



**Carmen Lopes Elias**

**Efeito das alterações globais e escala espacial nas comunidades de diatomáceas dos rios temperados. Implicação na biomonitorização**

**Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment**





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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 Salomé Fernandes Pinheiro de Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, e co-orientação da Doutora Maria João Feio, Cientista Convidada do Centro de Ciências do Mar e do Ambiente (MARE) do Departamento de Ciências da Vida da Universidade de Coimbra.

Este trabalho teve apoio financeiro da Fundação para a Ciência e a Tecnologia e do POCH - Programa Operacional Capital Humano, participado pelo Fundo Social Europeu e por fundos nacionais do Ministério da Educação e Ciência de Portugal, através da bolsa de doutoramento SFRH/BD/68973/2010 e dos projetos de investigação PTDC/AAC-AMB/105297/2008 e PTDC/AAC-AMB/105411/2008.



Dedico este trabalho aos meus pais, Gilberto e Maria Irene, irmã Gilberta, sobrinhos e ao Daniel.



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## **acknowledgements**

I would like to thank my supervisor Professor Salomé Almeida for her scientific guidance and for the good working conditions that made this work possible. For her best efforts in helping me during every task of this work and for all the time she spent during discussions on the most varied issues. Thank you for being always there.

I also express my gratitude to my co-supervisor Doctor Maria João Feio, first of all, for the kind way she has received me at Coimbra and secondly for her confidence on this work. Then, for being always positive and never give up when the problems occurred. For being the glass half-full...

I would like to thank to Ana Raquel Calapez for all the support provided during the endless field work. Without her help things would have been much more difficult. Thank you Raquel!

To Ana Luís and Cristina Delgado for their moral support and friendship. All the doubts in the diatom identification process were much softened after fruitful discussions with them.

I've much appreciated the friendly atmosphere provided by all the colleagues that are or have been at our Phycology Laboratory at the University of Aveiro, in particular Sandra Calado for being always there.

To Rui Rocha for the help provided during the construction of the experimental facility (artificial channels).

To Daniel, for all the support, patience and for being there in all the good and bad moments.

Finally, to my parents that always offered me the best conditions to reach this stage of my life.



## palavras-chave

Diatomáceas, rios de litoral, avaliação biológica da qualidade da água, Condição de menor perturbação, condição de referência, índice multimétrico de diatomáceas, *traits*, experiência de mesocosmos, variabilidade inter-continental, substratos, macroinvertebrados.

## resumo

As diatomáceas são as algas que se encontram mais frequentemente e em maior abundância em cursos de água doce. São conhecidas pela capacidade dos diferentes taxa responderem a variações das condições ambientais dos rios e por serem frequentemente o único elemento de qualidade biológica representativo da flora aquática em pequenos rios e ribeiras. Em consequência destas características, e de acordo com a Directiva Quadro da Água, as diatomáceas são presentemente um dos elementos biológicos obrigatórios na avaliação da qualidade ecológica dos rios europeus. No entanto, ainda existe algum desconhecimento acerca dos efeitos das alterações globais e da escala espacial nas diatomáceas. Para colmatar esta lacuna, o presente trabalho tem como objetivo estudar o efeito de alterações globais relevantes e da escala espacial nas comunidades de diatomáceas de rios temperados assim como estudar a melhor forma de lidar com as suas implicações na biomonitorização. O efeito das alterações globais foi estudado em rios e ribeiras da região centro litoral de Portugal continental dado tratar-se de uma região densamente povoada e sujeita a impactos antropogénicos significativos. O efeito da escala espacial nas comunidades de diatomáceas foi investigado através de uma experiência laboratorial (mesocosmos) e também com recurso a informação estatística de comunidades de diatomáceas de várias regiões temperadas, incluindo as costas ocidentais dos Estados Unidos da América e da Austrália. De modo atingir o objetivo principal deste trabalho, três questões globais foram colocadas: (1) Como lidar com as influências antrópicas históricas na biomonitorização?; (2) Será que as comunidades de diatomáceas reflectem os eventos extremos causados pelas alterações climáticas? Uma comparação com as comunidades de macroinvertebrados; (3) Como são as comunidades de diatomáceas influenciadas pela escala espacial (pequena escala: habitats; grande escala: inter-continental). Verificou-se uma influência antropogénica considerável nos rios e ribeiras da região centro litoral de Portugal continental assim como a inexistência de verdadeiros locais de referência. Mesmo os locais considerados como estando em condições de menor perturbação apresentaram concentrações de nutrientes consideravelmente elevadas na água. Uma vez que as fortes pressões antropogénica (p.e., alteração da vegetação ripária, condição morfológica e carga de sedimentos) não permitiram o estabelecimento de condições de referência adequadas para os rios desta região, foi proposto um procedimento alternativo baseado numa combinação de dois métodos – modelação e filtros ambientais. A aplicação



deste procedimento na definição de condições de referência revelou-se útil para lidar com os efeitos das alterações globais na biomonitorização com recurso a comunidades de diatomáceas e macroinvertebrados. De facto, como seria de esperar, as comunidades observadas em rios sujeitos a alterações antropogénicas revelaram-se menos homogéneas do que as previstas em condições de referência para esses mesmos rios, *i.e.*, através da aplicação dos referidos filtros ambientais. Em conjunto com o desenvolvimento deste novo procedimento, foi também proposto um novo índice multimétrico de diatomáceas. Este novo índice revelou-se fortemente correlacionado com as pressões seleccionadas, fornecendo uma avaliação da qualidade biológica mais abrangente do que o índice oficial adotado por Portugal, o *Indice de Polluosensibilité spécifique* (IPS). Para responder à segunda questão global apenas um subconjunto de ribeiras da região litoral de Portugal foi selecionado, o qual engloba as ribeiras cujos leitos secaram durante uma vaga de calor invulgar que ocorreu em Portugal em 2011/2012. Verificou-se que, de facto, quer as comunidades de diatomáceas quer de macroinvertebrados foram afetadas pelo evento de seca extrema tendo-se observado alterações ao nível das proporções de *traits*, composição e a classificação da qualidade biológica. No entanto, as diatomáceas apresentaram uma recuperação significativamente mais rápida que os macroinvertebrados. Os tipos de substratos usados na experiência de mesocosmos realizada neste trabalho (duro vs. macio; pequena escala espacial) também mostraram afetar as comunidades de diatomáceas, uma vez que houve diferenças quer na sua composição quer nas proporções de *traits*. Esta resposta ao tipo de substrato foi mais evidente em condições físico-químicas controladas do que aquela sugerida pelas diferenças entre as comunidades epipsâmicas e epilíticas dos rios monitorizados neste trabalho. Não obstante, durante a experiência de mesocosmos verificou-se que ao nível de IPS as diferenças entre substratos não eram relevantes quando comparadas com as que se haviam verificado nos rios. As diferenças observadas entre as comunidades de diatomáceas dos vários continentes (Europa, América e Austrália) sugerem que variações ao nível da grande escala também ocasionam diferenças nas comunidades, apesar de influenciados pelo mesmo tipo de clima. Isto sugere que outros factores para além do clima (p.e., geologia, processos biogeográficos históricos e hidrologia) podem ter contribuído para as diferenças intercontinentais verificadas ao nível da composição das comunidades de diatomáceas, a todos os níveis taxonómicos estudados. De um modo geral, os resultados obtidos durante este estudo trazem novas informações e novas abordagens para lidar com a biomonitorização. No entanto, mais trabalho será necessário a fim de investigar, por exemplo, os efeitos do aumento da temperatura previstos devido ao aquecimento global na avaliação da qualidade da água com base em comunidades de diatomáceas.



## keywords

Diatoms, lowland streams, biological water quality assessment, Least Disturbed Condition, reference condition, multimetric diatom index, traits, mesocosm experiment, inter-continental variability, substrate, macroinvertebrates.

## abstract

Diatoms are generally the most frequent and abundant algae in streams and are known by their responsiveness to changes in the environmental conditions of streams. Diatoms are frequently the only biological quality element representative of the aquatic flora in small streams. As a result, diatoms are nowadays a mandatory biological element in the evaluation of the ecological quality of European rivers, according to the Water Framework Directive. Nevertheless, there is still a relative lack of knowledge of key effects of global changes and spatial scale on diatoms. To bridge this gap, this work aims to study the effect of relevant global changes as well as spatial scale on diatom communities of temperate rivers and to deal with its implications in bioassessment. The relevant global changes were assessed in streams from the central Portuguese littoral region which is highly populated and suffers from high anthropogenic impacts. The effect of spatial scale on diatom communities was investigated by a laboratorial experiment (mesocosm experiment) and by using diatom data from temperate regions of the United States of America and Australia. In order to achieve the main aim, three global questions were raised: (1) How to deal with the historical anthropogenic influences in the bioassessment?; (2) Are extreme events due to climatic changes reflected in diatom communities? A comparison with macroinvertebrate communities; (3) How are diatom communities influenced at the spatial scale (small-scale: habitats; large-scale: inter-continental). A strong anthropogenic influence on the streams and rivers of the Portuguese littoral region was confirmed as well as the lack of true reference sites. Sites in the Least Disturbed Condition (LDC) had considerable high nutrient concentrations. As the strong anthropogenic pressures (*e.g.*, alteration of the riparian vegetation, morphological condition and sediment load) prevented an adequate definition of reference conditions for streams in this area an alternative procedure to define suitable reference conditions was investigated, consisting of a combination of modelling and filter approach. This procedure is suitable to deal with the implications of global changes on diatom and on macroinvertebrate communities. The communities observed in the different streams were less homogeneous than those predicted for the same streams under reference conditions (*i.e.*, through the filter approach), as would be expected in sites having variable levels of anthropogenic change. Along with the development of this new approach a new multimetric diatom index was developed. This index produced strong correlations with the selected pressures, providing a more comprehensive





assessment of biological quality than the Índice de Poluosensibilidade  
Específica (IPS) officially adopted for Portugal. To answer the second  
global question a smaller set of streams were used within the  
Portuguese littoral region that were affected by an unusual drought  
event that led to the complete drying of the stream channels. Diatoms,  
as well as macroinvertebrates, were affected by the drought event  
considering trait proportions, community's composition and  
bioassessment classifications. However, the diatom communities had a  
faster recovery response than macroinvertebrates. The type of substrate  
– hard and soft – representing the small spatial scale affected diatom  
communities by changing its composition and trait proportions but not  
water quality classification in a mesocosm experiment. However, the  
differences found in the epipsammic and epilithic diatom communities in  
the streams that were used to answer the first global question were  
more evident than those found in the mesocosm experiment. In fact,  
with the mesocosm results, and under the same physical and chemical  
conditions the IPS differences between substrates disappeared  
contrasting with the differences that were found in the streams.  
Additionally, the diatom communities were different between continents  
with the same type of climate, suggesting that diatom communities are  
also influenced at the large-scale (even at the order level). Therefore,  
constraints other than climate are likely to have contributed to the inter-  
continental differences in diatom community composition found at all  
taxonomical levels (e.g., geology, historical biogeographic processes  
and hydrology). On the whole, the results obtained during this study  
bring new information and new approaches to deal with the  
bioassessment. However, some more work must be done in order to  
investigate e.g., based in the global warming predictions, the effects of  
the temperature increase, on the water quality assessment based on  
diatom communities.

# Table of contents

|  |      |
|--|------|
| Table of contents.....   | i    |
| List of Figures .....  | v    |
| List of Tables .....   | x    |
| List of Publications .....   | xiii |
| Chapter 1 - Introduction .....   | 1    |
| 1.1 Diatoms .....  | 1    |
| 1.2 Global changes and implication on river ecosystems .....   | 4    |
| 1.3 Implications of global changes on diatom communities .....   | 6    |
| 1.4 Dealing with global changes and spatial scale in bioassessment .....   | 8    |
| 1.5 Aims of the present work.....  | 11   |
| Chapter 2 - Determining useful benchmarks for the bioassessment of highly disturbed areas based on diatoms .....                             | 21   |
| 2.1 Introduction.....  | 23   |
| 2.2 Methods .....  | 25   |
| 2.2.1 Study area and sampling sites .....  | 25   |
| 2.2.2 Sampling collection and processing.....  | 26   |
| 2.2.3 Abiotic characterisation of the sites.....   | 27   |
| 2.2.4 Data analyses.....   | 28   |
| 2.2.4.1 Typology .....   | 28   |
| 2.2.4.2 Establishment of the LDC.....  | 28   |
| 2.2.4.3 Least Disturbed Biological Condition (LDBC) based on diatom assemblages .....  | 29   |
| 2.2.4.4 Establishing new benchmarks for ecological assessment.....   | 32   |
| 2.3 Results .....  | 32   |
| 2.3.1 Typology.....  | 32   |
| 2.3.2 Establishment of the LDC.....  | 34   |
| 2.3.3 LDBC based on diatom assemblages.....  | 36   |
| 2.3.4 Establishing new benchmarks for ecological assessment.....   | 40   |
| 2.4 Discussion .....   | 41   |
| 2.5 Conclusions .....  | 45   |
| Chapter 3 - Predicting reference conditions for river bioassessment by incorporating boosted trees in the environmental filters method ..... | 53   |
| 3.1 Introduction.....  | 55   |
| 3.2 Materials and methods.....   | 57   |
| 3.2.1 Study area and data source .....   | 57   |

|  |   |     |
|--|---|-----|
| 3.2.2  | Diatom and macroinvertebrate sampling and processing.....                         | 60  |
| 3.2.3  | Conceptual model development .....  | 61  |
| 3.2.4  | Defining abiotic reference conditions .....                                       | 61  |
| 3.2.5  | Predicting the lowland reference community: diatoms and macroinvertebrates .....  | 64  |
| 3.2.6  | Quality assessment and diatom index development .....                             | 65  |
| 3.3  | Results .....   | 67  |
| 3.3.1  | Defining abiotic reference conditions .....                                       | 67  |
| 3.3.2  | Predicting reference communities for lowland streams .....                        | 68  |
| 3.3.3  | Quality assessment and index development .....                                    | 72  |
| 3.4  | Discussion.....   | 75  |
| 3.5  | Conclusions.....  | 79  |
| Chapter 4 - From perennial to temporary streams: an extreme drought as a driving force of    |   |     |
| freshwater communities' traits.....  |   | 89  |
| 4.1  | Introduction.....   | 90  |
| 4.2  | Material and methods.....   | 92  |
| 4.2.1  | Study sites.....  | 92  |
| 4.2.2  | Climate characterisation of the study period in the Atlantic-temperate area ..... | 92  |
| 4.2.3  | Sample collection and processing.....   | 93  |
| 4.2.4  | Biological traits.....  | 95  |
| 4.2.5  | Statistical analyses.....   | 97  |
| 4.3  | Results .....   | 99  |
| 4.3.1  | Trait patterns of diatom communities .....  | 99  |
| 4.3.2  | Trait patterns of macroinvertebrate communities .....                             | 103 |
| 4.4  | Discussion.....   | 107 |
| 4.5  | Conclusions.....  | 112 |
| Chapter 5 - Extreme drought effect and recovery patterns in benthic communities of temperate |   |     |
| streams .....  |   | 119 |
| 5.1  | Introduction.....   | 121 |
| 5.2  | Methods .....   | 122 |
| 5.2.1  | Study sites.....  | 122 |
| 5.2.2  | Sample collection and processing.....   | 124 |
| 5.2.3  | Community analyses and biological metrics .....                                   | 125 |
| 5.3  | Results .....   | 126 |
| 5.3.1  | Abiotic characterisation of the sites.....  | 126 |
| 5.3.2  | Macroinvertebrate patterns .....  | 128 |
| 5.3.3  | Diatom patterns .....   | 131 |

|   |   |     |
|---|---|-----|
| 5.4   | Discussion .....                                    | 134 |
| 5.5   | Conclusion .....                                    | 136 |
| Chapter 6 - Influence of the colonizing substrate on diatom assemblages and implications for<br>bioassessment - a mesocosm experiment ..... |   |     |
| 6.1   | Introduction .....                                  | 147 |
| 6.2   | Methods .....                                       | 148 |
| 6.2.1   | Experimental system description .....               | 148 |
| 6.2.2   | Experimental Set-up.....                            | 149 |
| 6.2.3   | Water, periphyton sampling and treatment.....       | 151 |
| 6.2.4   | Selection and calculation of biological traits..... | 152 |
| 6.2.5   | Data analysis.....                                  | 153 |
| 6.3   | Results .....                                       | 153 |
| 6.3.1   | Physical and chemical parameters .....              | 153 |
| 6.3.2   | Chlorophylls.....                                   | 154 |
| 6.3.3   | Periphyton assemblages.....                         | 155 |
| 6.3.4   | Biological traits.....                              | 161 |
| 6.4   | Discussion .....                                    | 162 |
| 6.5   | Conclusions .....                                   | 164 |
| Chapter 7 - Do similar climatic patterns in different continents lead to similar benthic diatom<br>assemblages? .....                       |   |     |
| 7.1   | Introduction .....                                  | 173 |
| 7.2   | Methods .....                                       | 174 |
| 7.2.1   | Study area and site selection.....                  | 174 |
| 7.2.2   | Diatom dataset.....                                 | 175 |
| 7.2.3   | Data analyses.....                                  | 176 |
| 7.3   | Results .....                                       | 176 |
| 7.3.1   | Species-level .....                                 | 176 |
| 7.3.2   | Genus-level.....                                    | 188 |
| 7.3.3   | Order-level.....                                    | 192 |
| 7.4   | Discussion .....                                    | 194 |
| 7.5   | Conclusion .....                                    | 197 |
| Chapter 8 - Final remarks.....  |   |     |
| Appendix I – Description of the lithology categories found in the Portuguese lowland sites – Chapter 3..<br>.....                           |   |     |
|   |   | 215 |
| Appendix II – Results of the filters approach, diatoms – Chapter 3 .....  |   |     |
|   |   | 219 |
| Appendix III – Results of the filters approach, macroinvertebrates – Chapter 3 .....  |   |     |
|   |   | 225 |

|  |     |
|--|-----|
| Appendix IV – Experimental set– Chapter 6..... | 229 |
|--|-----|

