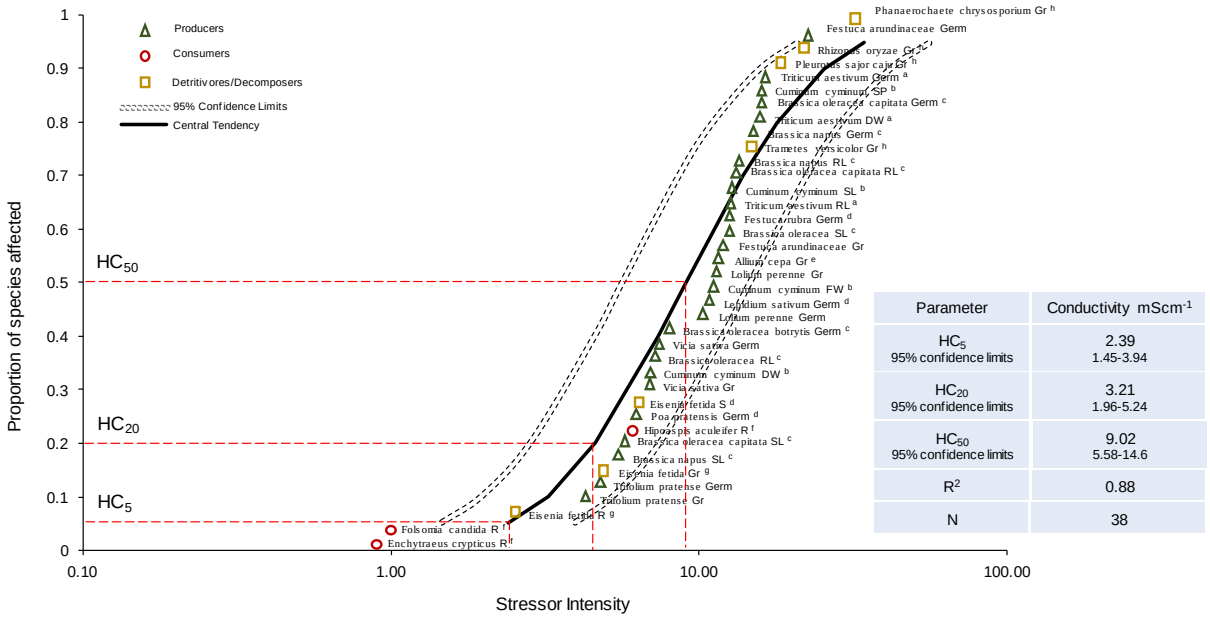
Parameter	Conductivity mScm^{-1}
HC_5 95% confidence limits	0.98 0.53-1.80
HC_{20} 95% confidence limits	2.32 1.28-4.18
HC_{50} 95% confidence limits	5.71 3.19-10.2
R^2	0.90
N	33

Figure 3: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to sublethal levels of sodium chloride (NaCl). Values correspond to median effective conductivities collected at Chapter II, III, IV rom the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 1 for further details). Superscripts are as follows: [a]Koeypudsa, 2005; [b]Stanley, 1974; [c]Simmons, 2012; [d]Reynoso et al., 1982; [e]Paradise, 2009; [f]Zalizniak et al., 2006; [g]Kolkmeier, 2013; [h]Loureiro et al., 2013. Abbreviations on the curve are as follows: DW-dry weight; Gr-growth; Em-emergence; R-reproduction; EH-egg hatching; F-feeding; M-morphology; EP-egg production; FI-fluorescence inhibition. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points.



Parameter	Conductivity mScm^{-1}
HC_5 95% confidence limits	2.26 1.64-3.13
HC_{20} 95% confidence limits	4.96 3.65-6.74
HC_{50} 95% confidence limits	11.3 8.34-15.2
R^2	0.97
N	18

Figure 4: Species Sensitive Distribution curve (SSD) assembled for freshwater species exposed to sublethal levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter II, III, IV from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 2 for further details). Superscripts are as follows: [a]Roache et al., 2006. Abbreviations on the curve are as follows: Gr-growth; EA-enzymatic activity; Em-emergence; F-feeding; M-morphology. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2- coefficient of determination of the curve; N-number of data points.