## List of Figures

|   |    |
|---|----|
| Figure 1.1 - a) Two frustules of <i>Caloneis</i> , as seen in gilde view; b) one valve of <i>Caloneis</i> , as seen in valve view. Photograph from the sample collected in Vala da Corujeira (Casal dos Netos). Scale bar: 10 $\mu\text{m}$ (for both figures).....   | 2  |
| Figure 1.2 - Hierarchical interrelationships among proximate (resources and abiotic stresses), intermediate, and ultimate (climate, geology, land use) determinants of benthic algal assemblage composition and biomass. Source: Stevenson (1997). ....   | 3  |
| Figure 1.3 - Percentage of good ecological status or potential of classified rivers and lakes in Water Framework Directive river basin districts. Source: EEA (2015). ....  | 6  |
| Figure 2.1 - Location of the study sites (grey circles) in the central Portugal catchments (above left, in grey). The rivers and streams represented (grey lines) are those presently included in the L type.....   | 26 |
| Figure 2.2 - Hierarchical classification of the sites based on the abiotic characterisation variables. Three groups were formed.....  | 33 |
| Figure 2.3 - Principal Component Analysis of the study sites plotted in relation to the abiotic characterisation variables. The groups obtained in the hierarchical classification of the sites (Figure 2.2) are identified: square-Group I, black triangle-Group II, white triangle-Group III, diamond-V80. ....   | 34 |
| Figure 2.4 - Boxplots showing the distribution of selected numerical pressure variables for the least disturbed samples selected after Step 1 (only class 1 - minimal or 2 - minor disturbance). Outliers/Extremes: see section 2.2.4.2. ....   | 38 |
| Figure 2.5 - Hierarchical classification of the diatom assemblages based on the least disturbed sites. Two groups were formed: black triangle-Group A, white triangle-Group B. The sites (triangles) are identified with the first letter of the catchment to which they belong - Vouga (V), Mondego (M), Lis (L), Ribeiras do Oeste (O), and Ribeiras do Noroeste (NO) - and with a number that is the site number. .... | 39 |
| Figure 2.6 - EQR based on the new IPS median reference values (squares) and in original IPS median reference values (circles) used presently in the official monitoring programme of the sampling sites from Group A (a) and B (b). Dashed vertical lines separates samples selected as being in the least disturbed condition (LDC) from the ones considered to be impaired. Spring samples. ....                        | 41 |
| Figure 3.1 - Map of Portuguese rivers showing locations of the lowland sites (white circles) and the remaining study sites (grey circles). ....   | 58 |
| Figure 3.2 - Conceptual model showing the abiotic variables that directly influence the composition of diatom and macroinvertebrate communities (connections via dashed lines) and those useful to estimate values of other abiotic variables (connections via solid lines). See Table 3.1 for variable descriptions.....   | 62 |

|   |     |
|---|-----|
| Figure 3.3 - Linear regression of observed nitrate concentrations ( $\text{NO}_3^-$ ; $\text{mg}\cdot\text{L}^{-1}$ ) for the entire country and all seasons against actual values predicted by boosted trees. ....   | 68  |
| Figure 3.4 - Comparison of observed nitrate concentrations ( $\text{NO}_3^-$ ; $\text{mg}\cdot\text{L}^{-1}$ ) for the lowland sites in the training dataset (triangles) and reference values predicted by boosted trees (circles).....   | 68  |
| Figure 3.5 - Multidimensional scaling (MDS) ordination of observed (circles) and predicted (triangles) diatom communities from the lowland sites (a) and detailed view of the dashed area (b). The sites considered to be in the least disturbed condition are distinguished by grey shading. ....  | 70  |
| Figure 3.6 - Multidimensional scaling (MDS) ordination of observed (circles) and predicted (triangles) macroinvertebrate communities from the lowland sites. The sites considered to be in the least disturbed condition are distinguished by grey shading.....   | 71  |
| Figure 3.7 - Ecological quality ratios (EQR) for diatom indices based on (a) the presently used IPS and median reference values and (b) the new multimetric diatom index (MDI) and median reference values from the LDC samples. 1-Site L03; 2-Site NO103; 3-Site O92; 4-Site L98; 5-Site L01; 6-Site M06; 7, 8, 9-Site M18; 10, 11-Site M09; 12-V117. Dashed lines indicate quality class boundaries. ....                               | 74  |
| Figure 3.8 - Ecological quality ratios (EQR) for macroinvertebrate indices based on (a) the presently used IPTI median reference values and (b) the new IPTI median reference values from the LDC samples. 1-Site L03; 2-Site NO103; 3-Site O92; 4-Site L98; 5-Site L01; 6-Site M06; 7, 8, 9-Site M18; 10, 11-Site M09; 12-Site V117. Dashed lines indicate quality class quality boundaries.....   | 75  |
| Figure 4.1 - Location of the study sites in continental Portugal in the Atlantic-temperate climate (black triangles) and in the Mediterranean climate (black circles).....  | 93  |
| Figure 4.2 - Mean precipitation of the (a) Atlantic-temperate climate and (b) Mediterranean climate sites recorded during the study. Sampling occasions: T1, temperate spring before drought; TAD, temperate first sample after water return; T2, temperate spring after drought. Data from the Portuguese Institute of Sea and Atmosphere (IPMA, I.P.; <a href="http://www.ipma.pt">http://www.ipma.pt</a> ).....                        | 94  |
| Figure 4.3 - Multidimensional Scaling Analysis (MDS) ordinations of the diatom traits for the groups of samples: (a) temperate spring before drought (T1), first temperate sample after water return (TAD) and Mediterranean spring (M); and (b) temperate spring before drought (T1), temperate spring after drought (T2) and Mediterranean spring (M). T1, black triangle; TAD, white circle; T2, white triangle; M, black square. .... | 100 |
| Figure 4.4 - Canonical Analysis of Principal Coordinates (CAP) based on proportions of diatom traits found in: (a) temperate spring before drought (T1) and first temperate sample after water return (TAD); (b) temperate spring before drought (T1) and after drought (T2); and (c) temperate spring before drought (T1) and Mediterranean streams. T1, black triangle; TAD, white circle; T2, white triangle; M, black square. ....    | 101 |
| Figure 4.5 - Multidimensional Scaling Analysis (MDS) ordinations of the macroinvertebrate traits for the groups of samples: (a) temperate spring before drought (T1), first temperate sample after water return (TAD) and Mediterranean spring (M); (b) temperate spring before drought (T1),   |     |

temperate spring after drought (T2) and Mediterranean spring (M). T1, black triangle; TAD, white circle; T2, white triangle; M, black square. .... 104

Figure 4.6 – Canonical Analysis of Principal Coordinates (CAP) based on proportions of macroinvertebrate traits found in: (a) temperate spring before drought (T1) and first temperate sample after water return (TAD); (b) temperate spring before drought (T1) and after drought (T2); (c) temperate spring before drought (T1) and Mediterranean streams. T1, black triangle; TAD, white circle; T2, white triangle; M, black square. .... 105

Figure 4.7 - Mean relative abundance of diatom (**bold underlined**) and macroinvertebrate traits found in the three groups of samples: black triangle, temperate spring before drought (T1); white circles, first temperate sample after water return (TAD); white triangle, temperate spring after drought (T2). Logarithmic scale. .... 107

Figure 5.1 - Locations of the study stream sites in Portugal (black triangles) and the precipitation gauge station (square) used in this study. .... 123

Figure 5.2 - Total monthly precipitation (mm) in the central territory region, Portugal, recorded during the study period (bars) and the historical mean monthly precipitation for the period of 1980-2013 (line). Black arrows represent the sampling events, and the open arrow indicate the time when the streams completely dried out. .... 124

Figure 5.3 - Multidimensional Scaling Analysis ordination for the macroinvertebrate (A) and diatom (B) communities of all study sites sampled in the pre-drought (spring 2011, s1: black triangles) and post-drought (spring 2012, s6: white triangles) conditions. Site codes are also indicated (Al: Alcoa; An: Ança; B: Boialvo; E: Eiras and S: Serra). .... 130

Figure 5.4 - Macroinvertebrate metrics for all study sites (mean  $\pm$  SE) at each sampling event from spring 2011 (s1) until spring 2012 (s6); a) total number of individuals, b) number of taxa (richness), c) Pielou's evenness, d) EPT taxa, e) number of Diptera families (Diptera richness), f) number of Coleoptera families (Coleoptera richness) and g) IPTI index (expressed in Ecological Quality Ratios). .... 131

Figure 5.5 - Diatom metrics for all study sites (mean  $\pm$  SE) at each sampling event from spring 2011 (s1) until spring 2012 (s6); a) number of taxa (richness), b) Shannon-Wiener diversity index, c) Pielou's evenness and d) IPS index (expressed in Ecological Quality Ratios). .... 134

Figure 6.1 - Cross-section of one of the twelve modular mesocosm systems used in the laboratory experiments. See Appendix IV. .... 150

Figure 6.2 - (a) Chlorophyll-*a* (Chl-*a*), (b) chlorophyll-*c* (Chl-*c*) and (c) chlorophyll-*b* (Chl-*b*) concentrations (mean $\pm$ standard deviation) of the algal assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b, c) do not significantly differ ( $p>0.05$ ; PERMANOVA pairwise test). .... 155

Figure 6.3 - Diatom density (cells/cm<sup>2</sup>) (mean $\pm$ standard deviation) found in the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment



means labelled with the same letter (a, b, c) do not significantly differ ( $p > 0.05$ ; PERMANOVA pairwise test). ..... 156

Figure 6.4 - Multidimensional Scaling Analysis (MDS) ordination of diatom assemblages at day 14 (white symbols) and 35 (black symbols) on the tile (triangles) and in the sand (squares). ..... 158

Figure 6.5 - Diatom diversity ( $H'$ ) (mean  $\pm$  standard deviation) found in the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b) do not significantly differ ( $p > 0.05$ ; PERMANOVA pairwise test). ..... 160

Figure 6.6 - Diatom-based IPS index (mean  $\pm$  standard deviation) obtained from the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b) do not significantly differ ( $p > 0.05$ ; PERMANOVA pairwise test). ..... 161

Figure 6.7 - Number of valves *per*  $\text{cm}^2$  (mean  $\pm$  standard deviation) found in the diatom assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels with the trait life-form categories: (a) Mobile, (b) Planktonic, (c) Pad, (d) Stalk and (e) Adnate. Treatment means labelled with the same letter (a, b, c, d) do not significantly differ ( $p > 0.05$ ; PERMANOVA pairwise test). ..... 162

Figure 7.1 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at species level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares). ..... 177

Figure 7.2 - Mean number ( $\pm$ SD) of species *per* sample in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ ). ..... 178

Figure 7.3 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the species that contributed most to within-region average similarity were found (Table 7.1). For each species, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). *Cocconeis placentula* was not significantly different between USA and AUS but the differences were not tested for the other two comparisons (PORT-USA and PORT-AUS). ..... 182

Figure 7.4 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at the genus level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares). ..... 188

Figure 7.5 - Mean number ( $\pm$ SD) of genera *per* sample in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ ). ..... 189

Figure 7.6 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the genera that contributed most to within-region average similarity were

found (Table 7.3). For each genus, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). *Navicula* was significantly different between PORT and AUS but the differences were not tested for the other two comparisons (PORT-USA and USA-AUS)..... 191

Figure 7.7 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at the order level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares)..... 192

Figure 7.8 - Mean number ( $\pm$ SD) of orders *per* sample found in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ )..... 193

Figure 7.9 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the orders that contributed most to within-region average similarity were found (Table 7.5). For each order, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). Cocconeidales was significantly different between POR and AUS but the differences were not tested for the other two comparisons (PORT-USA and USA-AUS). Licmophorales was not significantly different between USA and AUS but the differences were not tested for the other two comparisons (PORT-USA and PORT-AUS). ..... 194

## List of Tables

|   |    |
|---|----|
| Table 2.1 - Abiotic characterisation variables measured or calculated for each site, units, and transformation applied for the PCA. ....  | 27 |
| Table 2.2 - Abiotic pressure variables (water chemical and physical conditions and land use) used for the selection of the least disturbed condition.....   | 30 |
| Table 2.3 - Mean (SD, standard deviation) of the abiotic characterisation variables for each abiotic group. ....  | 35 |
| Table 2.4 - Thresholds for the Least Disturbed Abiotic Condition (LDAC) for water chemical and physical conditions and land use. ....   | 36 |
| Table 2.5 - Most representative diatom species (contributing to up to 81% of the Bray-Curtis similarity) the two groups (A and B) obtained by SIMPER analysis with respective average abundance. In bold are the species that were more abundant to each group. ....  | 39 |
| Table 3.1 - Characterization and pressure variables included in the study, with ranges of values for Portuguese lowland sites (n=218 samples from 62 sites). ....   | 59 |
| Table 3.2 - Abiotic pressure variables for which values were altered to predict reference communities.....  | 63 |
| Table 3.3 - PERMANOVA results for pairwise comparisons among observed diatom communities and predicted reference communities. Obs. LDC-observed communities at sites in nominally Least Disturbed Condition; Obs. nLDC-observed communities in non-Least Disturbed Condition sites; Pred. LDC-predicted communities in Least Disturbed Condition sites; Pred. nLDC-predicted communities in non-Least Disturbed Condition sites.....            | 69 |
| Table 3.4 - PERMANOVA results for pairwise comparisons among observed macroinvertebrate communities and predicted reference communities. Obs. LDC-observed communities at sites in nominally Least Disturbed Condition; Obs. nLDC-observed communities in non-Least Disturbed Condition sites; Pred. LDC-predicted communities in Least Disturbed Condition sites; Pred. nLDC-predicted communities in non-Least Disturbed Condition sites..... | 72 |
| Table 3.5 - Pearson correlation coefficient between the pressure variables on the first three components from principal components analysis (PCA) of data for all seasons and spring only and each of the original variables. The three highest coefficients for each PC are in bold font. BOD <sub>5</sub> -biochemical oxygen demand, COD-chemical oxygen demand. ....  | 73 |
| Table 3.6 - Coefficients of Spearman rank correlation between the PCA axis scores (all seasons; Table 3.5) and diatom metrics, used to determine weighting factors (wf). *- p < 0.05.....   | 73 |
| Table 3.7 - Median reference values of the MDI and its constituent metrics, and weighting factors (wf), for MDI (EQR) calculation (Equation 3.2). ....  | 74 |
| Table 3.8 - New class boundaries for biological quality assessment of lowland streams using the new diatom index (MDI) and the modified IPTI macroinvertebrate method.....  | 74 |

|   |     |
|---|-----|
| Table 3.9 - Metric and index reference values for IPTIs calculation (Equation 3.1).....   | 74  |
| Table 4.1 - Predictions and justification for expected behaviour of diatom trait categories in response to an extreme drought event in temperate rivers. Arrows indicate changes in category proportion expected in the temperate samples after drought (TAD and T2) compared with temperate samples before drought (T1) (up arrow, higher proportion; down arrow, lower proportion). .....   | 96  |
| Table 4.2 - Predictions and justification for expected behaviour of macroinvertebrate trait categories in response to an extreme drought event in temperate rivers. Arrows indicate changes in category proportion expected in the temperate samples after drought (TAD and T2) compared with temperate samples before drought (T1) (up arrow, higher proportion; down arrow, lower proportion). .....  | 97  |
| Table 4.3 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different diatom biological trait categories of communities T1 (temperate spring before drought), TAD (first sample after water return) and M (Mediterranean). Only the trait categories where statistical differences were verified shown. Relationships shown in bold have $p < 0.05$ . .....              | 99  |
| Table 4.4 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different diatom biological trait categories of communities T1 (temperate spring before drought), T2 (temperate spring after drought) and M (Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have $p < 0.05$ . .....            | 102 |
| Table 4.5 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different macroinvertebrate trait categories of communities T1 (temperate spring before drought), TAD (temperate first sample after water return and M (Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have $p < 0.05$ . ..... | 103 |
| Table 4.6 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different macroinvertebrate trait categories of communities T1 (temperate spring before drought), T2 (temperate spring after drought and M (Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have $p < 0.05$ . .....             | 106 |
| Table 5.1 - Mean values ( $\pm$ standard error) of the measured and calculated variables for each study site.....   | 127 |
| Table 5.2 - Macroinvertebrate taxa contributing 88.3% to the mean dissimilarity (SIMPER analysis) between s1 and s6 (spring 2011 pre-drought and spring 2012 post-drought, respectively) with their percentage contribution, average dissimilarity and standard deviation (SD). The increase (I) or decrease (D) in the mean abundance of each taxon from s1 to s6 is also shown. ....  | 129 |

Table 5.3 - Diatom taxa contributing 65.4% to the mean dissimilarity (SIMPER analysis) between s1 and s6 (spring 2011 pre-drought and spring 2012 post-drought, respectively), with their percentage contribution, average dissimilarity and standard deviation (SD). The increase (I) or decrease (D) in the mean abundance of each taxon from s1 to s6 (spring 2011 pre drought and spring 2012 post-drought) is also shown..... 133

Table 6.1 - Mean ( $\pm$ standard deviation) of the abiotic variables measured in the eleven artificial channels throughout 35 days of the colonization experiment (from day 0 to 35). BDL-Value below detection limit. .... 154

Table 6.2 - Diatom species that contributed more to the group average similarity (up to 80% of cumulative abundance of the Bray–Curtis similarity) developing on tiles (T) and sand (S) substrates at day 14 and 35 obtained by SIMPER analysis..... 158

Table 6.3 - Diatom species that contributed more to the group average dissimilarity and that were only present in one of the substrates at the same sampling moment, day 14 and 35 (up to 80% of cumulative abundance of the Bray–Curtis similarity) obtained by SIMPER analysis..... 159

Table 7.1 - Diatom species that contributed most to average assemblage similarity in PORT-Portugal, USA-United States of America and AUS-Australia (up to 90% of cumulative contribution, Bray–Curtis similarity: SIMPER analysis). The values in bold indicate the species that contributed the most to average similarity within each region. .... 179

Table 7.2 - Diatom species that contributed most to average dissimilarity between AUS-Australia, PORT-Portugal and USA-United States of America (up to 80% of cumulative dissimilarity, Bray–Curtis similarity: SIMPER analysis).The region code opposite a species indicates that the species was found only in that region among the two regions being compared. .... 183

Table 7.3 - Diatom genera that contributed most to average similarity in AUS-Australia, PORT-Portugal, and USA-United States of America (up to 90% of cumulative similarity, Bray–Curtis similarity: SIMPER analysis). The values in bold indicate the genera that contributed most to average similarity within each region..... 189

Table 7.4 - Diatom genera that contributed most to average dissimilarity between AUS-Australia, PORT-Portugal and USA-United States of America (up to 80% of cumulative dissimilarity, Bray–Curtis similarity: SIMPER analysis).The region code opposite a genus indicates that the genus was found only in that region among the two regions being compared..... 191

Table 7.5 - Diatom orders that contributed most to average similarity in AUS-Australia, PORT-Portugal, and USA-United States of America (up to 90% of cumulative similarity, Bray–Curtis similarity: SIMPER analysis). The values in bold indicate the orders that contributed most to average similarity within each region..... 193

## List of Publications

This thesis is based on, and includes, the following articles:

- Elias, C.L., Calapez, A.R., Almeida, S.F.P., Feio, M.J., 2015. Determining useful benchmarks for the bioassessment of highly disturbed areas based on diatoms. *Limnologica* 51, 83-93 – Chapter 2
- Elias, C.L., Calapez, A.R., Almeida, S.F.P., Chessman, B., Simões, N., Feio, M.J., 2016. Predicting reference conditions for river bioassessment by incorporating boosted trees in the environmental filters method. *Ecological Indicators* 69, 239-251 – Chapter 3
- Elias, C.L., Calapez, A.R., Almeida, S.F.P., Feio, M.J., 2015. From perennial to temporary streams: an extreme drought as a driving force of freshwater communities' traits. *Marine and Freshwater Research* 66, 469-480 – Chapter 4
- Calapez, A.R., Elias, C.L., Almeida, S.F.P., Feio, M.J., 2014. Extreme drought effect and recovery patterns in benthic communities of temperate streams. *Limnetica* 33, 281-296 – Chapter 5
- Elias, C.L., Rocha, R.J.M., Feio, M.J., Figueira, E., Almeida, S.F.P., (Submitted to *Aquatic Ecology*). Influence of the colonizing substrate on diatom assemblages and implications for bioassessment - a mesocosm experiment – Chapter 6
- Elias, C.L., Almeida, S.F.P., Chessman, B., Potapova, M., Feio, M.J., (Submitted to *Hydrobiologia*). Do similar climatic patterns in different continents lead to similar benthic diatom assemblages? – Chapter 7



## Chapter 1 - Introduction

### 1.1 *Diatoms*

Diatoms (Class Bacillariophyceae sensu Ruggiero et al., 2015) are considered the major constituents of benthic and planktonic algal communities (Mann and Droop, 1996) even with a higher species-richness (Mann, 1999). They are very common and abundant in almost all aquatic habitats (freshwater and marine) and also in terrestrial habitats (Mann and Droop, 1996; Stoermer and Smol, 1999). This group of algae is characterized by many features but especially by their siliceous cell walls, composed of two valves that together with the girdle bandas form the frustule (Figure 1.1). Due to the size, shape, and ornamentation of its cell walls, diatoms are relatively easily distinguished to species and subspecies levels. Also, due to its siliceous nature, diatom frustules are preserved in sediments and record habitat history (Stevenson and Pan, 1999).

Diatoms are the most species-rich components of river and stream ecosystems. They play an important role in food webs (*e.g.*, they are the food for many macroinvertebrates and fish), in the oxygenation of surface waters, and constitute a linkage between biogeochemical cycles (*e.g.*, nutrient uptake and remineralization) (Mulholland, 1996; Stevenson and Pan, 1999). In rivers and streams diatoms are present either in the water column (potamoplanktonic diatom communities) or in the benthos (growing attached to surfaces-rocks, aquatic plants, sand grains or living free in the sediment) (Round et al., 1990). However, diatom communities found in small streams are mostly benthic (Patrick, 1977).