Parameter	Conductivity mScm^{-1}
HC_5 95% confidence limits	2.39 1.45-3.94
HC_{20} 95% confidence limits	3.21 1.96-5.24
HC_{50} 95% confidence limits	9.02 5.58-14.6
R^2	0.88
N	38

Figure 5: Species Sensitive Distribution curve (SSD) assembled for soil species exposed to increased levels of sodium chloride (NaCl). Values correspond to median lethal conductivities collected at Chapter VII and VIII from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 3 for further details). Superscripts are as follows: [a]Ibrahim et al., 2016; [b]Rebey et al., 2017; [c]Jamil et al., 2005; [d]Robidoux and Deslile, 2001; [e]Joutti et al., 2003; [f]Pereira et al., 2015; [g]Gozyte, 2011; [h]Venâncio et al., 2017. Abbreviations on the curve are as follows: Gr-growth; Germ-germination; SP-seed production; W-dry weight; RL-root length; SL-shoot length; FW-fresh weigth; S-survival; R-reproduction. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2-coefficient of determination of the curve; N-number of data points.

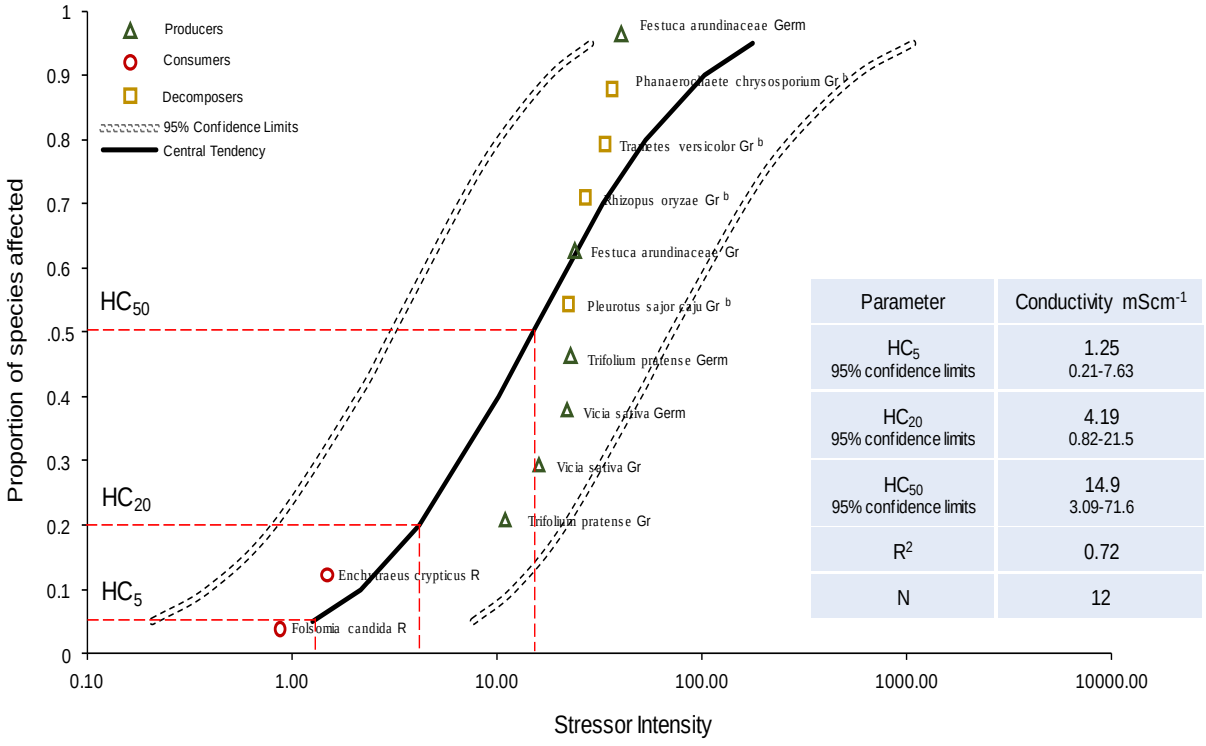


Figure 6: Species Sensitive Distribution curve (SSD) assembled for soil species exposed to increased levels of natural seawater (SW). Values correspond to median lethal conductivities collected at Chapter VII and VIII from the present thesis and data collected from the current literature only for species that are recorded for tempered regions (identified with superscripts; please see Supplementary Table 4 for further details). Superscripts are as follows: [a]Pereira et al., 2015; [b]Venâncio et al., 2017. Abbreviations on the curve are as follows: Gr-growth; Germ-germination; S-survival; R-reproduction. Abbreviations on the table are as follows: HC_x-Hazard Concentration that affect X% of the species; R^2: coefficient of determination of the curve; N-number of data points.

Soils salinization may pose an added risk to freshwater ecosystems

Considering the above mentioned and the connectivity between freshwater and terrestrial compartments, it is foreseen an additional risk to freshwater ecosystems resulting from the salinization impacts on adjacent soil ecosystems.

In soils, major ions (like sodium and chloride) may interfere with normal ionic changes, for instance sodium easily substitutes other essential elements (like calcium and magnesium) and chloride, which by being negatively charged, can easily be transported with porewater to adjacent freshwater ecosystems (Löfgren, 2001). In addition, the presence of these ions in porewater may