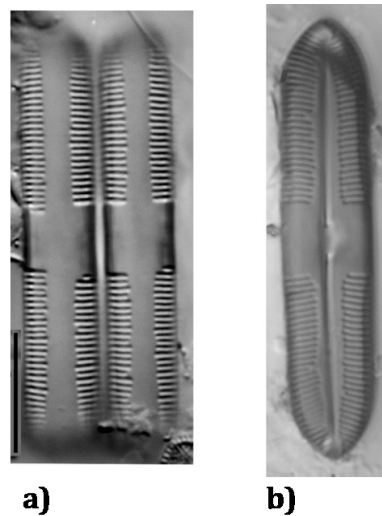


Figure 1.1 - a) Two frustules of *Caloneis*, as seen in gilde view; b) one valve of *Caloneis*, as seen in valve view. Photograph from the sample collected in Vala da Corujeira (Casal dos Netos). Scale bar: 10  $\mu\text{m}$  (for both figures).

The structure and function of diatom communities are regulated by many abiotic and biotic factors, at different spatial scales (from single sand grains to across continents) (Biggs, 1996). Climate, geology, biogeography and land use are ultimate factors, at large spatial scale, that affect the resources (*e.g.*, light, nutrients and space), biotic factors (*e.g.*, competitors and herbivores) and abiotic stressors (*e.g.*, pH, temperature and toxic substances) that directly affect the function and structure of benthic algal communities (Stevenson, 1997) (Figure 1.2). Environmental factors, expressed at multiple scales, determine the composition and structure of local biological communities as these variables can be seen as ‘filters’ through which species in the regional species pool must ‘pass’ to potentially be present at a given site (Keddy, 1992; Poff, 1997; Quist et al., 2005). The suite of taxa existing at a site under reference conditions is visualised as resulting from a natural, top-down filtering process applied to a regional pool of taxa, whereby only those taxa with appropriate traits are able to pass the natural filters (Chessman and Royal, 2004). Thus, if diatoms were well dispersed in sites with similar environmental conditions we would find the same species in all sites (Virtanen and Soininen, 2012) as the reference condition is, accordingly with Reynoldson et al. (1997), ‘the condition that is representative of a group of minimally disturbed sites organized by selected physical, chemical, and biological characteristics’.

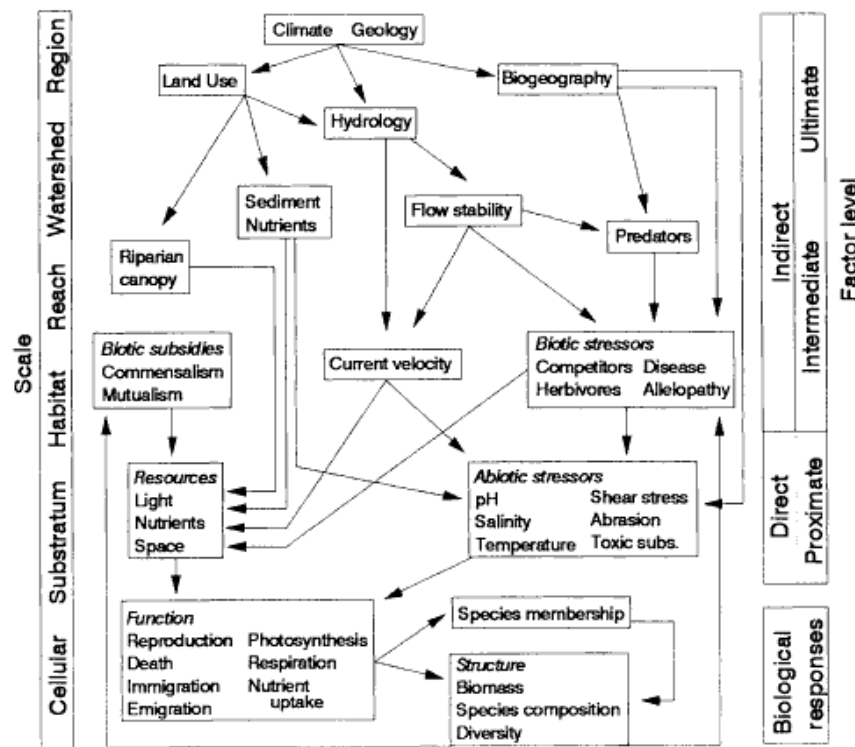


Figure 1.2 - Hierarchical interrelationships among proximate (resources and abiotic stresses), intermediate, and ultimate (climate, geology, land use) determinants of benthic algal assemblage composition and biomass. Source: Stevenson (1997).

Diatoms, like other organisms, have been considered to be ubiquitous. In contrast to larger organisms (*e.g.*, macroinvertebrates Heino and Soininen, 2007), diatoms may be easily distributed via wind, currents and animal vectors (*e.g.*, Kristiansen, 1996), which result in wider distributions in comparison to metazoans (*e.g.*, Finlay et al., 2002; Heino and Soininen, 2007).

Some large-scale studies (Potapova and Charles, 2002; Soininen, 2004; Soininen et al., 2004) have demonstrated that factors related to spatial variables may also influence diatom distribution patterns (Virtanen and Soininen, 2012) even at the genus level (Vyverman et al., 2007). The increasing incidence of apparent regional endemisms (*e.g.*, in the diatom floras of Australia) and the increasing evidences pointing to a strong regionalization of diatom floras in the Antarctic and sub-Antarctic regions, mirroring the biogeographical regions that have been recognized for macroorganisms, are in favour of the prevalence of restricted distribution patterns among diatom species (Vyverman et al., 2007; Vyverman et al., 2010). Factors related to historical processes (*i.e.*, colonization and extinction, dispersion and migration) constrain global patterns in regional and local diatom composition and diversity (Vyverman et al., 2007).

## **1.2 Global changes and implication on river ecosystems**

Freshwater is a fundamental resource for both nature and humans (Naiman and Dudgeon, 2011). However, freshwater ecosystems are among the most altered systems on Earth (Naiman and Dudgeon, 2011). Due to the high number of species *per* area found in these ecosystems the decline in its biodiversity is far greater than in terrestrial or marine ecosystems (Sabater et al., 2013). Within these ecosystems running waters are unique in their unidirectional flow (Malmqvist and Rundle, 2002). Such characteristic shapes the morphology of river channels, makes running waters inherently variable in space and time and has led to a biota that is highly adapted to dynamic conditions (Malmqvist and Rundle, 2002).

The unidirectional nature of rivers and streams makes these systems unique, however also aggravates its vulnerability. Almost any activity within a river catchment has the potential to cause environmental change and any pollutant entering a river is likely to exert effects for a large distance downstream (Malmqvist and Rundle, 2002). Running waters provide a multitude of benefits for humans, such as sources of water, means of power generation and waste disposal, routes for navigation and places for leisure activities (Malmqvist and Rundle, 2002). Due to this, river banks have been inhabited for millennia and until nowadays are densely populated. With such intense human pressure on rivers and increasing demand for water over the past century (Gleick, 2000), it is not surprising that nowadays few streams or rivers remain unaltered (Frissell and Bayles, 1996; Malmqvist and Rundle, 2002; Wiens, 2002; Naiman and Dudgeon, 2011). In fact, some of these ecosystems have been so starkly and extensively modified that their return to a natural state is almost impossible (Frissell and Bayles, 1996). The main factors causing such modifications in running waters are ecosystem destruction (*e.g.*, urban and agricultural expansion), physical habitat (*e.g.*, hydrology, channel morphology) and water chemistry alteration (*e.g.*, industrial emissions) and direct species additions or removals (*e.g.*, aquaculture/aquariums) (Malmqvist and Rundle, 2002). An indirect factor that is presently, and will in the future be expected to cause more modifications in the rivers is the increase in the use of coal, oil and gas worldwide. This induces higher greenhouse gases emissions (carbon dioxide- CO<sub>2</sub>, methane- CH<sub>4</sub> and nitrous oxide- N<sub>2</sub>O) that are resulting in a global transformation of the Earth's atmosphere and climate without precedents (Sabater et al., 2013; IPCC, 2014).

Due to climate changes many freshwater species have shifted their geographic ranges, seasonal activities, migration patterns, abundances and species interactions as in many regions, changing precipitation or melting snow and ice are altering hydrological systems, affecting water resources in terms of quantity and quality (IPCC, 2014). At high rates of

warming, major groups of terrestrial and freshwater species are unable to move fast enough to stay within the spatially shifting climate envelopes to which they are adapted (IPCC, 2014).

Despite all the climate changes that have already been verified, forecasts predict a doubling in atmospheric CO<sub>2</sub> concentration by the end of this century and a rise in world mean air temperature between 1.8 and 4.0 °C (IPCC, 2007). In central Europe, heat waves are predicted to be more frequent, more intense and longer in duration. Heavy winter precipitation is also predicted to increase in central and northern Europe, and decrease in the south (IPCC, 2007; IPCC, 2013). The southern part of Europe will also suffer a predicted decrease in summer precipitation (Beniston et al., 2007). These changes in precipitation, temperature and consequently evapotranspiration rates will affect volumes of surface water runoff. Model simulations predict a 10-30% decrease in annual runoff in southern Africa, southern Europe and in the western United States by the mid-century (Milly et al., 2005; IPCC, 2007). In contrast, other models predict a decrease in annual runoff in southern Europe that may reach as much as 50% of the current levels (Arnell, 1999). Typically temperate and rainy climate areas (*e.g.*, central Europe) will become more similar to Mediterranean climate areas that are characterised by mild wet winters and hot dry summers (Giorgi and Lionello, 2008), thus suggesting its expansion to northern regions. These changes in the world climate will be exacerbated by other pressures that are presently affecting the streams and rivers (*e.g.*, increase in the length of time that threshold concentrations of nutrients are exceeded) (Ockenden et al., 2016).

Europe's running waters are no exception and are affected by a range of pressures, including water pollution, water scarcity, floods, and modifications to water bodies that affect morphology and water flow (EEA, 2015). In order to try to reverse this situation, in 2000 the European Community published the Water Framework Directive (WFD: European Commission, 2000, Directive 2000/60/EC). This directive aims to incorporate into a legally binding instrument the key principles of integrated river basin management bringing together economic and ecological perspectives (COM (2012) 670 final).

In the WFD, water management accounts for the water needs of both humans and nature (European Commission, 2000). The WFD established that all European Union Member States (MS) shall protect, enhance and restore the aquatic environment through the implementation of programmes of measures developed and implemented as part of river basin management plans in order to maintain or achieve good water status by 2015 (European Commission, 2000). This status would allow aquatic ecosystems to recover and to deliver the ecosystem services that are necessary to support life and economic activity that depend on water (COM (2012) 670 final). The good water status goal is achieved when both good chemical and ecological status of water bodies is attained (European Commission, 2000; Guidance Document No. 10, 2003). However, 2015 reports state that the MS still have a long way to go

before the quality of all European Union waters is good enough, due to decades of previous degradation and persisting ineffective management and that the WFD objective will only likely be met by 53% of surface water bodies (Figure 1.3) (EEA, 2015). This constitutes a modest improvement, as in 2009 43% of surface water bodies were in good or high ecological status, and is far from meeting policy objectives. Rivers and transitional waters are on average in a worse condition than lakes and coastal waters.

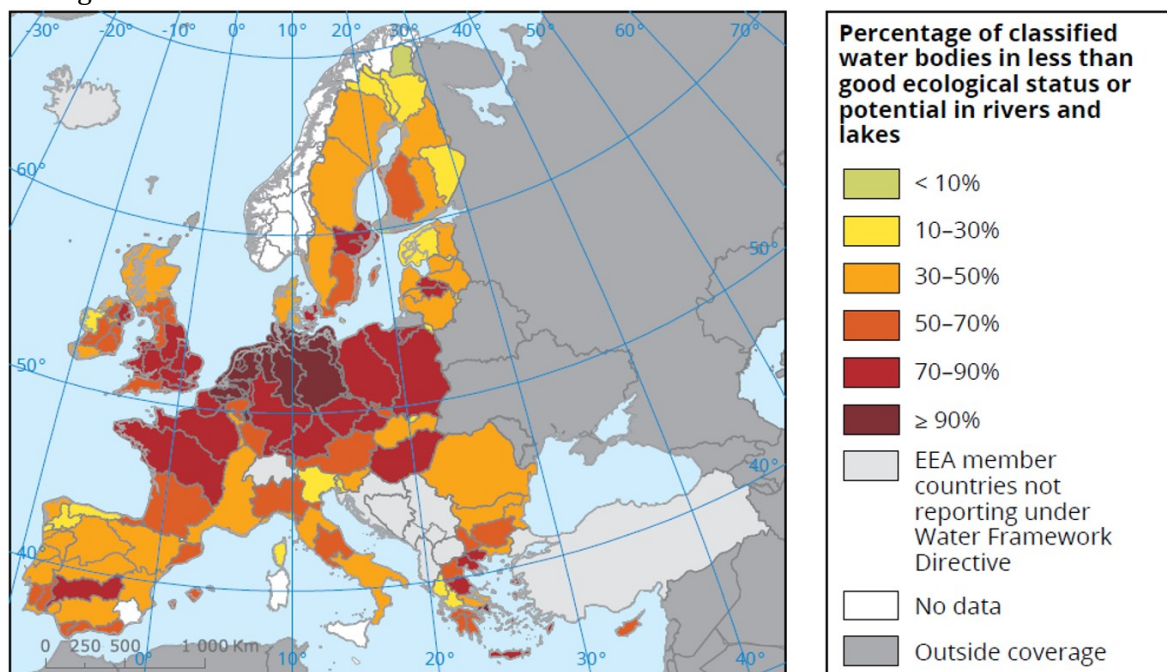


Figure 1.3 - Percentage of good ecological status or potential of classified rivers and lakes in Water Framework Directive river basin districts. Source: EEA (2015).

### 1.3 Implications of global changes on diatom communities

Environmental factors determine the composition and structure of local biological diatom communities. Therefore, any anthropogenic changes to these environmental factors (*e.g.*, pH, salinity, nutrients, suspended sediments, flow) may affect the diatom communities as aquatic organisms are known to integrate all the biotic and abiotic changes that occurred in their habitat (Lowe and Pan, 1996).

Pan et al. (1999) showed that diatom communities are strongly controlled by geomorphic and disturbance factors (*e.g.*, land cover/use in watersheds and riparian condition). The degradation of riparian vegetation can lead, for example, to change in water temperature patterns. The streams are more exposed to sunlight and, therefore, more influenced by atmospheric temperature (Poole and Berman, 2001). Among the sources that contribute to sedimentation and turbidity in streams are agriculture, forestry, mining, road

construction, and urban activities (Henley et al., 2000). Land use in the catchment can also influence the water quality as it may cause changes in nutrient load. However, this influence depends on intrinsic factors of the catchment, such as the transport capacity of the watershed, the influence of riparian buffers, climatic and geomorphological basin features, and the existence of additional pressures (Pardo et al., 2012). The loss of riparian vegetation also facilitates the runoff of sediments into the streams, as these areas are important sediment sinks (Daniels and Gilliam, 1996; Sheridan et al., 1999). Increase in the sediment load and consequent increase in turbidity of streams can cause a reduction in algae growth due to the decrease of light availability for photosynthesis (Henley et al., 2000). In addition, sediment deposition can also promote smothering and stability of substrata to which diatoms attach. Hence, non-motile and particularly chain-forming diatoms cannot establish easily, further pushing the assemblage towards single celled and motile taxa (Jones et al., 2014).

Nutrient enrichment of freshwaters is widespread. The main sources of nitrogen and phosphorus include point source emissions from urban wastewater treatment plants and industry, and diffuse emissions from agricultural production (EEA, 2015). The projected climate changes have also been associated with likely increases in sediment and nutrient loads. For example, the expected increase in winter rainfall due to climate changes is likely to enhance the annual loads of total phosphorus and total reactive phosphorus load from land to water (Ockenden et al., 2016).

The alterations in nutrient concentrations can potentially cause changes in the diatom communities as different diatoms have different ranges and tolerances. The nutrient enrichment can benefit certain taxa, while others become rarer (Lange et al., 2011). Tolerant species can become dominant in the communities (*e.g.*, *Cocconeis placentula*, *Encyonema minuta* and *Fragilaria vaucheria*) whereas *Achnanthyidium minutissimum* and *Rossithidium petersenii* show the opposite pattern (Lange et al., 2011).

Current velocity variations over both space and time is typical of all streams and periphytic algae are exposed to these variations. Therefore, current affects directly (*e.g.*, dislodging of species loosely attached to substrata) and indirectly (*e.g.*, affecting the distribution of macroinvertebrate grazers) benthic algal metabolism and distribution (Stevenson, 1996). Diatoms are known to be relatively susceptible to disturbance by current (Stevenson, 1996; Ghosh and Gaur, 1998). Floods and droughts are also important hydrologic events in running water ecosystems. Therefore, in-stream biological communities have developed adaptations that range from the avoidance of individual floods or droughts to life-history strategies that are synchronised with long-term flow patterns (Lytle and Poff, 2004). The traits developed by species enable them to survive, exploit and even depend on disturbance as a result of a long evolutionary period (Lytle and Poff, 2004). Thus, the native communities of drought-prone systems are the result of a multi-habitat filter process that

selects organisms with a set of traits that enable them to either survive to droughts (resistance traits) or to re-colonise and recruit after the drought breaks (resilience traits) (Poff, 1997; Bond et al. 2008). However, the communities of rivers exposed to rapid changes in natural flow conditions (*i.e.*, magnitude, frequency, duration, timing and predictability) are deprived of such an evolutionary period. Changes in the natural flow of typically permanent streams due to extreme events or climate change may therefore exert stronger effects on the ecological structure and functioning of stream communities (Lake, 2000).

#### **1.4 Dealing with global changes and spatial scale in bioassessment**

Diatoms are valuable indicators of environmental conditions in rivers and streams, because they respond directly and sensitively to many physical, chemical, and biological changes in river and stream ecosystems, namely nutrient concentrations (Stevenson and Pan, 1999). In fact, diatom community characteristics (composition and abundance) have been used to assess the ecological integrity of rivers and streams due to three basic reasons: their importance in ecosystems, their utility as indicators of environmental conditions, and their ease of use (Stevenson and Pan, 1999). The frequent use of diatoms as biological indicators has led to the development of several systems in order to interpret the data (Kelly et al., 1995). Diatom autecological indices were developed to infer levels of pollution (mostly nutrients) based on the species' composition of communities and their ecological preferences and tolerances (IPS-Cemagref, 1982; CEE-Descy and Coste, 1990; EPI-D – Dell'Uomo, 2004; IBD – Prygiel and Coste, 1998, among others). However, several studies have demonstrated that diatoms also respond to important hydromorphological, chemical (besides nutrients), and physical changes in the environment (*e.g.*, Potapova and Charles, 2003; Almeida and Feio, 2012; Feio et al., 2014).

In result of this sensitivity to environmental conditions, diatoms are nowadays a mandatory biological element in the evaluation of ecological quality of European rivers, according to the WFD (European Commission, 2000; Leira and Sabater, 2005). In association with other Biological Quality Elements - BQE (other aquatic flora, benthic invertebrate fauna and fish fauna), and the hydromorphological, chemical and physical elements that support them (European Commission, 2000; Ferreira et al., 2011) they provide a holistic picture of the ecological status of the aquatic environment (Leira and Sabater, 2005; SWD (2012) 379 final). The ecological status of each water body is determined by the quality element having the lowest status class, according to the one-out-all-out principle. This principle is at the heart of integrated river basin management that addresses all pressures and impacts on aquatic environment. It ensures that the negative impact of the most dominant pressure on

the most sensitive quality element is not averaged out and obscured by minor impacts of less severe pressures or by less sensitive quality elements responding to the same pressure.

From all types of substrates found in rivers, most studies with diatoms focus on epilithon (Winter and Duthie, 2000), as hard surfaces are the preferred substrates used in water quality assessment (Kelly et al., 1998). However, other substrates like submerged macrophytes and sediments are also commonly found and may be dominant in some stream and river sections (Kelly et al., 1998; Elias et al., 2015). Some studies indicate that benthic diatom species present different biological characteristics that enable them to adapt to specific micro-habitats (Krejci and Lowe, 1986; Soininen and Eloranta, 2004). If these natural inter-substrate differences are reflected in water quality assessment metrics, this can potentially mask responses of algal communities to stresses associated with human activities and may interfere with water quality assessments based on the knowledge of these responses (Winter and Duthie, 2000; Potapova and Charles, 2005; Bere and Tundisi, 2011; Mendes et al., 2012). Some studies have tested the effect of different substrates in water quality assessments and have not verified significant differences (Kitner and Poulícková, 2003; Potapova and Charles, 2005; Mendes et al., 2012). However, these studies were conducted under natural conditions where the communities are shaped simultaneously by many environmental factors, which may lead to confounding effects (Stevenson and Pan, 1999). In addition the substrate effect is difficult to detect in large-scale, coarse resolution studies, when the role of other factors, such as inter-stream differences in hydrology, physical habitat, and chemistry become more important than the role of substrate (Potapova and Charles, 2005). Even when different diatom communities of the same river are compared and differences are verified (Cetin, 2008) it is difficult to ensure that the communities have been exposed to the same environmental variations at the same time or even at the same developmental phase. Additionally, it is impossible to avoid contamination between substrates with diatoms migrating between habitats in wadeable streams.

The definition of type specific reference conditions is an important step in the process of assessing the ecological quality status of rivers. The ecological quality status is obtained by quantifying the deviation in composition and abundance of BQEs in a water body from the composition and abundance expected under reference conditions for that type of water body (*e.g.*, mountain or lowland type) (Schaumburg et al., 2004). However, a representative suite of appropriate reference conditions has to be identified for each type of water body (Schaumburg et al., 2004; Pardo et al., 2012) to permit an interpretation of measured values (*e.g.*, biological indices) that takes account of natural spatial differences in the composition and abundance of BQEs (Hawkins et al., 2010). Under the WFD, the reference point is 'undisturbed' conditions with no or only 'very minor' human impacts on hydromorphological, physical and chemical elements (Guidance Document No. 10, 2003). However, reference



water bodies with only minor impacts are absent from many parts of Europe (and other regions of the world) because of historical anthropogenic alteration of landscapes and high population densities (Nijboer et al., 2004; Kelly et al., 2012; Feio et al., 2014). An example of an area where 'undisturbed' conditions is almost impossible to find is the central-western Portuguese littoral region. During the WFD implementation all this area was included in a single type, the Littoral (L type), since only a few reference sites were used. That probably resulted in the inclusion of a wide variety of streams in the same typology. Recognizing this limitation, the WFD allows the type-specific biological reference conditions to be either spatially based (via reference sites), based on modelling (predictive models or hindcasting methods), or derived from a combination of these methods and even based in paleolimnological data (European Commission, 2000). Where it is not possible to use these methods, Member States may use expert judgement to establish such conditions (European Commission, 2000).

As in many situations it is difficult to dissociate the obvious effect of climate change, increase in temperature, from other anthropogenic or natural effects. Therefore, as the diatom communities are known to respond well to several environmental factors is very likely that the effects of climate change will be reflected in the presently used autecological indices. The prevention of higher negative impacts on diatom communities due to climate changes probably passes through the reduction of non-climatic stressors, such as habitat modification, overexploitation, pollution and invasive species, to increase the inherent capacity of ecosystems and their species to adapt to a changing climate (IPCC, 2014). Adaptive water management techniques, including scenario planning, learning-based approaches and flexible and low-regret solutions, can help adjust to uncertain hydrological changes due to climate change and their impacts (limited evidence, high agreement). Strategies include adopting integrated water management, augmenting supply, reducing the mismatch between water supply and demand, reducing non-climate stressors, strengthening institutional capacities and adopting more water-efficient technologies and water-saving strategies (IPCC, 2014). Results suggest that, for example, in order to build resilience in stream health and help mitigate potential increases in diffuse agricultural water pollution due to climate change, land management practices should target controllable risk factors, such as soil nutrient status, soil condition and crop cover (Ockenden et al., 2016). Actions may also include maintenance of genetic diversity, assisted species migration and dispersal, manipulation of disturbance regimes (*e.g.*, fires, floods) (IPCC, 2014).

## **1.5 Aims of the present work**

In view of the current knowledge on diatoms communities and their use as indicators of rivers ecological quality, the present thesis aimed to answer to the following global questions:

- How to deal with the historical anthropogenic influences in bioassessment?

In order to achieve this goal, the central-western Portuguese littoral region, a highly impacted area subjected to different stressors (*e.g.*, agriculture and urbanization), was defined as case study. The following chapters resulted from this global question:

Chapter 2 - Determining useful benchmarks for the bioassessment of highly disturbed areas based on diatoms

Chapter 3 - Predicting reference conditions for river bioassessment by incorporating boosted trees in the environmental filters method

- Are extreme events due to climatic changes – reflected in diatom communities? A comparison with macroinvertebrate communities.

In order to answer this question, four streams located in the central-western Portuguese littoral region, affected by a severe and uncharacteristic drought event from the end of summer 2011 to the winter of 2012, were defined as case study. The drought effects in the diatom and macroinvertebrate communities from temperate streams were also compared with those from Mediterranean systems to assess possible shifts in the communities. Two chapters resulted from this second global question:

Chapter 4 - From perennial to temporary streams: an extreme drought as a driving force of freshwater communities' traits

Chapter 5 - Extreme drought effect and recovery patterns in benthic communities of temperate streams

- How are diatom communities influenced at the spatial scale (small-scale: habitats; large-scale: inter-continental).

In order to answer this question, an indoor controlled experiment was performed and diatom data from streams and rivers from three continents with similar climate were defined as case study. Two chapters resulted from this third global question:

Chapter 6 - Influence of the colonizing substrate on diatom assemblages and implications for bioassessment - a mesocosm experiment

Chapter 7 - Do similar climatic patterns in different continents lead to similar benthic diatom assemblages?

### Abbreviations

|        |   |
|--------|---|
| BQE    | Biological Quality Elements   |
| L type | In general terms the L type (littoral type) are a set of lowland coastal streams with a small to medium drainage area located all in the central-western Portuguese littoral region |
| MS     | Member States, European Union   |
| WFD    | Water Framework Directive   |

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## Chapter 2 - Determining useful benchmarks for the bioassessment of highly disturbed areas based on diatoms

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Published in *Limnologica* 2015, 51, 83–93.

### Abstract

Modern ecological assessments of running waters are based on the a priori definition of ecological benchmarks, given by reference-quality sites. Such benchmarks are established at the level of ecoregions, typologies, or site. Yet, in highly disturbed regions, such as coastal areas of European countries, the assessment of streams' water quality based on the reference condition concept is very difficult, due to the lack of undisturbed sites. Among others, the reduced number of reference sites may have as a consequence the definition of imprecise ecological benchmarks. Here we tested the hypotheses that (1) the increase in the number of potential reference sites (2) the definition of more precise abiotic thresholds using the least disturbed condition approach (LDC), and (3) the use of diatom assemblages, as the most ubiquitous element in lowland areas, would result in refinement and eventual sub-division of existing river types of a highly disturbed area, such as the Portuguese centre-western region. For this purpose, abiotic data characterising natural conditions of 55 sites from a littoral highly disturbed region were used in a hierarchical classification analysis that revealed the existence of three different sub-groups. In addition, a three-step approach was used to define thresholds for the pressure variables in LDC. Based on these new thresholds, sites in LDC were selected. A hierarchical classification performed to the LDC diatom spring assemblages revealed the existence of two sub-groups, concordant with two of the abiotic sub-groups. Several species contributed to the dissimilarity between the two sub-groups (*e.g.*, *Achnanthes minutissimum* and *Karayevia oblongella*). Differences between the sub-groups were also found in the trait proportions of stalked species. New benchmark values for these two sub-groups, based on the scores of the official diatom index, the '*Indice de*

*Polluosensibilité Spécifique'* (IPS), were different from the previous reference value used. Yet, no biological benchmark values were established for one of the groups due to the absence of sites in the LDC.

Our study suggests that streambed substrate is an important characterisation variable in the river type definition and highlights that, in spite of the potential refinement in reference conditions and typology obtained, an alternative approach that does not require the use of reference sites should be explored in the future.

## **Keywords**

Least Disturbed Condition, lowland streams, natural variability, substrate, type-specific reference conditions

## 2.1 Introduction

Modern ecological assessments of streams and rivers are frequently based on the a priori definition of ecological benchmarks, given by reference-quality sites, to which assessed sites are matched in terms of assemblage composition and structure (Hawkins et al., 2010). Such ecological benchmarks have been established at the level of ecoregions, typologies or site-specific (Hawkins et al., 2010).

In Europe, and according to the Water Framework Directive (WFD; European Commission, 2000), type-specific reference conditions must be defined (Schaumburg et al., 2004; Pardo et al., 2012) for each water body and biological quality element (BQE; phytoplankton, macrophytes and phytobenthos, benthic invertebrate fauna, and fish fauna). The classification in types is an attempt to organise the abiotic variability of streams (or water bodies) in order to determine areas that are homogeneous regarding a certain number of features, such as climate, altitude, geology, or morphology, that strongly influence the distribution and community composition of aquatic biota (Wimmer et al., 2000). However, while the suitability of such an approach was often investigated and well accepted for macroinvertebrates (*e.g.*, Rawer-Jost et al., 2004; Verdonschot and Nijboer, 2004; Verdonschot, 2006; but see Lorenz et al., 2004), the same is not always true for other biological elements.

Among the BQEs that are primary producers, phytobenthos is frequently the only available community in small streams, due to the absence of true phytoplankton and macrophyte vegetation (Eloranta and Soininen, 2002). Diatoms have been selected by most countries in Europe as representative of such a group (Almeida and Feio, 2012; Kelly et al., 2012; Feio et al., 2014). Several studies have demonstrated that diatoms respond to important hydromorphological, chemical, and physical changes in the environment (*e.g.*, Potapova and Charles, 2003; Almeida and Feio, 2012; Feio et al., 2014). Yet, diatoms are also known to react differently than other aquatic elements to streams' abiotic features. For example, in a study conducted by Feio et al. (2007), the macroinvertebrates revealed to be highly associated with the substrate type, diversity of habitats, and water velocity, while diatoms revealed to be highly associated with geology and stream size. That is one of the main problems of the WFD typological approach, as it assumes a community concordance between the BQEs (the use of the same river types to define ecological benchmarks for all BQEs), which is difficult precisely because the various elements respond differently to various water body and catchment features (Paavola et al., 2003; Dolph et al., 2011).

In addition, the establishment of true reference conditions may be a problem in Europe, and also in many other regions of the world, due to a long history of anthropogenic alteration

of landscapes and high population densities (Nijboer et al., 2004; Kelly et al., 2012; Feio et al., 2014). Therefore, alternatives to the use of pristine sites to establish benchmarks for the ecological assessment have been discussed and proposed by several authors in recent years (Reynoldson et al., 1997; Stoddard et al., 2006; Hawkins et al., 2010; Birk et al., 2012; Pardo et al., 2012).

Among those, the concept of least disturbed condition (LDC), according to Stoddard et al. (2006), seems potentially useful to define ecological benchmarks for the central-western Portuguese littoral region. As in other lowland/coastal regions, this area has been suffering from intensive land use, urbanisation, industry, and agriculture, and historical data aren't available. To establish the LDC for these regions, a careful definition of what are presently the 'best' conditions, based on a set of explicit criteria, is needed (Stoddard et al., 2006). The 'best' (best available) for the present region are sites with anthropogenic disturbances concerning changes in hydromorphological characteristics such as riparian vegetation, hydrological regime, channelisation, sediment load, and nutrient concentrations. However, applying this concept in highly disturbed regions may result in a reduced number of sites, and consequently in the need to enlarge the target area to include more reference sites. This was what we believe occurred during the implementation of the WFD in Portugal. By then, 15 river types (INAG, 2008) were established by the Portuguese water authority (Portuguese Water Institute, now Agência Portuguesa do Ambiente); however, the entire central-western Portuguese littoral region was included in a single type, the Littoral (L type), since only a few reference sites were used. That probably resulted in the inclusion of a wide variety of streams (see section 2.2.1) in the L type, with different communities and potentially different reference values for water quality assessment. If reference values are inappropriate, an incorrect quality rating of sites is likely with the attribution of too high or too low quality classes.

Here we discuss and test strategies to overcome the problem of inadequate reference conditions in such highly disturbed regions. Our main hypotheses were, first, that the increase of the sampling sites density, then the refinement of benchmark criteria (to LDC), and the use of diatom assemblages (as the most ubiquitous biological element in this region) could lead to (1) the alteration of the presently accepted reference values for water quality assessment for the L type based on diatoms, and (2) the redefinition of the L type, with its possible division. In order to avoid forcing the biological to the abiotic data or vice-versa, we analysed independently the existence of abiotic (abiotic data only) and biological (diatom assemblages) sub-groups and assessed *a posteriori* the congruence of the sub-groups formed. For this purpose, we used data collected at 55 sites from this region to (1) test the coherence of the L type concerning its abiotic characteristics – *i.e.*, test the existence of sub-groups; (2)

establish and apply criteria for the selection of sites on the LDC; (3) verify if the least disturbed diatom assemblages validate the abiotic sub-groups formed in (1), using both taxonomic composition and diatom traits – *i.e.*, if the abiotic grouping of sites (typology) matches the biological grouping; (4) if the sub-groups could be used to define more adequate benchmarks for the biological assessment of streams based on diatoms – namely, using the Portuguese mandatory diatom index for the region in study, the '*Indice de Polluosensibilité Spécifique*' (IPS); and (5) recommend, if needed, new types for this region based on abiotic and biological information.

## **2.2 Methods**

### **2.2.1 Study area and sampling sites**

The central-western Portuguese littoral region has an Atlantic-temperate climate characterised by mild temperatures, moderate summers and winters, and precipitation values above 2,800 mm·year<sup>-1</sup> (Agência Portuguesa do Ambiente, 2007).

It comprises the catchments of rivers Vouga (V), Mondego (M), Lis (L), and Tagus (T), and the Ribeiras do Oeste (O) and Ribeira do Noroeste (NO). The Ribeiras do Oeste and Ribeiras do Noroeste are small streams discharging directly into the sea, located in the south-western part of the study area (Figure 2.1). In general, the L type refers to coastal lowland rivers (mean 40 m a.s.l.) with a wide range of drainage areas (from 10 to 5386 km<sup>2</sup>), including small tributaries and coastal streams but also lowland sections of the main rivers Mondego and Vouga (INAG, 2008). In the Portuguese context, these streams are located in an area with a high mean annual temperature (15 °C) and intermediate mean annual precipitation (900 mm) (INAG, 2008). The lithology has mixed characteristics but includes large limestone areas.

The study sites cover most of the existing natural variability in the littoral region and also include other water bodies that were originally not included in the L type (*e.g.*, streams from Tagus catchment) but have apparently similar environmental characteristics, regarding geology, sediment type, channel morphology, and climate, among others (as described above) (Figure 2.1). We used data from 31 sites sampled between 2004 and 2006 in spring for the implementation of the WFD in Portugal by the national water agency (INAG, IP). In addition, we collected 132 new samples from 36 sites in 2011 and 2012, covering spring, autumn and winter. Thus, a total of 163 samples from 55 different sites were used (as some of the sites were common in 2004/2006 and 2011/2012). At all sites, diatom assemblages and environmental data were collected following the same procedures (see section 2.2.2).



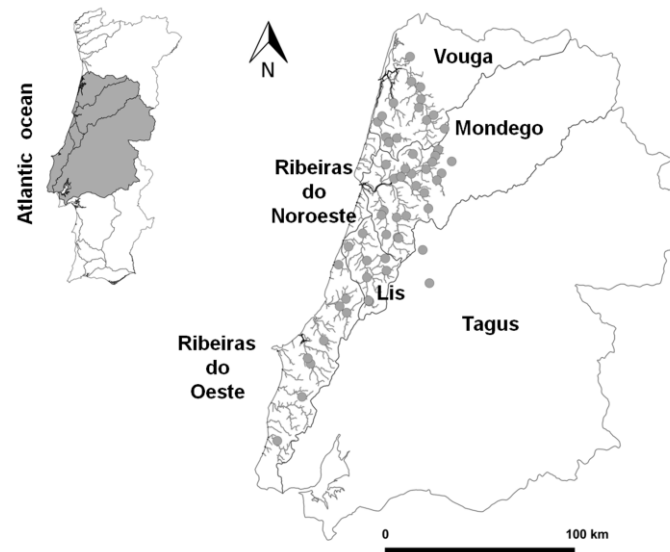


Figure 2.1 - Location of the study sites (grey circles) in the central Portugal catchments (above left, in grey). The rivers and streams represented (grey lines) are those presently included in the L type.

### 2.2.2 Sampling collection and processing

Sampling and treatment of the diatom assemblages were performed according to the European standards (European Committee for Standardisation 2003, 2004, 2006). The sampled substrate was preferably hard substrate (stone or rock); otherwise sediment (sand) was sampled. The sediment was used according to the study by Mendes et al. (2012), who validated the use of alternative substrates for water quality assessment with diatoms. For the epilithic biofilm sampling, an area of  $\approx 100 \text{ cm}^2$  (five stones) was scraped with a toothbrush from the upper surface of submerged stones. In epipsammon sampling, a volume of about 50 mL of streambed sediment was collected using a syringe. Samples were preserved with formaldehyde (4%). In order to ensure ecological reliability of the samples, the proportion of living and dead cells was assessed under a light microscope. The samples were then oxidised with concentrated nitric acid and potassium dichromate for about 24 hours. Thereafter, permanent slides were mounted using Naphrax®, and a minimum of 400 diatom valves for each sample were counted and identified to the lowest taxonomic category possible. The identification was normally done to species or infra-specific rank using a light microscope (100x objective and 1.32 numerical aperture) and Krammer and Lange-Bertalot's floras (1986, 1988, 1991a, 1991b) and Krammer's (2000, 2001, 2009) floras.

### 2.2.3 Abiotic characterisation of the sites

*In situ* measurements of pH, conductivity ( $\mu\text{S}\cdot\text{cm}^{-1}$ ), and percentage of dissolved oxygen (DO; %) were made using a field meter (Multiparameter Probe 3430 WTW). Water samples were collected for posterior laboratory determination of nutrients and chemical oxygen demand. Stream data on hydromorphological characteristics (*e.g.*, substrate) and a set of pressure categorical variables adapted from Pont et al. (2006) were assessed at each site (Table 2.1 and Table 2.2). Other data (latitude, altitude, runoff, mean annual temperature and precipitation, drainage area, slope, lithology, % artificial areas, % intensive and extensive agriculture) were obtained from cartographic sources (Table 2.1 and Table 2.2) (1:25,000 digital military maps, Instituto Geográfico do Exército, Portugal; Atlas do Ambiente Digital: Agência Portuguesa do Ambiente 2007; Corine Land Cover, 1990).

The abiotic variables were divided into characterisation and pressure variables. The first have very little or minimal influence of anthropogenic activities, such as altitude, lithology or precipitation and were used to analyse the streams' typology (Table 2.1). The pressure variables (Table 2.2) are those highly influenced by human activities, such as nutrients (*e.g.*, nitrates, phosphates, sulphates), dissolved oxygen, or hydrological regime and were used to determine the LDC.