cause the mobilization of other elements that might be as well transported to nearby freshwater ecosystems (like metals; e.g., Löfgren, 2001). Furthermore, salinized soils may be more prone to erosion events, which can lead to salts and soil particles being washed into the nearest freshwater systems. Though this runoff/mobilization process of salts is depending on several characteristics of the soils (e.g. permeability, composition, ion exchange capacity, topography), it is possibly that water reservoirs constitute the final recipients. For instance, mining exploitation reveals salt rich scobs that are washed with rains, contributing significantly to the increase of the conductivity on the nearest freshwater systems like rivers (e.g., Cañedo-Argüelles et al., 2012; Cañedo-Argüelles et al., 2013).

Very low salinity levels may pose a significant risk to terrestrial and freshwater low-lying coastal ecosystems

The employment of assessment factors to the lowest values found in a toxicity dataset or the use of median HC_x (Hazard Concentrations) for derivation or recommendation of water quality guidelines has already been studied and found to be protective for natural populations (e.g., Hose and Van den Brink, 2004; Jin et al., 2012). Here the maximum acceptable conductivity environmental quality standard (MAC-EQS) were computed by using these two methodologies for the dataset obtained with SW both for the freshwater and soil biota (Table 1; European Commission, 2011). The MAC-EQS values to be employed in risk derivation were computed for soils and for freshwater. For freshwater, was taken in account, lethal effects (considering that saline intrusion may occur due to sudden events that will dramatically increase the salinity levels) and also sublethal effects (considering gradual intrusions of seawater).

Table 1: Median values of Hazard Concentrations that protect 95% of the species (HC_5) obtained through the Species Sensitivity Distribution curves (SSD) method for natural seawater (SW). MAC – Maximum Acceptable Concentrations. L(E)C_{50}- Median lethal or effective conductivity ($mScm^{-1}$).

		Soils	Freshwater	
			Lethal	Sublethal
MAC-EQS	HC_5 ($mScm^{-1}$)	1.25	2.78	2.26
	Lowest L(E)C_{50}/10 ($mScm^{-1}$)	0.09	0.51	0.17

Computed HC_x (first methodology to estimate MAC-EQS) for both compartments revealed to be very low, when comparing with conductivity of natural seawater (SW ≈ 52 $mScm^{-1}$), which means that both compartments are probably very sensitive to increased salinity levels. The HC_5 values (the most common suggested endpoint, to protect 95% of the species), were of 2.78 and 2.26 $mScm^{-1}$ for freshwater (considering lethal and sublethal effects caused by SW) and of 1.25 $mScm^{-1}$ for soil (considering also SW; Table 1), which corresponds to approximately 4.3% and 2.4% of SW conductivity, respectively.