Table 2.1 - Abiotic characterisation variables measured or calculated for each site, units, and transformation applied for the PCA.

| Abiotic characterisation variables  |
|---|
| Latitude ( $\log (x+1)$ )   |
| Altitude (m; square root) <sup>a</sup>  |
| Runoff, mean annual (mm; square root) <sup>a</sup>  |
| Temperature, mean annual air temperature ( $^{\circ}\text{C}$ ; $\log (x+1)$ ) <sup>a</sup> |
| Precipitation, mean annual (mm; $\log (x+1)$ ) <sup>a</sup>                                 |
| Drainage area ( $\text{km}^2$ ; $\log (x+1)$ ) <sup>a</sup>                                 |
| Slope of the drainage area (%; square root) <sup>a</sup>                                    |
| Lithology (categorical; square root) <sup>a</sup>   |
| Dominant substrate (in $\approx 500$ m from the site %): <sup>b</sup>                       |
| Bedrock (BE; arcsin)  |
| Boulder (BO; arcsin)  |
| Cobble (CO; arcsin)   |
| Gravel/Pebble (GP; arcsin)  |
| Sand (SA; arcsin)   |
| Silt (SI; arcsin)   |

<sup>a</sup> Based on Atlas do Ambiente (Agência Portuguesa do Ambiente, 2007); <sup>b</sup> Based on Environment Agency (2003).

#### 2.2.4 Data analyses

##### 2.2.4.1 Typology

To verify the coherence of the Portuguese L type in terms of abiotic characteristics, a hierarchical classification (Euclidean distance; Unweighted Pair Group Method with Arithmetic mean – UPGMA) was performed using data from the abiotic characterisation variables of all sites. A principal components analysis (PCA) was also performed to determine which of the abiotic characterisation variables best characterised the different groups (translated in the position of sites along the PCA axes). These were performed based on abiotic characterisation data of the 55 sampling sites (Table 2.1). The variables with non-normal distribution were transformed to achieve normality and comparable measurement scales (normalising variables) (Table 2.1). This normalisation consists in subtracting to each entry the mean for the variable and then dividing by the standard deviation (Primer 6 & PERMANOVA+; PRIMER-E Ltd., Plymouth, UK; Clarke and Gorley, 2006; Anderson et al., 2008). Mean values and standard deviations were calculated for the variables explaining most of the variability in PC1 and PC2.

##### 2.2.4.2 Establishment of the LDC

For the establishment of the LDC we applied a three-step approach based on Feio et al. (2014) to the pool of 163 samples.

*Step 1.* Samples with class 1 and 2 (minimal and small disturbance), according to Pont et al. (2006), in all abiotic categorical pressure variables (for more detail see Table 2.2) were kept and the remaining were eliminated. The selection based only on class 1 in all the categorical variables was not considered due to the absence of samples in such conditions.

*Step 2.* Based on Step 1 selection, the distribution of numerical variables was characterised using boxplots. The minimum and maximum thresholds for the LDC, hereinafter called least disturbed abiotic condition (LDAC), were established based on all the samples from Step 1, after excluding outliers and extremes. We considered as outliers and extremes the values  $> 75^{\text{th}} \text{ percentile} + k (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$  or  $< 25^{\text{th}} \text{ percentile} - k (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$ , being  $k=1.5$  to outliers and  $k=3$  for extremes.

These thresholds characterise the LDC for littoral streams and can be used as thresholds to select least disturbed sites.

*Step 3.* All samples were screened against the thresholds defined in Step 2, and those that were within the limits were considered as representatives of the LDAC for Littoral type. All samples of the same site should pass this step, otherwise they were all eliminated.

#### 2.2.4.3 *Least Disturbed Biological Condition (LDBC) based on diatom assemblages*

The spring samples obtained from Step 3 were used to analyse the biological consistency of the L type and the possible existence of sub-groups regarding diatom assemblages. For this purpose, a hierarchical classification (Bray-Curtis dissimilarity measure, flexible UPGMA; square root transformation, relative abundance  $\geq 1\%$ ) was performed (PRIMER 6 & PERMANOVA+). Additionally, a SIMPER analysis was performed to see which taxa contributed the most to the Bray-Curtis dissimilarity (up to 80% cumulative contribution) between the resulting groups. As some of the sites had more than one spring sample, the number of valves in those samples was previously averaged.

Benthic diatoms use different strategies to resist the environmental pressures. For instance, variations in life forms are strongly influenced by current velocity (Rimet and Bouchez, 2012). The use of this trait can provide new insights on the characterisation of river types. Thus, in addition to the taxonomic composition, we used a functional indicator of diatom assemblages, the trait life-form, and respective categories: mobile, adnate, pedunculate (pad and stalked), non-colonial, and colonial, according to Rimet and Bouchez (2012). Therefore, each species was assigned to all the trait categories that it could display, according to the bibliography. For each sample, the relative abundance of all the species presenting a given trait category was calculated. To obtain the trait abundance of each trait category in each sub-group, the average of all the samples from the same group was also calculated. Significant differences between the trait categories of the groups were checked with an ANOSIM (Analysis of similarity, PRIMER 6 & PERMANOVA+).

Table 2.2 - Abiotic pressure variables (water chemical and physical conditions and land use) used for the selection of the least disturbed condition.

| Abiotic pressure variables  |
|---|
| Categorical variables   |
| Connectivity, degree of alterations in longitudinal connectivity at the stream reach (minimal disturbances: no alterations related to the presence of artificial barriers (dams, weirs, or other); small disturbances: slight alterations (presence of a minor dam or weir) and allowing for water to flow over it) (category) <sup>a</sup>         |
| Riparian vegetation, changes in the integrity of the riparian corridor due to cut of vegetation or presence of alien species (minimal disturbances: no alteration; small disturbances: slight alteration with good riparian forest cover with only a few and isolated alien species) (category) <sup>a</sup>  |
| Sediment load, changes in the natural concentration of sediments transported by the stream (minimal disturbances: gravel, pebble, and boulder particles have <5% of their surface covered by fine sediment; small disturbances: gravel, pebble, and boulder particles have 5–25% of their surface covered by fine sediment) (category) <sup>a</sup> |
| Hydrological regime, deviation from the natural discharge (minimal disturbances: >90% of the mean annual discharge and >90% of the natural duration of flood periods; small disturbances: >30% of the mean annual discharge and >75% of the natural duration of flood periods) (category) <sup>a</sup>  |
| Morphological condition, changes in the channel morphology and loss of natural habitats (minimal disturbances: no alterations or negligible; small disturbances: all habitats are present) (category) <sup>a</sup>  |
| Acidification and toxicity, changes in the natural acidification and oxygenation level of the water (minimal disturbances: no alterations; small disturbances: occasional deviation from the natural condition) (category) <sup>a</sup>   |
| Organic contamination and nutrient enrichment, evidences of organic substances and nutrients in the water (minimal disturbances: no evidence of eutrophication or organic load; small disturbances: small evidences of eutrophication or organic load) (category) <sup>a</sup>  |
| Numerical variables   |
| pH  |
| % Dissolved oxygen (DO)   |
| Ammonium (N; mg·L <sup>-1</sup> )   |
| Nitrates (NO <sub>3</sub> <sup>-</sup> ; mg·L <sup>-1</sup> )   |
| Total phosphorus (P; mg·L <sup>-1</sup> )   |
| Phosphates (PO <sub>4</sub> <sup>3-</sup> ; mg·L <sup>-1</sup> )  |
| Sulphates (SO <sub>4</sub> <sup>2-</sup> ; mg·L <sup>-1</sup> )   |
| Chemical oxygen demand (COD; mg·L <sup>-1</sup> )   |
| Conductivity (μS·cm <sup>-1</sup> )   |
| % Artificial areas, in the catchment (urban areas continuous and discontinuous, industrial and commercial zones, communication infrastructures and networks, mines, etc.) <sup>b</sup>  |

% Intensive agriculture, in the catchment (corresponding to a high potential impact from agricultural activities: arable land (including irrigated land), permanent crops (with associated annual crops), vineyards, orchards, olive groves, complex cultivation patterns)<sup>b</sup>

% Extensive agriculture, in the catchment (lower potential impact from agricultural activities: pastures, land principally occupied by agriculture, with significant areas of natural vegetation, agro-forestry areas)<sup>b</sup>

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<sup>a</sup> Variables on a five-classes scale: 1-minimally disturbed from the natural state to 5-completely disturbed. Adapted from Pont et al. (2006); <sup>b</sup> Based on Atlas do Ambiente (Agência Portuguesa do Ambiente, 2007).

#### 2.2.4.4 *Establishing new benchmarks for ecological assessment*

Diatom taxa abundance was used to calculate the IPS of the least disturbed spring samples with the OMINIDIA software (Lecointe et al., 1993). As there were some sites with more than one sample, the respective IPS values were averaged for the remaining data analysis. For each sub-group (obtained from the diatom assemblages of the LDC sites), the median IPS reference value was calculated. Only spring samples were used because only this season is used in the Portuguese official monitoring programme; consequently, only spring reference values are available. To obtain the ecological quality ratios (EQRs) for each site, the observed IPS value was divided by the reference IPS value (*i.e.*, the median of each sub-group). The EQR gives the deviation of the observed biological parameter in a given water body (type) to the values of that parameter in reference conditions applicable to that water body. The EQRs were calculated for reference and disturbed sites (all spring samples) and compared with the values obtained from the use of the original median (17.4, as used presently in official monitoring programme) (INAG, 2009). The attribution of a sub-group to the disturbed sites was done according to the groups established in the abiotic hierarchical classification.

## 2.3 **Results**

### 2.3.1 *Typology*

Cluster analysis (Figure 2.2) showed that, based on abiotic data, the L type could be subdivided into three sub-groups. A small group of sites (Group I) clearly segregated from the remaining, and two other bigger groups (Group II and III). Only one site (V80) was not included in any group because it was very different from the remaining (outlier) (Figure 2.2).

The two streams from the Tagus catchment (T90 and T91) that presently are not included in the L type, as we expected, were similar to other streams that are considered L type (Figure 2.2).

The first two axes explained 48.2% of sites' abiotic variability (PC1=30.1%, PC2=18.1%). The variables with the highest contribution to PC1 were runoff, precipitation, slope, and channel substrate (sand and cobble) (eigenvectors between 0.314 and 0.424), while drainage area (eigenvectors = 0.483) and lithology (eigenvectors = 0.380) contributed more to PC2 (Figure 2.3). The substrate sand contributed almost equally to PC1 and PC2 (eigenvectors of 0.314 and 0.344, respectively).

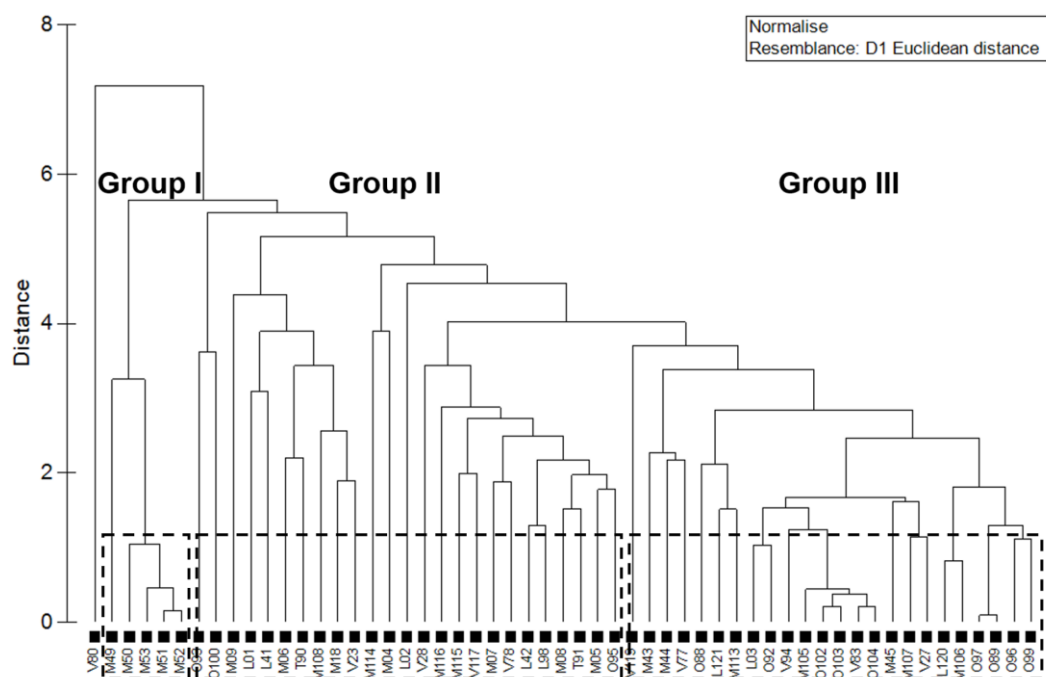


Figure 2.2 - Hierarchical classification of the sites based on the abiotic characterisation variables. Three groups were formed.

The PCA confirmed the segregation of the abiotic groups. Group I (Figure 2.3) was clearly segregated from the remaining over PC2. This group includes only sites from the Mondego River with a large drainage area (ca. 5000 km<sup>2</sup>) (Table 2.3). The other two groups appeared segregated towards the negative or the positive sides of the PC1 axis (Figure 2.3). The streams of these two groups differed in slope, altitude, mean annual runoff and precipitation values, and stream substrate (namely, cobble and sand) (Figure 2.3). In Group II, we found streams in a higher altitude with higher average values of precipitation and runoff than in Group III (Table 2.3) and streambed mainly of cobbles and gravel/pebble, while in Group III the streams have predominantly sandy bottoms (Table 2.3). Over the second axis there was also a segregation of the samples by catchment (*i.e.*, latitudinal gradient). In the positive side of this axis, we found samples from Ribeiras do Oeste and Lis catchment (the most southern sites); in the negative side, we found samples from Mondego and Vouga catchments (the most northern sites) (Figure 2.3).



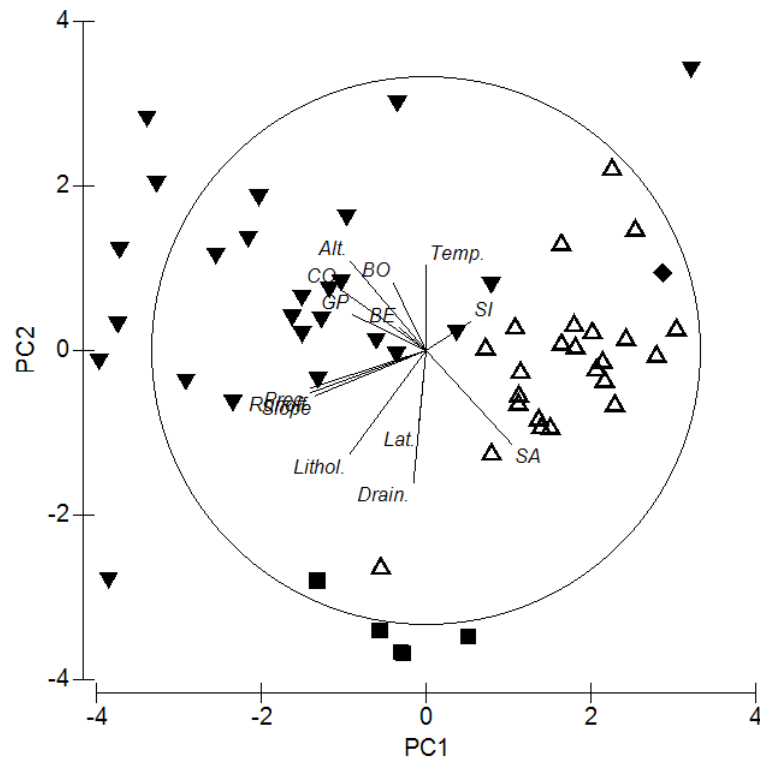


Figure 2.3 - Principal Component Analysis of the study sites plotted in relation to the abiotic characterisation variables. The groups obtained in the hierarchical classification of the sites (Figure 2.2) are identified: square-Group I, black triangle-Group II, white triangle-Group III, diamond-V80.

### 2.3.2 Establishment of the LDC

Only 30 samples (from 13 sites) were selected after Step 1, corresponding to 18.4% of the total. The pressure categorical variables that contributed more to sample elimination were: riparian vegetation (58.9%), morphological condition (37.4%), and sediment load (33.1%). Contrarily, toxic acidification (3.1%) and hydrological regime (12.3%) were the categories with less contribution for sample elimination.

In Step 2, the distribution of values for the numerical variables of the samples selected in Step 1 revealed several outliers and extremes, especially for ammonium, nitrates, phosphates, and sulphates (*e.g.*, Figure 2.4). This means that these were sites in good hydromorphological condition but with an excess of nutrients. The samples corresponding to such values were also eliminated, except if they were outliers or extremes for just one of the pressure variables. The LDAC of littoral streams is thus represented by the thresholds determined with the remaining samples and presented in Table 2.4.

Table 2.3 - Mean (SD, standard deviation) of the abiotic characterisation variables for each abiotic group.

|  | Group I            | Group II   | Group III  |
|--|--------------------|--|--|
| Latitude   | 40.1742 to 40.2204 | 38.7048 to 40.6355                                       | 39.1350 to 40.7537                                       |
|  | Mean               | Mean   | Mean   |
| Altitude (a.s.l; m)                              | 9.52±10.46         | 84.89±56.93  | 31.19±21.09  |
| Runoff, mean annual (mm)                         | 466.72±70.12       | 537.71±210.31  | 324.05±101.19  |
| Temperature, mean annual air temperature (°C)    | 15.00±0.00         | 15.50±0.97   | 15.33±0.48   |
| Precipitation, mean annual (mm)                  | 1160.00±89.44      | 1256.00±267.83   | 983.33±130.77  |
| Drainage area (km <sup>2</sup> )                 | 4995.20±543.74     | 97.13±183.43   | 70.09±60.60  |
| Slope of the drainage area (%)                   | 15.26±0.50         | 12.38±4.75   | 6.08±3.30  |
| Lithology <sup>a</sup>                           | 17-100.00%         | 1-8.00%, 3-24.00%, 5-8.00%, 6-28.00%, 7-4.00%, 11-28.00% | 1-50.00%, 3-8.33%, 5-16.67%, 6-16.67%, 7-4.17%, 11-4.17% |
| Dominant substrate (in ≈ 500 m from the site %): |                    |  |  |
| Bedrock (BE)                                     | 0.00               | 2.34±7.01  | 0.00   |
| Boulder (BO)                                     | 0.00               | 7.47±17.06   | 0.00   |
| Cobble (CO)                                      | 0.00               | 25.81±28.57  | 0.00   |
| Gravel/Pebble (GP)                               | 16.00±35.78        | 51.88±30.20  | 3.48±8.47  |
| Sand (SA)  | 84.00±35.78        | 11.13±15.86  | 81.04±33.33  |
| Silt (SI)  | 0.00               | 0.57±2.86  | 2.56±7.12  |

<sup>a</sup> 1-Sands, sand dunes, gravels, rolled stones, little consolidated sandstones, clays; 3-Limestones, dolomitic limestones, marly limestones, marls; 5-Conglomerates, sandstones, limestones, dolomitic limestones, marly limestones, marls, carbonaceous schist's, schist's; 6-Sandstones, more or less marly limestones, sands, gravels, clays conglomerates, limestones, dolomitic limestones, marls; 7-Red stoneware, conglomerates, marls, limestones generally dolomitic; 11-Schists and related rocks; 17-Granites and related rocks.

Table 2.4 - Thresholds for the Least Disturbed Abiotic Condition (LDAC) for water chemical and physical conditions and land use.

| Abiotic pressure variables                                       | Threshold    |
|--|--------------|
| pH   | 6.7 – 8.1    |
| % Dissolved oxygen (DO)  | 56.4 – 116.5 |
| Ammonium (N; mg·L <sup>-1</sup> )                                | ≤ 0.40       |
| Nitrates (NO <sub>3</sub> <sup>-</sup> ; mg·L <sup>-1</sup> )    | ≤ 6.14       |
| Total-P (P; mg·L <sup>-1</sup> )                                 | ≤ 1.18       |
| Phosphates (PO <sub>4</sub> <sup>3-</sup> ; mg·L <sup>-1</sup> ) | ≤ 1.14       |
| Sulphates (SO <sub>4</sub> <sup>2-</sup> ; mg·L <sup>-1</sup> )  | ≤ 10.9       |
| COD (mg·L <sup>-1</sup> )  | ≤ 33.90      |
| Conductivity (μS·cm <sup>-1</sup> )                              | ≤ 482.0      |
| Artificial areas (in the catchment; %) <sup>a</sup>              | ≤ 8          |
| Intensive agriculture (in the catchment; %) <sup>a</sup>         | ≤ 51         |
| Extensive agriculture (in the catchment; %) <sup>a</sup>         | ≤ 17         |

<sup>a</sup> See description for these variables on Table 2.2

After the three steps, 24 samples from 11 sites were considered in the LDC. From those 24 samples, 54.2% were from spring, 25.0% from winter, and 20.8% from autumn. Twelve LDC spring samples from nine sites were retained for biological analyses. All these samples corresponded to Groups II and III of the abiotic classification (above). All samples from Group I, the large river, failed Step 1 due to hydrological (connectivity, hydrological regime) or morphological alterations, which are caused by the presence of large dams upstream and channelisation of some parts of the river.

### 2.3.3 LDBC based on diatom assemblages

The cluster analysis of the spring diatom least disturbed assemblages (Figure 2.5) identified two distinct groups (Group A and B). Among the species that most contributed to the 81.2% average dissimilarity between the two groups (SIMPER) were *Achnantheidium minutissimum* (Kützing) Czarnecki, *Karayevia oblongella* (Østrup) Aboal, *Hippodonta pseudacceptata* (H. Kobayasi) Lange-Bertalot, Metzeltin & A. Witkowski, and *Nitzschia dissipata* var. *media* (Hantzsch) Grunow (Table 2.5).

There was a total agreement between the two groups formed by the diatom assemblages of the LDC sites (Figure 2.5) and two of the abiotic groups (Figure 2.2). The LDC sites of Group A were all inserted in the abiotic Group II (cluster), while the LDC sites in the Group B were all inserted in the abiotic Group III (cluster). Group A includes small stony streams with higher precipitation, slope, and runoff. The lithology of the area is quite varied, and includes six different categories (category 1, 3, 5, 6, 7, and 11, see Table 2.3 for category definition).

Species such as *A. minutissimum*, *Eolimna minima* (Grunow) Lange-Bertalot & W. Schiller, *Cocconeis euglypta* Ehrenberg, *Amphora pediculus* (Kützing) Grunow ex A. Schmidt, and *Gomphonema pumilum* (Grunow) E. Reichardt & Lange-Bertalot were more abundant in this group. In Group B we found sandy streams, low precipitation and runoff values, and lithology categories mainly siliceous (Table 2.3). Group B was characterised by higher abundances of species such as *K. oblongella*, *H. pseudacceptata*, *N. dissipata* var. *media*, *Nitzschia palea* (Kützing) W. Smith, and *Hippodonta capitata* (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski (Table 2.5). In general, both groups were composed mostly by neutrophil to alkaliphilic species.

The biotic groups also presented differences regarding the trait life-form. Group A presented higher average abundances of mobile (92.7%) and stalked (64.4%) species. In Group B the trait categories with higher average abundances were mobile (80.2%) and pad (26.4%). Both groups presented high values of non-colonial species (90.7% Group A and 78.5% Group B). The two groups only presented statistically significant differences in the trait category stalked, with higher average abundance in Group A than in Group B (Global  $R=0.76$ ,  $P<0.02$ ).

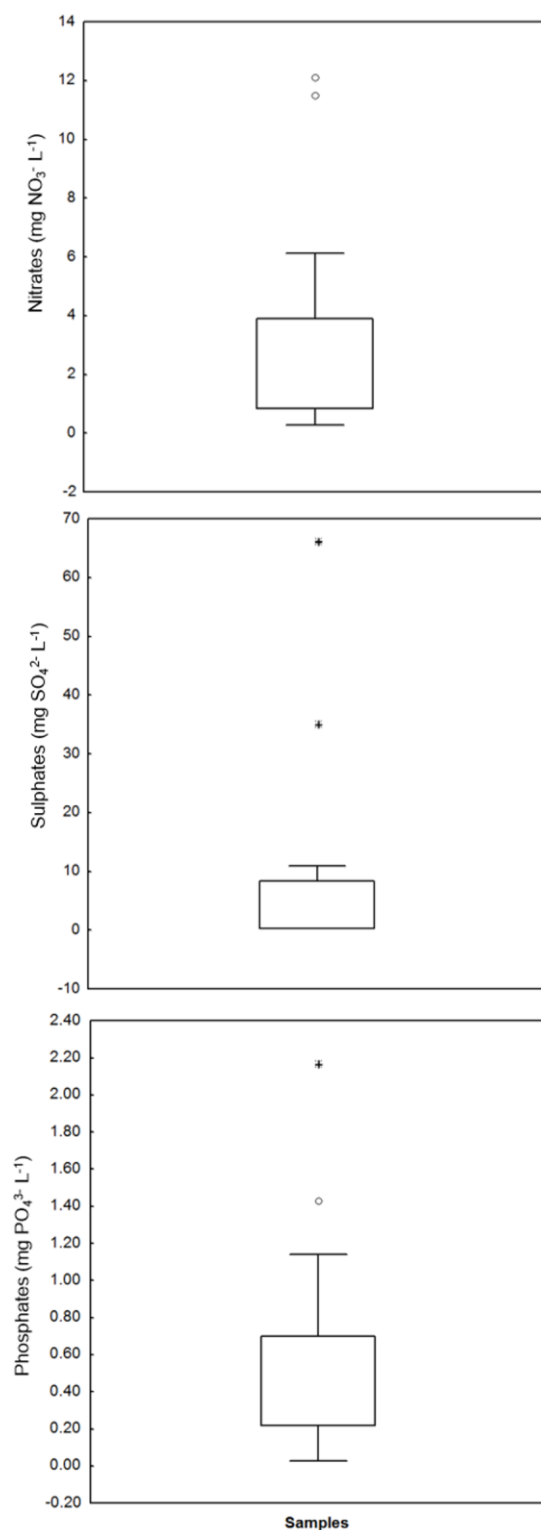


Figure 2.4 - Boxplots showing the distribution of selected numerical pressure variables for the least disturbed samples selected after Step 1 (only class 1 - minimal or 2 - minor disturbance). Outliers/Extremes: see section 2.2.4.2.

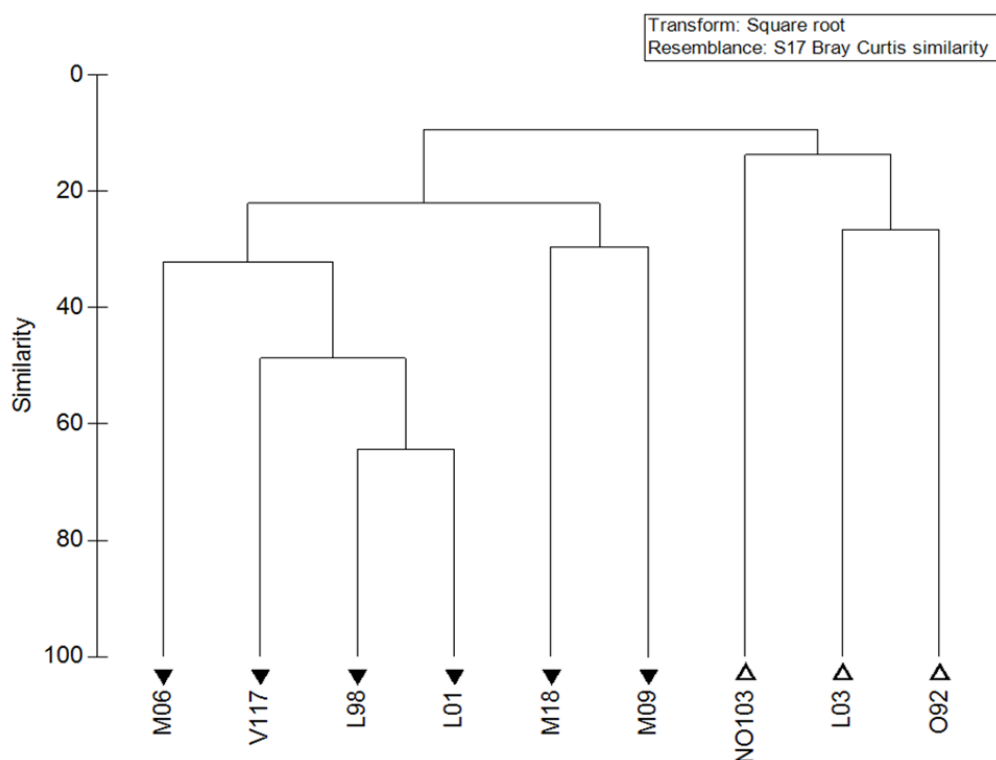


Figure 2.5 - Hierarchical classification of the diatom assemblages based on the least disturbed sites. Two groups were formed: black triangle-Group A, white triangle-Group B. The sites (triangles) are identified with the first letter of the catchment to which they belong - Vouga (V), Mondego (M), Lis (L), Ribeiras do Oeste (O), and Ribeiras do Noroeste (NO) - and with a number that is the site number.

Table 2.5 - Most representative diatom species (contributing to up to 81% of the Bray-Curtis similarity) the two groups (A and B) obtained by SIMPER analysis with respective average abundance. In bold are the species that were more abundant to each group.

|   |                       |                              | Group A     | Group B     | % of       |
|---|-----------------------|------------------------------|-------------|-------------|------------|
|   |                       |                              | Average     | Average     | cumulative |
|   |                       |                              | abundance   | abundance   | abundance  |
| <i>Achnantheidium</i>                   | <i>minutissimum</i>   | (Kützing)                    | <b>5.61</b> | 0.90        | 10.64      |
| <i>Czarnecki</i>                        |                       |                              |             |             |            |
| <i>Karayevia</i>                        | <i>oblongella</i>     | (Østrup) Aboal               | 0.67        | <b>3.51</b> | 17.35      |
| <i>Hippodonta</i>                       | <i>pseudacceptata</i> | (H. Kobayasi)                | 0.00        | <b>1.98</b> | 21.73      |
| Lange-Bertalot, Metzeltin & A. Witkowsk |                       |                              |             |             |            |
| <i>Nitzschia</i>                        | <i>dissipata</i>      | var. <i>media</i> (Hantzsch) | 0.00        | <b>1.88</b> | 25.71      |
| Grunow                                  |                       |                              |             |             |            |
| <i>Eolimna</i>                          | <i>minima</i>         | (Grunow) Lange-Bertalot &    | <b>2.23</b> | 0.46        | 29.56      |
| W. Schiller                             |                       |                              |             |             |            |
| <i>Nitzschia</i>                        | <i>palea</i>          | (Kützing) W. Smith           | 0.00        | <b>1.61</b> | 32.81      |
| <i>Cocconeis</i>                        | <i>euglypta</i>       | Ehrenberg                    | <b>1.52</b> | 0.00        | 35.95      |
| <i>Hippodonta</i>                       | <i>capitata</i>       | (Ehrenberg) Lange-           | 0.00        | <b>1.40</b> | 38.77      |
| Bertalot, Metzeltin & Witkowski         |                       |                              |             |             |            |
| <i>Gomphonema</i>                       | <i>parvulum</i>       | (Kützing) Kützing            | 0.38        | <b>1.51</b> | 41.60      |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|   |             |             |       |
|---|-------------|-------------|-------|
| <i>Geissleria decussis</i> (Østrup) Lange-Bertalot & Metzeltin                  | 0.00        | <b>1.14</b> | 44.13 |
| <i>Amphora pediculus</i> (Kützing) Grunow ex A. Schmidt                         | <b>1.18</b> | 0.00        | 46.60 |
| <i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot                | <b>1.08</b> | 0.72        | 49.06 |
| <i>Fragilaria gracilis</i> Østrup   | 0.19        | <b>1.08</b> | 51.48 |
| <i>Navicula gregaria</i> Donkin   | 0.49        | <b>1.21</b> | 53.82 |
| <i>Achnantheidium rivulare</i> Potapova & Ponader                               | <b>1.23</b> | 0.00        | 56.15 |
| <i>Cocconeis placentula</i> var. <i>lineata</i> (Ehrenberg) Van Heurck          | <b>1.21</b> | 0.00        | 58.45 |
| <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer                        | <b>1.04</b> | 0.00        | 60.58 |
| <i>Achnantheidium subatomoides</i> (Hustedt) O. Monnier, Lange-Bertalot & Ector | 0.00        | <b>0.99</b> | 62.07 |
| <i>Planothidium frequentissimum</i> (Lange-Bertalot) Lange-Bertalot             | 0.20        | <b>1.07</b> | 64.75 |
| <i>Placoneis clementis</i> (Grunow) E. J. Cox                                   | 0.00        | <b>1.03</b> | 66.79 |
| <i>Sellaphora seminulum</i> (Grunow) D. G. Mann                                 | 0.00        | <b>1.04</b> | 68.71 |
| <i>Staurosirella pinnata</i> (Ehrenberg) D. M. Williams & Round                 | 0.00        | <b>0.88</b> | 70.59 |
| <i>Planothidium dauai</i> (Foged) Lange-Bertalot                                | 0.00        | <b>0.87</b> | 72.45 |
| <i>Nitzschia</i> sp.  | 0.00        | <b>0.76</b> | 74.13 |
| <i>Achnantheidium atomoides</i> Monnier. Lange-Bertalot & Ector                 | <b>0.88</b> | 0.00        | 75.81 |
| <i>Gomphonema minutum</i> (C. Agardh) C. Agardh                                 | <b>0.73</b> | 0.00        | 77.24 |
| <i>Hippodonta lesmonensis</i> (Hustedt) Lange-Bertalot, Metzeltin & Witkowski   | 0.00        | <b>0.61</b> | 78.59 |
| <i>Parlibellus protracta</i> (Grunow) Witkowski, Lange-Bertalot & Metzeltin     | 0.00        | <b>0.59</b> | 79.90 |
| <i>Encyonema minutum</i> (Hilse) D. G. Mann                                     | <b>0.63</b> | 0.00        | 81.21 |

#### 2.3.4 Establishing new benchmarks for ecological assessment

For LDC samples, the IPS values ranged between 11.3 and 19.9, with Group A presenting higher IPS values (median 18.2) than Group B (median of 13.7). While the median values of Group A were close to the one presently used in official monitoring (17.4), the median of Group B was much lower. The application of these medians resulted in a slight decrease of EQR values of LDC samples in Group A (that ranged 0.69 – 1.09) and a considerable increase of EQR in Group B (that ranged 0.82 – 1.16) compared to the EQR values resultant from the application of the official median for L type (Figure 2.6). For disturbed sites, the application of

the group medians also resulted in comparable increases/decreases of the EQR values (Figure 2.6).

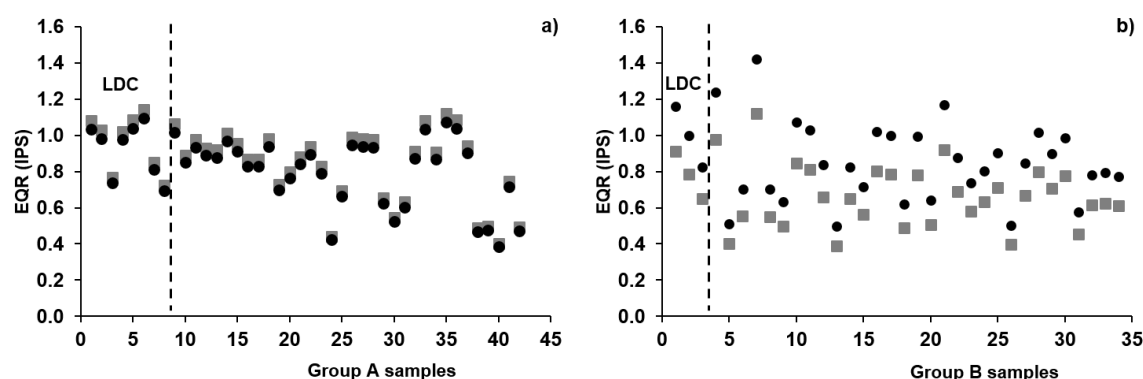


Figure 2.6 - EQR based on the new IPS median reference values (circles) and in original IPS median reference values (squares) used presently in the official monitoring programme of the sampling sites from Group A (a) and B (b). Dashed vertical lines separates samples selected as being in the least disturbed condition (LDC) from the ones considered to be impaired. Spring samples.

## 2.4 Discussion

In this study, we confirmed the environmental variability of streams present in the littoral Portuguese coastal region in terms of environmental characteristics. In a small geographic area, we were able to differentiate three sub-groups of streams with differences in mean annual precipitation, mean annual runoff, slope, altitude, size of the drainage area, lithology, and stream substrate. Since these variables are used in the definition of the WFD typology (European Commission, 2000), those groups could be considered different types. One of the groups formed is composed exclusively of sites from Mondego River. The environmental variables that contributed more to the segregation of this group (lithology and size of the drainage area) are geochemical and hydrological factors known to influence the diatom assemblages (Potapova and Charles, 2002; Lorenz et al., 2004; Feio et al., 2009; Almeida and Feio, 2012). Therefore, despite the lack of LDAC and consequently LDBC sites for this group, we predict that the diatom assemblages would also be different from the remaining groups. We also verified that streams which presently are not classified as L type (*e.g.*, streams from the Tagus catchment) should be included in this type, as they weren't segregated from the remaining in terms of environmental characteristics.

Studies such as that by Pan et al. (1999) showed that diatom assemblages are strongly controlled by geomorphic and disturbance factors (*e.g.*, land cover/use in watersheds and riparian condition). Here, the main alterations in the littoral streams were related to the riparian vegetation, morphological condition, and sediment load, which was expected, as the



main pressures in the region are agriculture and deforestation. The degradation of riparian vegetation in the majority of the L type streams can lead, for example, to change in water temperature patterns. The streams are more exposed to sunlight and, therefore, more influenced by atmospheric temperature (Poole and Berman, 2001). In Feio et al. (2014), degradation of the riparian vegetation was also among the main disturbances found in Mediterranean streams. The sediment load is a measure of deviation from the natural condition either in the water column or in the streambed. Among the sources that contribute to stream sedimentation and turbidity are agriculture, forestry, mining, road construction, and urban activities (Henley et al., 2000). The loss of riparian vegetation also facilitates the runoff of sediments into the streams, as these areas are important sediment sinks (Daniels and Gilliam, 1996; Sheridan et al., 1999). Increase in the sediment load and consequent increase in turbidity of streams can cause a reduction in algae growth due to the decrease of light availability for photosynthesis (Henley et al., 2000).

Land use in the catchment can greatly influence the quality of the streams as it may cause changes in nutrient load. However, this influence depends on intrinsic factors of the catchment, such as the transport capacity of the watershed, the influence of riparian buffers, climatic and geomorphological basin features, and the existence of additional pressures (Pardo et al., 2012). The littoral Portuguese region is densely populated but nonetheless, it was possible to establish a threshold of 8% of the catchment covered by artificial areas for the LDC. However, this value is still high in comparison with other thresholds established in other studies (*e.g.*, <0.4% reference and >0.8% rejection threshold in Pardo et al., 2012). Agriculture (intensive and extensive) is quite relevant in the region, which is reflected in the thresholds of 17% (extensive) and 51% (intensive). The threshold found in Feio et al. (2014) for the intensive agriculture in Mediterranean countries was only of 11%, but in Pardo et al. (2012) the rejection threshold (for a reference site) for northern Europe was very close to ours (*i.e.*, 50%). Also, Allan (2004) stated that streams remain in good condition in agricultural catchments as long as the extent of agriculture (including intensive and extensive) doesn't exceed 30% – 50%. Our values of total agriculture (intensive plus extensive) for the least disturbed sites are close to this limit, as they ranged from 5.8 to 53.5%.