Calculation MAC-EQS based on the lowest EC_{50} divided by a safety factor of 10 were lower than those computed through the HC_5 methodology. A safety factor of only 10 was employed as EC_{50} values were available for several species representing different trophic levels. This second methodology showed to be more protective of salinization effects on coastal low-lying terrestrial and freshwater ecosystems. The risk quotient here presented was based on the MAC-EQS computed through EC_{50}/10 (considered as the PNEC-predicted non-effect concentration). For the predicted effect conductivities (PEC), two examples found in literature were used (Table 2). The first example is related with a saline intrusion scenario mainly through surface seawater intrusion (e.g., Baixo Vouga Lagunar; IDAD, 2008). Three sampling points were chosen (2A, 3A and 11B), with freshwater characteristics, but that occasionally present high salinity levels (above 15 $mScm^{-1}$) due to high fluctuations of the tide (IDAD, 2008). The other is related to a scenario of saline intrusion through groundwater supplies (Almada aquifer). Analysis on water samples from this

aquifer have shown high $rCl/rHCO_3$ ratios (an indication of saline intrusion) for two sites (F3A and P8A, with salinity levels of 6.29 and 4.71, respectively) (Ferreira, 2012).

No values were found in the literature relatively to salinization of Portuguese soils due to seawater intrusion.

Table 2: Risk quotient for freshwater ecosystems based on the ratio between salinity levels detected in field studies (values considered the Predicted Effect Concentrations – PEC) and PNEC (Predicted Non-Effect Concentrations) computed through the lowest effect concentration (LC or EC_{50}) for seawater (SW) divided by a safety factor of 10.
[a]Based on the $rCl/rHCO_3$ ratio – rapid increments on this index is an indication of saline intrusion; Ferreira, 2012.
[b]Sampling points with freshwater characteristics but which occasionally present high salinities due to tidal elevations or due to water scarcity; IDAD, 2008.

Location/ Type of intrusion	Sampling Sites	Conductivity $mScm^{-1}$ (PEC)	RISK QUOTIENT
Groundwater Almada (Tagus river southern margin)	F3A[a]	6.29	>1
	P8A[a]	4.71	> 1
Superficial Baixo Vouga Lagunar (Vouga river mouth)	2A[b]	>15	> 1
	7A[b]	>15	> 1
	11B[b]	>30	> 1

Risk quotient is usually calculated by the PEC/PNEC ratio. If the ratio > 1 then, the ecosystems are at risk, in this case, by salinization. The risk value calculated for the freshwater compartment was well above 1 (Table 2), meaning that these coastal freshwater ecosystems are at high risk.

Despite no risk quotient was possible to calculate for the soil compartment (no PEC value was found in the literature), the low MAC-EQS values presented at Table 1, are indicative that the terrestrial compartment might be also highly susceptible to small increases in salinity due to seawater intrusions.

Mesocosms experiments showed that natural communities may be more resilient to small salinity changes than could be computed by standard toxicity methodologies

The values of MAC-EQS computed in the previous section showed that freshwater and soil ecosystems would be at risk at conductivity values higher than 0.17 and 0.09 $mScm^{-1}$, respectively. Assessment of salinization effects using natural communities (e.g., outdoor mesocosms) are more realistic examples of such scenarios of exposure and provide insights on community's resilience that are not envisaged by standard laboratorial and controlled approaches. Furthermore, as complex systems involving long term exposure, they can encompass many of the responses focused along the chapters of this thesis in an integrated manner: sensitivity of the ecological receptors (Chapter II and III), ability of species to increase tolerance through phenotypic plasticity mechanisms (Chapter II to V), influence on inter-species relations (Chapter V).