Under pristine conditions, streams should have low concentration of nutrients (namely, phosphorous-P, nitrogen-N, and sulphur-S). Therefore, the values obtained here for littoral LDC were high. Several studies have proposed lower maximum values for these compounds (see Feio et al., 2014). The high values of N ( $0.40 \text{ mg}\cdot\text{L}^{-1}$ ) and P ( $1.18 \text{ mg}\cdot\text{L}^{-1}$ ) that we found in the streams were probably related with the high percentages of agriculture (such as rice fields in Mondego or large orchards in the Ribeiras do Oeste) found in the catchments, since

this is one of the major contributors of non-point source pollution (Bernot et al., 2006). In spite of these broad thresholds, by the end of the three-step approach used for LDC selection, the approach was in fact very strict, as only 14.7% of the samples were accepted. This high elimination rate of samples revealed that this region is under high anthropogenic pressure and that the approach we used for determining the LDC, through the increase in the sampling site density and the refinement of the benchmark criteria, may not be sufficient to define the reference condition. Other methods have been proposed to infer the LDC such as the use of a stressor gradient approach, which uses the relationship between the stressor gradient and biological indicators to predict LDC for the biological indicators (Angradi et al., 2009) or the quite similar alternative benchmarking (Birk et al., 2012) based on the notion of aquatic communities with similar low levels of impairment. Yet, none of these approaches assure that the existent communities are not already significantly altered, especially when the best sites are already far from the pristine conditions.

The large river sites included in our study were all eliminated after failing Step 1, due to hydromorphological modifications, specifically in the hydrological regime, connectivity, and/or morphological alterations. These are two common problems of large rivers (Seegert, 2000; Nilsson et al., 2005), which often require the establishment of alternative benchmarks (Birk et al., 2012), or ultimately their inclusion in the category of highly modified water bodies when their recovery to the Good status is not possible (European Commission, 2000). However, concerning the large river in this study (Mondego River), care must be taken as the number of samples was very small and the screening abiotic metrics may have been unsuitable for large rivers (Angradi et al., 2009). Criteria based on screening abiotic metrics, such as riparian vegetation and sediment load, are likely to be less reliable for screening large rivers compared to smaller rivers (Angradi et al., 2009).

We found two distinct diatom assemblages (two sub-groups) for the least disturbed sites. These two groups were in accordance with two of the abiotic groups formed. The abiotic characterisation variables relevant for the difference between Groups II and III were mean annual runoff, slope, mean annual precipitation, substrate (stone vs. sand), and lithology. These variables are recognised to be important for determining diatom species composition, and therefore the biological groups were in total agreement with the natural division of the assemblages (Groups A and B) (Potapova, 1996; Potapova and Charles 2002; Almeida and Feio, 2012). Most of these variables reflected a longitudinal distribution of the streams: sites in Group A were found in more inland streams while the ones from Group B were found in more lowland streams close to the coast. Thus, despite the different approaches (abiotic and biotic), we verified a complete agreement between these two classifications. This agreement was also verified in some studies, even with other BQEs (Lorenz et al., 2004; Rawer-Jost et al., 2004; Verdonschot and Nijboer, 2004; Sandin and

Verdonschot, 2006) but not always (Sanchez-Montoya et al., 2007; Feio et al., 2014). In the study conducted by Feio et al. (2014), for example, the streams of one of the abiotic groups formed (temporary streams) were spread into at least three biological groups for all elements studied (diatoms, macrophytes, and macroinvertebrates). However, this lack of agreement was probably due to the broad scale of the variables for the definition of the types. In Sanchez-Montoya et al. (2007), the largest difference found between the abiotic classification and the macroinvertebrates communities was also in the ecotype comprising the temporary streams, which was probably due to the large variability in salinity and hydrology found in those streams.

Given the abiotic differences of the two groups of streams in terms of slope, runoff, and substrate, we expected differences in diatoms' trait life form. In Group A the species should have the capacity to attach to the substrate in order to resist the higher current velocities associated with higher slopes and runoff values; in Group B the species should be able to overcome the abrasion of moving grains that can detach larger taxa (Townsend and Gell, 2005) or be able to move (Soininen and Eloranta, 2004) in order to avoid the entrapment by the sand grains. Indeed, Group A had higher abundance of stalked species than Group B. This difference confirms that the species from Group A are exposed to higher velocities, and that the streambed substrate is more stable than the streambed substrate found in streams of Group B. However, contrary to what we were expecting, the abundance of mobile species was similar in both groups, revealing the importance of this trait category in both groups of streams.

As the sites used to calculate the median IPS in Groups A and B were under similar anthropogenic disturbances (LDC), it was to be expected that they presented similar IPS values. However, Group A presented a higher median IPS value than Group B. In particular, the high abundance of a sensitive species, *Achnanthes minutissimum*, in Group A may be the cause of such differences. In some of the samples from Group A, *A. minutissimum* reached an abundance of approximately 70%. However, in Group B this particular species only reached a maximum abundance of 7.3%. As the number of counted valves was similar in all the samples, the diatom assemblages from Group B were generally more diverse and showed higher evenness. The sensitive and less sensitive species contributed equally to the IPS calculation in Group B, while in Group A the IPS calculation was highly influenced by *A. minutissimum* abundances. Therefore, the use of a single IPS benchmark value for both groups (Group A and B) to calculate EQR values would lead to erroneous classifications, particularly in the case of Group B. The division by a higher median than by the appropriate one (as done presently) results in lower ratios, and therefore potential erroneous attribution of lower quality to sites of good quality. These Type II errors (false negatives) can have

important consequences, as they imply a higher investment than the one needed for streams' restoration (Johnson et al., 2006). The two groups are indeed abiotically different, suggesting the existence of sub-groups within the L type; however, due to the small number of LDC sites, particularly for Group B, we must be cautious in our interpretations.

## **2.5 Conclusions**

The contribution of the stream substrate for the definition of the groups suggests that this variable should have been included in the typological variables during the WFD implementation to allow the construction of relevant types for diatoms. In fact, Lorenz et al. (2004) included the predominant type of substrate in the determination of stream typologies in Germany. This study also revealed that there is a wider abiotic variability and corresponding differences in diatom assemblages within the L type, as it was defined. However, we would still need a higher number of sites in LDC to achieve an adequate redefinition of reference conditions for all potential sub-groups, which is not feasible, as we already performed an intensive search. We must also take into consideration that type redefinition must consider other BQEs. Therefore, even if the LDC approach can help improve the present bioassessment methods, other alternatives that do not require the use of reference sites, such as modelling the reference conditions, are still needed, as the present LDC is far from the desired reference condition (Stoddard et al., 2006; Angradi et al., 2009).

### **Abbreviations**

|      |   |
|------|---|
| BQE  | Biological Quality Elements                   |
| EQR  | Ecological Quality Ratios                     |
| IPS  | <i>Indice de Polluosensibilité Spécifique</i> |
| LDAC | Least Disturbed Abiotic Condition             |
| LDBC | Least Disturbed Biotic Condition              |
| LDC  | Least Disturbed Condition                     |
| PCA  | Principal Components Analysis                 |
| WFD  | Water Framework Directive                     |

### **Acknowledgements**

This study was possible due to the financial support of the Foundation for Science and Technology (Portugal) through the projects AQUAWEB (PTDC/AAC-AMB/105297/2008),

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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FRAMEFFECTIVE (PTDC/AAC-AMB/105411/2008), and programmes POPH and QREN (FSE and national funds of MEC) and the PhD scholarship SFRH/BD/68973/2010 of the first author. We would like to thank the Portuguese Water Institute (INAG I.P., now Agência Portuguesa do Ambiente) for making data available, to the logistic support at IMAR-CMA, Department of Life Sciences, University of Coimbra, and at GeoBioTec Research Centre and Biology Department of Aveiro, University of Aveiro.

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## Chapter 3 - Predicting reference conditions for river bioassessment by incorporating boosted trees in the environmental filters method

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Published in Ecological Indicators 2016, 69, 239-251.

### Abstract

Contemporary bioassessment methods for water bodies require the description of ‘reference conditions’ representing an absence or only ‘very minor’ presence of human impacts on hydromorphological, physical and chemical properties. However, minimally disturbed reference sites are lacking in many European regions and other parts of the world because of pervasive anthropogenic influences. Here we describe the use of environmental filters modelling, incorporating boosted trees (BT), to derive reference data for abiotic variables and biological communities (diatoms and macroinvertebrates) for rivers in a highly disturbed region (Portuguese central-western lowland area), where minimally disturbed reference sites are non-existent. We also revise quality class boundaries for diatom and macroinvertebrate bioassessment in this region, and develop a new multimetric diatoms index (MDI). The new index includes not only the ‘*Indice de Polluosensibilité Spécifique*’ (IPS) based on species’ sensitivity to organic pollution and nutrients, but also the numbers of total and sensitive taxa. Our approach predicted significantly different communities under reference conditions from those observed, with a higher median reference number of taxa *per* site than the observed number (69 against 27 for diatoms; 53 against 22 for macroinvertebrates). In addition, the predicted communities for both biological groups were more similar among sites than the observed communities. Adjustment of index calculation

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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and quality class boundaries to incorporate the new reference data resulted in more stringent site assessments that were better correlated with human pressures than assessments with previous methods. This study brings new insight to solve the problem of an absence of minimally disturbed reference sites.

### **Keywords**

Freshwater, macroinvertebrates, diatoms, reference condition, multimetric diatom index

### 3.1 Introduction

Running waters provide a multitude of benefits for humans, such as sources of water, means of power generation and waste disposal, routes for navigation and places for leisure activities (Malmqvist and Rundle, 2002). With intense human pressure on rivers and increasing demand for water over the past century (Gleick, 2000), it is not surprising that nowadays few streams or rivers remain unaltered (Frissel and Bayles, 1996; Malmqvist and Rundle, 2002; Wiens, 2002; Naiman and Dudgeon, 2011). In fact, some of these ecosystems have been so starkly and extensively modified that their return to a natural state is almost impossible (Frissel and Bayles, 1996).

In the European Union (EU), with the implementation of the Water Framework Directive (WFD: European Commission, 2000), water management accounts for the water needs of both humans and nature. The WFD main goals are to achieve good chemical and ecological status of water bodies and prevent deterioration (Guidance Document No. 10, 2003). In order to pursue these goals, ecological status is assessed by quantifying the deviation in composition and abundance of Biological Quality Elements (BQEs) in a water body from the composition and abundance expected under reference conditions for that type of water body (*e.g.*, mountain or lowland type) (Schaumburg et al., 2004). A representative suite of appropriate reference conditions has to be identified for each type of water body (Schaumburg et al., 2004; Pardo et al., 2012) to permit an interpretation of measured values (*e.g.*, biological indices) that takes account of natural spatial differences in the composition and abundance of BQEs (Hawkins et al., 2010).

Under the WFD, the reference point is ‘undisturbed’ conditions with no or only ‘very minor’ human impacts on hydromorphological, physical and chemical elements (Guidance Document No. 10, 2003). However, reference water bodies with only minor impacts are absent from many parts of Europe (and other regions of the world) because of historical anthropogenic alteration of landscapes and high population densities (Nijboer et al., 2004; Kelly et al., 2012; Feio et al., 2014a). Recognizing this limitation, the WFD allows the reference condition to be either spatially based (via reference sites), based on modelling (predictive models or hindcasting methods), or derived from a combination of these methods and even based in paleolimnological data (European Commission, 2000).

Several alternative ways to establish useful reference conditions for the ecological assessment of streams have been proposed and discussed in recent years (*e.g.*, Reynoldson et al., 1997; Stoddard et al., 2006; Tison, et al., 2007; Hawkins et al., 2010; Birk et al., 2012; Pardo et al., 2012; Feio et al., 2014a). However, recent evaluations of ecological status have compared assemblage composition and structure between assessment sites and ‘least

disturbed' reference sites (Hawkins et al., 2010). Such assessments do not fully express deviations from undisturbed conditions because of the ubiquity of human impacts, many of which are not easily detected and quantified (Chessman and Royal, 2004). To address this problem, Chessman and Royal (2004) proposed an alternative approach to establish a biological reference condition that does not rely on reference sites. This approach is based on the hypothesis that key environmental variables (filters), expressed at multiple scales, determine the composition and structure of local biological communities (Keddy, 1992; Poff, 1997; Quist et al., 2005). The suite of taxa existing at a site under reference conditions is visualised as resulting from a natural, top-down filtering process applied to a regional pool of taxa, whereby only those taxa with appropriate traits are able to pass the natural filters (Chessman and Royal, 2004). Thus, knowledge of the environmental tolerances of the taxa in the regional pool will allow prediction of the suite of taxa that should occur at a test site under reference conditions, provided that reference values of the environmental filters can be estimated for that site (Hawkins et al., 2010).

Diatoms and macroinvertebrates are key components of aquatic ecosystems, belonging to three trophic levels (primary producers, primary and secondary consumers), and are among the obligatory BQEs for rivers in the WFD. However, to our knowledge the environmental filters' approach has not been used to predict the occurrence of diatom species or macroinvertebrates at the genus rank. For the present work, we hypothesised that the environmental filters approach could be used to establish biological reference conditions for Portuguese lowland streams and rivers that better represent communities existing before the effects of human activities, *i.e.*, correspond to a higher level of naturalness, than the presently used Least Disturbed Condition (LDC) based only on reference sites. In addition, we further developed the filters' approach of Chessman and Royal (2004) by using the statistical learning method of boosted trees to derive reference values of abiotic environmental filters, because we believe that the 'best available' values presently used are in some cases far from the reference ones required by the WFD (*i.e.*, pre-human-disturbance conditions). We also derived reference values for abiotic variables from the literature.

Finally, in order to use such predictions of WFD reference communities in biomonitoring of lowland streams, we established new reference values and quality class boundaries for the official Portuguese multimetric index for benthic macroinvertebrates and a new multimetric index for diatoms. The new index includes the present official diatom index used in Portugal, the '*Indice de Polluosensibilité Spécifique*' - IPS (Cemagref, 1982), based on species sensitivity to pollution in general and in addition, the total number of species observed and the number of sensitive species. We suggest that more weight should be given to sensitive species in the assessment of biological quality because an increase in the number of such species is a less

ambiguous indicator of improvement in water quality than changes in relative abundance (Stevenson et al., 2008).

In summary, the main aims of this work were: 1) prediction of WFD biological reference conditions for macroinvertebrates and diatoms for lowland streams of the central-western Portuguese region by extending the environmental filters' approach of Chessman and Royal (2004) with boosted trees; 2) development and testing of a new multimetric diatom index; and 3) establishment of new reference values and class boundaries for bioassessment methods based on diatoms and macroinvertebrates for this region.

## **3.2 Materials and methods**

### **3.2.1 Study area and data source**

The Portuguese central-western region comprises the lowland areas of the catchments of the Vouga (V), Mondego (M) and Tagus (T) rivers, and the entire catchment of the Lis River (L), the Ribeiras do Oeste (O) and the Ribeiras do Noroeste (NO). Mondego River is the largest river entirely within Portuguese territory with a length of 227 km from mouth to source and a catchment area of 6670 km<sup>2</sup> (Feio et al., 2007). Tagus River is an international river with its source in Spain and a catchment area in Portugal of 24850 km<sup>2</sup>. Vouga River, located north of the Mondego River, has a catchment area of 3600 km<sup>2</sup> and measures 136 km from mouth to source (Feio et al., 2007). Lis River has the smallest catchment of 945 km<sup>2</sup> and is 40 km long from mouth to source (Feio et al., 2007). The Ribeiras do Oeste and Ribeiras do Noroeste are small lowland streams (mean 40 m a.s.l.) with a small/medium drainage area (mean 180 km<sup>2</sup>) (INAG, 2008a). The streams and rivers of this region were included in a single official river type during the implementation of the WFD which was the Littoral type (Elias et al., 2015).

In this study, we used a database (AQUAWEB: [www.aquaweb.pt](http://www.aquaweb.pt)) of records of diatom and macroinvertebrate community composition and abiotic variables covering the whole of Portugal and a wide variety of natural conditions from mountains (up to 2000 m a.s.l.) to lowland sedimentary areas, from northern schist and granite dominant lithology to southern more alkaline and sedimentary lithology and from temperate Atlantic to Mediterranean climates. It characterised both natural site attributes, such as geomorphology and climate, and also human pressures (Figure 3.1, Table 3.1). We used data from 549 diatom samples and 984 macroinvertebrate samples from 506 sites, of which 167 diatom samples (55 sites) and 218 macroinvertebrate samples (62 sites) were from rivers and streams in the lowland region of central Portugal. These data comprised samples collected from 1993 to 2012 (during the four seasons).



In a previous study considering organic pollution and nutrients, hydromorphological alterations and land use, nine sites in the Portuguese lowland region were considered to be in LDC (Elias et al., 2015): Canadas (L03), Casal dos Netos (NO103), São Pedro de Muel (O92), Caldelas (L98), Alcaria (L01), Almagreira (M06), Botão (M18), Foz do Ceira (M09) and Avelãs de Cima (V117). Despite being considered in the least disturbed condition these sites present, however, high levels of nutrients (*e.g.*, nitrates and phosphates concentrations reaching  $5.1 \text{ mg NO}_3^- \cdot \text{L}^{-1}$  and  $0.83 \text{ mg PO}_4^{3-} \cdot \text{L}^{-1}$ , respectively) and minimal and small disturbance, according to Pont et al. (2006) in riparian vegetation and morphology. Samples from these sites, hereinafter treated as LDC samples and referred to by the site codes above, were used for comparative purposes to determine whether the new reference conditions represented an improvement on least disturbed conditions.

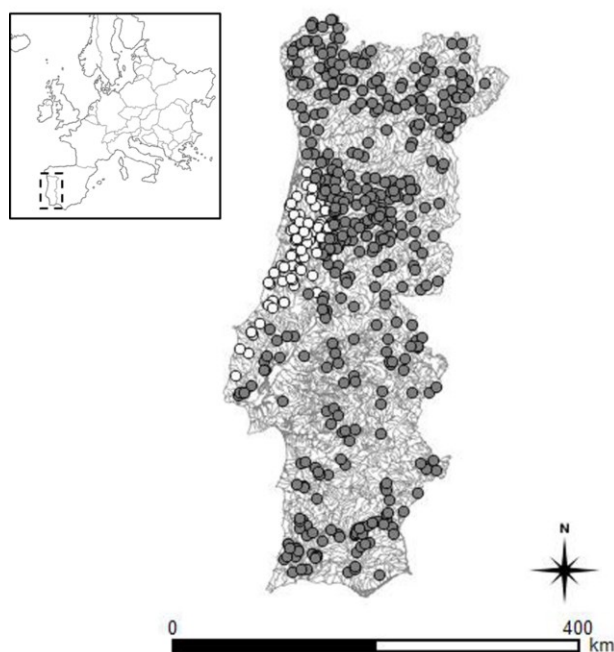


Figure 3.1 - Map of Portuguese rivers showing locations of the lowland sites (white circles) and the remaining study sites (grey circles).