The mesocosms experiments that were performed during this work have shown that natural communities may cope with salinity levels higher than the MAC-EQS values computed in the previous section. Abundance and species richness of natural communities of macroinvertebrates and zooplankton were affected by a pulse of increased salinity (between 2 and 15 $mScm^{-1}$). However, though the invertebrate community was exposed to salinity values that were higher than the LC_{50} computed for some of the exposed species, all species were able to persist in the mesocosms during the whole experiment. The interaction with several other factors (e.g., environmental variables such as temperature, nutrients or light) may, somehow and until a certain limit, have decreased the effects of salinity to these biota [for instance, algae may benefit from small increases in phosphate concentrations in water (e.g., Steinman and Duhamel, 2017); increased water temperature may promote increments in the abundance of smaller zooplankton species (e.g., Rasconi et al., 2015); the presence of organic matter in the mesocosm could bind excessive ions present in seawater reducing its bioavailability]. Furthermore, the differential sensitivity observed in chapter IV for the different clonal lineages of *D. longispina*, may suggest that though the populations present in the mesocoms communities were able to recover their

abundances they could have undergone through genetic erosion by the elimination of the most sensitive genotypes (decreases in abundances) during the phase of exposure to increased. Furthermore, since exposure to salinity was long it could have occurred that organisms were able to activate phenotypic plasticity mechanisms that conferred them an increased tolerance to salinization. This type of response was observed for some species in Chapters II to V.

No mesocosms were performed in the present work with terrestrial communities, because they already exist in literature. Pereira (2014), using terrestrial mesocosms found that, at a salinity threshold of 4 $mScm^{-1}$, microorganisms inhabiting the soils may suffer adverse effects, namely reduced abundance due to decreasing reproduction rates (e.g. earthworms) or biomass (e.g. plants). Though, terrestrial ecosystems may also present some plasticity when confronted with saline stress. The patchiness or heterogeneity of soils may constitute an advantage in providing shelters for smaller organisms and if enough time is provided between salt stresses, soils species can recover, as shown by Pereira (2014) regarding springtails, mites and enchytraeids.

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Supplementary Information

Supplementary Table 1: Median effective conductivity values ($mScm^{-1}$) reported in the current literature and reported along the thesis work, regarding sodium chloride (NaCl) toxicity towards freshwater species. Data used to assemble the Species Sensitivity Distribution curves (SSD).

Group	Species	Measured Endpoint		Conductivity $mScm^{-1}$	Reference
Protozoa (Oomycota)	*Saprolegnia* sp. Strain Norway_N5	$EC_{50,14d}$	Colony radial growth	19.8	Koeypudsa, 2005
	Saprolegnia sp. Strain Norway_N33	$EC_{50,72h}$	Colony radial growth	29.7	
Protozoa (Ciliophora)	*Paramecium caudatum*	$LC_{50,72h}$	Mortality	8.0	Zalizniak et al., 2006
		$EC_{50,72h}$	Growth	8.0	
Algae (Chlorophyta)	*Pseudokerchneriella subcapitata*	$EC_{50,96h}$	Growth	2.7	Simmons, 2012
		$EC_{50,96h}$	Fluorescence	2.4	
	Chlamydomonas reinhardtii	$LC_{50,6d}$	Mortality	9.83	Reynoso et al., 1982
Algae (Bacillariophyceae)	*Nitzschia linearis*	$LC_{50,5d}$	Mortality	0.48	Patrick et al., 1968
Aquatic plants (Macrophytes)	*Lemna minor*	$EC_{50,48h}$	Survival	14.0	Simmons, 2012
		$EC_{50,7d}$		6.5	
	Myriophyllum spicatum	$EC_{50,\ 32d}$	Root weight	14.1	Stanley, 1974
Invertebrates (Cladocera)	*Daphnia magna*	$LC_{50,48h}$	Mortality	5.92	Ghazy et al., 2009
		$EC_{50,21d}$	Reproduction	8.00	Kolkmeier, 2013
	D. pulex	LC_{50}	Mortality	3.59	Gardner and Royer, 2010
	D. galeata	$LC_{50,48h}$	Mortality	5.70	Loureiro et al., 2013
		$EC_{50,21d}$	Reproduction	1.40	
	Scapholeberis mucronata	$LC_{50,48h}$	Mortality	0.74	Gokce and Turhan, 2014
	Simocephalus vetulus	$LC_{50,48h}$	Mortality	0.49	Gokce and Turhan, 2014
		$EC_{50,21d}$	Reproduction	2.26	Loureiro et al., 2013
Invertebrates (Oligochaete)	*Lumbriculus variegatus*	$LC_{50,96h}$	Mortality	15.0	Paradise, 2009
		$LC_{50,7d}$		11.8	
	Branchiura sowerbii	$EC_{50,6w}$	Growth	9.5	Kefford et al., 2006
		$LC_{50,72h}$	Mortality	< 6.4	
	Tubifex tubifex	$LC_{50,96h}$	Mortality	13.4	USEPA, 2008
Invertebrates (Gastropoda)	*Physa acuta*	$LC_{50,96h}$	Mortality	13.17	Paradise, 2009
		$LC_{50,30d}$		10.54	
		$EC_{50,30d}$	Egg production	3.3	
		$EC_{50,30d}$	Hatching success	7.98	
		$EC_{50,30d}$	Growth	3.15	

Supplementary Table 1 (Cont.): Median effective conductivity values ($mScm^{-1}$) reported in the current literature and reported along the thesis work, regarding sodium chloride (NaCl) toxicity towards freshwater species. Data used to assemble the Species Sensitivity Distribution curves (SSD).

Group	Species	Measured Endpoint		Conductivity $mScm^{-1}$	Reference
Arthropoda (Insecta)	*Centroptilum* sp.	$LC_{50,96h}$	Mortality	2	Zalizniak et al., 2006
	Culex sp.	$LC_{50,5\text{-}6d}$	Mortality	11.1	Thamer and Abdulsamad, 2005
Fish (Cyprinodontiformes)	*Gambusia affinis*	LC_{50}	Mortality	34.64	Wallen et al., 1957
Amphibia (Anura)	*Xenopus laevis*	LC_{50}	Mortality	2.61	Dougherty and Smith, 2006

Supplementary Table 2: Median effective conductivity values ($mScm^{-1}$) reported in the current literature and reported along the thesis work, regarding natural seawater (SW) toxicity towards freshwater species. Data used to assemble the Species Sensitivity Distributuon curves (SSD). [a]Tests performed with natural SW; [b]Tests performed with artificial SW.

Group	**Species**	**Measured Endpoint**		**Conductivity $mScm^{-1}$**	**Reference**
Aquatic plants (Macrophytes)	*Triglochin procerum*	$EC_{50,21d}$	Enzymatic activity	34.6	Roache et al., 2006[a]
		$EC_{50,21d}$	Dry weight	40.4	
Invertebrates (Cladocera)	*Daphnia magna*	$LC_{50,48h}$	Mortality	9.54	Ghazy et al., 2009[a]
		$LC_{50,21d}$		6.89	
	Diaphanossoma brachyurum	$LC_{50,48h}$	Mortality	1.98	Mohammed and Agard, 2007[b]

Supplementary Table 3: Median effective conductivity values ($mScm^{-1}$) reported in the current literature and reported along the thesis work, regarding sodium chloride (NaCl) toxicity towards soil species. Data used to assemble the Species Sensitivity Distribution curves (SSD).

Group	**Species**	**Measured Endpoint**		**Conductivity $mScm^{-1}$**	**Reference**
Plants (Monocotyledonous)	*Lolium perenne*	$EC_{50, 14d}$	Germination	10.5	Chapter VIII
		$EC_{50, 14d}$	Growth	11.5	
	Festuca arundinaceae	$EC_{50, 14d}$	Germination	23.1	Chapter VIII
		$EC_{50, 14d}$	Growth	12.2	
	F. rubra	$IC_{50, 7d}$	Germination	12.7	Robidoux and Delisle, 2001
	Poa pratensis	$IC_{50, 7d}$	Germination	6.33	
	Allium cepa	$EC_{50, 4d}$	Growth	11.9	Joutti et al, 2003
	Triticum aestivum (VAR: Yang)	$EC_{50, 10d}$	Germination	16.7	Ibrahim et al., 2005
		$EC_{50, 21d}$	Root length	12.7	
		$EC_{50, 21d}$	Total dry weight	16.0	
Plants (Dicotyledonous)	*Trifolium pratense*	$EC_{50, 14d}$	Germination	4.76	Chapter VIII
		$EC_{50, 14d}$	Growth	4.32	
	Vicia sativa	$EC_{50, 14d}$	Germination	7.45	Chapter VIII
		$EC_{50, 14d}$	Growth	6.94	
	Lepidium sativa	$IC_{50, 7d}$	Germination	10.9	Robidoux and Delisle, 2001
	Cuminum cyminum	$EC_{50, 14d}$	Height	12.9	Rebey et al., 2017
		$EC_{50, 14d}$	Fresh weight	11.4	
		$EC_{50, 14d}$	Dry weight	6.99	
		$EC_{50, 14d}$	Seed production	16.1	
	Brassica oleraceae capitata	$EC_{50, 14d}$	Germination	16.0	Jamil et al., 2005
		$EC_{50, 14d}$	Root length	13.3	
		$EC_{50, 14d}$	Shoot length	5.71	
	Brassica oleraceae botrytis	$EC_{50, 14d}$	Germination	8.12	
		$EC_{50, 14d}$	Root length	7.19	
		$EC_{50, 14d}$	Shoot length	12.6	
	Brassica napus	$EC_{50, 14d}$	Germination	15.1	
		$EC_{50, 14d}$	Root length	13.7	
		$EC_{50, 14d}$	Shoot length	5.56	
Invertebrates (Annelida)	*Enchytraeus crypticus*	$EC_{50, 28d}$	Reproduction	0.89	Pereira et al., 2015
	Eisenia fetida	$EC_{50, 14d}$	Survival	6.33	Robidoux and Delisle, 2001
		$EC_{50, 28d}$	Growth	4.97	Guzyte, 2011
		$EC_{50, 28d}$	Cocoon production	2.55	
Invertebrates (Arthropoda)	*Hipoaspis aculeifer*	$EC_{50, 14d}$	Reproduction	6.03	Pereira et al., 2015
	Folsomia candida	$EC_{50, 28d}$	Reproduction	0.98	
Wood decay fungi (Basidiomycetes)	*Phanaerochaete chrysosporium*	$EC_{50, 3d}$	Mycelial growth	32.1	Venâncio et al., 2017
	Lentinus sajor caju	$EC_{50, 8d}$	Mycelial growth	18.7	
	Trametes versicolor	$EC_{50, 8d}$	Mycelial growth	14.9	
Wood decay fungi (Zygomycetes)	*Rhizopus oryzae*	$EC_{50, 8d}$	Mycelial growth	22.0	

Supplementary Table 4: Median effective conductivity values ($mScm^{-1}$) reported in the current literature and reported along the thesis work, regarding natural seawater (SW) toxicity towards soil species. [a]Tests performed with natural SW. Data used to assemble the Species Sensitivity Distribution curves (SSD).

Group	**Species**	**Measured Endpoint**		**Conductivity $mScm^{-1}$**	**Reference**
Plants (Monocotyledonous)	*Festuca arundinaceae*	$EC_{50,\ 14d}$	Germination	41.6	Chapter VIII[a]
		$EC_{50,\ 14d}$	Growth	24.6	
Plants (Dicotyledonous)	*Trifolium pratense*	$EC_{50,\ 14d}$	Germination	23.5	Chapter VIII[a]
		$EC_{50,\ 14d}$	Growth	12.1	
	Vicia sativa	$EC_{50,\ 14d}$	Germination	22.6	Chapter VIII[a]
		$EC_{50,\ 14d}$	Growth	16.7	
Invertebrates (Annelida)	*Enchytraeus crypticus*	$EC_{50,\ 28d}$	Reproduction	1.45	Pereira et al., 2015[a]
Invertebrates (Arthropoda)	*Folsomia candida*	$EC_{50,\ 28d}$	Reproduction	0.86	
Wood decay fungi (Basidiomycetes)	*Phanaerochaete chrysosporium*	$EC_{50,\ 3d}$	Mycelial growth	37.4	Venâncio et al., 2017 [a]
	Lentinus sajor caju	$EC_{50,\ 8d}$	Mycelial growth	23.7	
	Trametes versicolor	$EC_{50,\ 8d}$	Mycelial growth	34.1	
Wood decay fungi (Zygomycetes)	*Rhizopus oryzae*	$EC_{50,\ 8d}$	Mycelial growth	28.5	