Table 3.1 - Characterization and pressure variables included in the study, with ranges of values for Portuguese lowland sites (n=218 samples from 62 sites).

| Variable  | Range                            |
|---|----------------------------------|
| Characterization variables  |                                  |
| Latitude (°N)   | 38.9264 - 40.7537                |
| Longitude (°W)  | 8.2394 - 9.3602                  |
| Precipitation (annual mean; mm) <sup>a</sup>  | 600 - 1600                       |
| Drainage area (km <sup>2</sup> ) <sup>a</sup>   | 0.71 - 5518.43                   |
| Air temperature (annual mean; °C) <sup>a</sup>  | 10.0 - 17.5                      |
| Slope of the drainage area (%) <sup>a</sup>   | 1.65 - 24.55                     |
| Lithology (categorical) <sup>a, b</sup>   | 1 - 17                           |
| Runoff (annual mean; mm) <sup>c</sup>   | 81.63 - 1034.71                  |
| Distance to the source (m) <sup>a</sup>   | 271 - 230098                     |
| Soil pH <sup>a</sup>  | 6.95 - 14.90                     |
| Alkalinity (CaCO <sub>3</sub> ; mg·L <sup>-1</sup> )  | 2.41 - 478.00                    |
| Water temperature (on sampling occasion; °C) <sup>d</sup>   | 3.8 - 24.0                       |
| Conductivity (μS·cm <sup>-1</sup> ) <sup>d</sup>  | 10 - 1388                        |
| pH (on sampling occasion) <sup>d</sup>  | 5.13 - 11.06                     |
| Mineralisation class (% high, medium and low) <sup>a, e</sup>   | 0 - 100                          |
| Cover of substrata (within ≈ 500 m of sampling; %) <sup>f</sup> :   |                                  |
| Bedrock   | 0 - 40                           |
| Boulder   | 0 - 90                           |
| Cobble  | 0 - 100                          |
| Gravel/Pebble   | 0 - 100                          |
| Sand  | 0 - 100                          |
| Silt  | 0 - 50                           |
| Clay  | 0 - 100                          |
| Earth   | 0 - 100                          |
| Pressure variables  |                                  |
| Dissolved oxygen (DO; % saturation) <sup>d</sup>  | 21 - 146                         |
| Ammonium (N; mg·L <sup>-1</sup> ) <sup>d</sup>  | 0.01 - 18.00                     |
| Nitrate (NO <sub>3</sub> <sup>-</sup> ; mg·L <sup>-1</sup> ) <sup>d</sup>   | 0.03 - 23.90                     |
| Nitrite (NO <sub>2</sub> <sup>-</sup> ; mg·L <sup>-1</sup> ) <sup>d</sup>   | 0.01 - 3.10                      |
| Total phosphorus (P; mg·L <sup>-1</sup> ) <sup>d</sup>  | 0.01 - 5.15                      |
| Phosphate (PO <sub>4</sub> <sup>3-</sup> ; mg·L <sup>-1</sup> ) <sup>d</sup>  | 0.01 - 4.56                      |
| Biochemical oxygen demand (BOD <sub>5</sub> ; mg·L <sup>-1</sup> ) <sup>d</sup>   | 0.50 - 16.00                     |
| Chemical oxygen demand (COD; mg·L <sup>-1</sup> ) <sup>d</sup>  | 1.01 - 289.00                    |
| Intensive agriculture (in the catchment; %) <sup>a</sup>  | 0.00 - 92.25                     |
| Extensive agriculture (in the catchment; %) <sup>a</sup>  | 0.00 - 50.22                     |
| Artificial areas (in the catchment; %) <sup>a</sup>   | 0.00 - 27.04                     |
| Natural and semi-natural areas (in the catchment; %) <sup>a, d</sup>  | 2.11 - 100.00                    |
| Riparian vegetation condition (change due to removal of natural vegetation or presence of alien species) (category) <sup>g, d</sup> | 1 (no change) - 5 (large change) |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|  |       |
|--|-------|
| Morphological condition (change in channel morphology and loss of natural habitats) (category) <sup>g, d</sup>                       | 1 - 5 |
| Sediment load (change in natural concentration of sediments transported by stream water) (category) <sup>g, d</sup>                  | 1 - 5 |
| Hydrological condition (changes from natural discharge) (category) <sup>g, d</sup>   | 1 - 5 |
| Acidification and toxicity (change from natural acidity and oxygenation of the water) (category) <sup>g, d</sup>                     | 1 - 4 |
| Organic contamination and nutrient enrichment (evidence of organic substances and nutrients in the water) (category) <sup>g, d</sup> | 1 - 4 |
| Connectivity (degree of alteration in longitudinal connectivity of the stream reach) (category) <sup>g, d</sup>                      | 1 - 5 |

<sup>a</sup> Based on Atlas do Ambiente (Agência Portuguesa do Ambiente, 2007); <sup>b</sup> See Appendix I for category description; <sup>c</sup> Calculated; see section 3.2.3 for description; <sup>d</sup> Variables used in the PCA; <sup>e</sup> See section 3.2.3 for class description; <sup>f</sup> Based on Environment Agency (2003); <sup>g</sup> Adapted from Pont et al. (2006).

### 3.2.2 Diatom and macroinvertebrate sampling and processing

Diatom sampling followed European standards of sampling and treatment (European Committee for Standardisation, 2003, 2004, 2006). Hard substrata (rocks/stones) were sampled in each site if present; otherwise sediment was sampled. For epilithic biofilms the upper surfaces of five submerged stones (an area of ~100 cm<sup>2</sup>) were scraped with a toothbrush and washed with running water, and for epipsammic biofilms a volume of about 50 mL of the upper surface of streambed sediment was collected with a syringe. Samples were preserved with formaldehyde (5-10% final concentration) and oxidised with concentrated nitric acid and potassium dichromate for about 24h at room temperature. Thereafter, permanent slide mounts in Naphrax® were prepared and a light microscope (100x objective and 1.32 numerical aperture) was used to count about 400 diatom valves *per* sample at species or infra-specific rank, mainly with Krammer and Lange-Bertalot's floras (1986, 1988, 1991a, 1991b) and Krammer (2000, 2001, 2009).

Benthic macroinvertebrate sampling followed the multi-habitat approach described in INAG (2008b). Briefly, each sample was a composite of six sub-samples from the most representative habitats (*e.g.*, stones, sand and submerged macrophytes), collected by kicking and sweeping approximately 1 m towards upstream with a hand net (500 µm mesh size; 0.25×0.25 m opening). Macroinvertebrate samples were preserved in formaldehyde (4%) and, after sorting, preserved in ethanol (75%) for later identification and counting. Identification, under a stereomicroscope (magnification 60x), was mostly to genus level except for Diptera (sub-family) and Annelida (class).

### 3.2.3 Conceptual model development

As a starting point, a conceptual model (Figure 3.2) was developed to represent the environmental variables (filters) that have the potential to directly influence the composition of diatom and macroinvertebrate communities and the indirect abiotic variables that are potentially useful to estimate reference values of the environmental filters. The set of direct variables that were selected from a literature review are those provided in Figure 3.2. These variables were chosen because both taxonomic composition and structure of diatom and macroinvertebrate communities are known to be largely influenced by climate, geology and water chemistry (Potapova, 1996; Wallace and Webster, 1996; Tison et al., 2005; Potapova and Charles, 2007; Smith et al., 2007; Feio et al., 2009; Almeida and Feio, 2012; Marzin et al., 2012). The indirect abiotic variables selected were mean annual precipitation, mineralization, soil pH and land-use alterations (as agriculture or artificial areas) (Figure 3.2). The variable mineralization reflects the geology of the soil (geological formations) and its contribution to the water mineralisation. This variable was divided in a three-class scale: low (composed essentially by acid rocks), medium (composed essentially by alkaline rocks) and high mineralisation (composed essentially by sedimentary rocks) (INAG, 2008a).

Values of the direct variables (Figure 3.2) were obtained from cartographic sources or assessed at each site, with the exception of the runoff values which were estimated from an annual water balance. Ignoring exchanges with other catchments, the water balance was expressed by the equation  $P=H+E$ , where  $P$  is annual precipitation,  $E$  is annual evapotranspiration and  $H$  is annual runoff (Hipólito and Vaz, 2011).  $P$  is an observed value and  $E$  was calculated with Turc's (1954) method.  $P$  and  $H$  values were validated against cartographic sources.

### 3.2.4 Defining abiotic reference conditions

Boosted trees (BT) technique was used to predict reference values of environmental filters. This technique aims to improve the performance of a single model by fitting many models and combining them for prediction (Elith et al., 2008). BT uses two algorithms: regression trees (models that relate a response to their predictors by recursive binary splits) and boosting (that works by sequentially applying a classification algorithm to reweighted versions of the training data and then taking a weighted majority vote of the sequence of classifiers thus produced) (Friedman et al. 2000; Elith et al., 2008). The general idea of BT is to compute a sequence of simple trees, where each successive tree is built for the prediction residuals of the preceding tree. This method builds binary trees (*i.e.*, partition the data into two samples at each split node) whose complexity is limited to 3 nodes only: a root node and two child nodes, *i.e.*, a single split. Thus, at each step of the boosting (boosting trees

algorithm), a simple (best) partitioning of the data is determined, and the deviations of the observed values from the respective means (residuals for each partition) are computed.

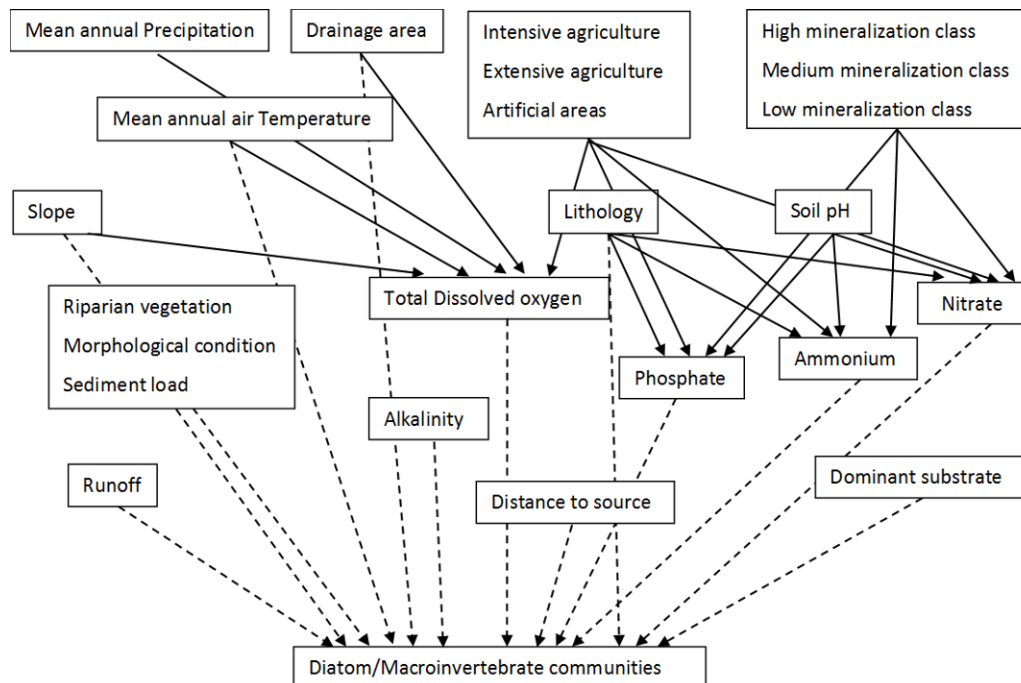


Figure 3.2 - Conceptual model showing the abiotic variables that directly influence the composition of diatom and macroinvertebrate communities (connections via dashed lines) and those useful to estimate values of other abiotic variables (connections via solid lines). See Table 3.1 for variable descriptions.

The next 3-node tree will then be fitted to those residuals, to find another partition that will further reduce the residual (error) variance for the data, given the preceding sequence of trees (STATISTICA 7, StatSoft Inc.). Such 'additive weighted expansions' of trees can eventually produce an excellent fit of the predicted values to the observed values, even if the specific nature of the relationships between the predictor variables and the dependent variable of interest is very complex (nonlinear in nature). Hence, the method of gradient boosting - fitting a weighted additive expansion of simple trees - represents a very general and powerful machine learning algorithm (STATISTICA 7, StatSoft Inc.). The BT method includes all the desirable properties of classification and regression trees (CART): (1) handling different types of predictor variables including numeric and categorical variables; (2) being invariant to monotonic transformations of the predictors, so that a priori data transformation or elimination of outliers is not necessary; (3) simply modelling complex interactions; and (4) managing missing values in the predictors with minimal loss of information (Leathwick et al., 2006; De'ath, 2007; Elith et al., 2008). Additionally, boosted trees overcome two of CART's weaknesses: poor prediction performance and interpretation

difficulties for large trees (De'ath, 2007; Elith et al., 2008). The analyses were carried out through the statistical software STATISTICA 7 (StatSoft Inc.).

The application of the BT to predicting the environmental data was performed in two steps. First, models were developed to predict observed concentrations of dissolved oxygen (DO), phosphates, ammonium and nitrate from linked abiotic explanatory variables in the conceptual model (Figure 3.2). For example, nitrate concentrations were modelled from lithology, land use, mineralization and soil pH. The model training data were the entire abiotic dataset, covering a wide variety of spatial and temporal conditions (all sampled Portuguese river types, various disturbance levels and all seasons), but with 10% of samples excluded randomly for model validation. Two thousand trees were run with a learning rate of 0.1. Model quality was evaluated through the  $R^2$  value of observed-predicted regression for the training set, with a minimum value for model acceptance of 0.5. The ratio of the Mean Squared Errors (MSEs) of the training and validation datasets was also calculated in order to test whether the value was close to one, which was considered optimal.

Second, the accepted models were used to predict reference values of DO and nutrients for the lowland streams in all seasons. For this purpose, a new dataset with manipulated data was created for the lowland samples, with the observed values of the model predictors replaced by values corresponding to undisturbed conditions. Values of the predictors related to land use were set to 0, *i.e.*, no or minor changes from natural conditions (Table 3.2), whereas values of climatic, topographic and geological predictors remained unchanged. This new dataset was then run as test data in the previous created boosted tree models to estimate reference DO and nutrient values. Values thus obtained for the lowland samples were used to build a complete dataset of abiotic reference values by adding the variables riparian vegetation, morphological condition and sediment load, with all values set to 1, corresponding to no or minor modifications, instead of the observed values. All other environmental filters not influenced by human disturbance remained unchanged.

Table 3.2 - Abiotic pressure variables for which values were altered to predict reference communities.

| Variable   | Reference value  |
|--|--|
| Total dissolved oxygen (DO; %)                                   | Observed value retained if $\geq 73.7$ and $\leq 127.9\%$ , otherwise replaced by 73.7% if $< 73.7$ or 127.9% if $> 127.9$ (from Feio et al., 2014a) |
| Ammonium (N; mg·L <sup>-1</sup> )                                | Observed value retained if $\leq$ to 0.09 mg·L <sup>-1</sup> , otherwise replaced by 0.09 (from Feio et al., 2014a)                                  |
| Nitrate (NO <sub>3</sub> ; mg·L <sup>-1</sup> )                  | Estimated by boosted tree analysis   |
| Phosphates (PO <sub>4</sub> <sup>3-</sup> ; mg·L <sup>-1</sup> ) | Observed value retained if $\leq$ to 0.06 mg·L <sup>-1</sup> , otherwise replaced by 0.06 (from Feio et al., 2014a)                                  |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|   |                     |
|---|---------------------|
| Intensive agriculture (in the catchment; %) | 0                   |
| Extensive agriculture (in the catchment; %) | 0                   |
| Artificial areas (in the catchment; %)      | 0                   |
| Riparian vegetation <sup>a</sup>            |                     |
| Sediment load <sup>a</sup>                  | Class 1 (no change) |
| Morphological condition <sup>a</sup>        |                     |

<sup>a</sup> Variable on a five-class scale described in Table 3.1.

### 3.2.5 Predicting the lowland reference community: diatoms and macroinvertebrates

We followed the environmental filters approach described by Chessman and Royal (2004) to predict the taxa expected under WFD reference conditions in lowland streams. First, taxa considered exotic/invasers were removed from the datasets, as such taxa are not expected under reference conditions. For diatoms, the species considered 'exotic/invaser' according to the '*Indice Biologique Diatomées*'-IBD (Prygiel and Coste, 2000) and Coste and Ector (2000) was *Achnanthes subhudsonis* (Hustedt) H. Kobayasi; for macroinvertebrates the species considered exotic were *Corbicula fluminea* and *Procambarus clarki*. Second, all taxa present in fewer than 5% of samples (27 samples for diatoms and 49 for macroinvertebrates) were eliminated from the observed and predicted lists as we considered that these taxa were insufficiently represented to determine their tolerance limits with confidence. We determined the upper and lower tolerance limits of each remaining taxon for each environmental filter (dashed lines in Figure 3.2). These limits were set as the range of values at which a taxon was present in the national dataset (all sites and seasons), after removing extremes, considered as values  $> 75^{\text{th}} \text{ percentile} + 3 \cdot (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$  or  $< 25^{\text{th}} \text{ percentile} - 3 \cdot (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$ .

Finally, we compared the abiotic reference values predicted for each lowland site (from the previous section 3.2.4) with the tolerance ranges for each taxon. If a taxon's tolerance ranges included all the abiotic reference values predicted for a given site, the taxon was included in the list of taxa predicted for that site under reference conditions. Predicted and observed taxon lists were compared for samples collected in spring (90 samples for diatoms and 122 for macroinvertebrates) because the LDC reference values currently used are based on spring data only. If a site had more than one spring sample, the samples were combined in order to obtain a single observed and predicted list *per* site (55 and 62 sites for diatoms and macroinvertebrates, respectively). As the filters approach predicts only presence or absence of taxa, the data from the observed lists of taxa were also converted to presence/absence.

Differences between the observed and predicted spring communities (diatoms and macroinvertebrates analysed separately) were depicted by non-metric multidimensional scaling (MDS) (Bray–Curtis dissimilarity measure) and statistically tested with a permutational multivariate analysis of variance (PERMANOVA global test) with unrestricted permutations. Additionally, a similarity percentage analysis, SIMPER (Bray–Curtis similarity measure) was performed to determine the within-community similarity of the observed and predicted communities. Additional PERMANOVA pairwise tests with unrestricted permutations were performed to compare LDC and non-LDC sites (see section 3.2.1). The reliability of the two-dimensional images of the multidimensional relationship between the samples is indicated by a stress value (Clarke and Warwick, 2001; Clarke and Gorley, 2006). Stress values < 0.05 are excellent, leaving little danger of misinterpretation, stress values < 0.1 correspond to a good representation of the similarities between the samples and stress values > 0.2 indicate that not too much reliance should be placed on details (Clarke and Warwick, 2001). All these analyses were done in PRIMER 6 & PERMANOVA.

### 3.2.6 Quality assessment and diatom index development

The official indices currently accepted in Portugal were used to compare biological quality assessments based on the LDC and filters reference conditions for all the lowland sites sampled in spring. These are the IPS index (Cemagref, 1982) for diatoms and the multimetric ‘Índice Português de Invertebrados’ (IPTIs–INAG, 2009; Ferreira et al., 2008) for macroinvertebrates (Equation 3.1).

$$IPTIs = \frac{\frac{N^{\circ}Fam.}{N^{\circ}Fam.ref.} \times 0.4 + \frac{EPT}{EPT.ref.} \times 0.2 + \frac{(IASPT-2)}{(IASPT-2).ref.} \times 0.2 + \frac{Log(Sel.EPTCD+1)}{Log(Sel.EPTCD+1).ref.} \times 0.2}{IPTIs\ reference}$$

Equation 3.1 - Macroinvertebrate index used for biological quality assessment of the Portuguese lowland streams. EPT=number of families belonging to Ephemeroptera, Plecoptera and Trichoptera; IASPT=value of the Iberian BMWP index divided by the number of families included in Iberian BMWP index determination; Log (Sel. EPTCD + 1)=Log<sub>10</sub> of 1 + the sum of abundances of individuals belonging to the families Chloroperlidae, Nemouridae, Leuctridae, Leptophlebiidae, Ephemerellidae, Philopotamidae, Limnephilidae, Psychomyiidae, Sericostomatidae, Elmidae, Dryopidae and Athericidae.

The IPS was included as a metric in a new multimetric index proposed here for the biological quality assessment based on diatoms (Equation 3.2). This new index also contains two other metrics: the number of sensitive taxa and total taxa richness (Equation 3.2). The metric ‘sensitive taxa’ includes diatoms with sensitivity value ≥ to 4.5 according to the IPS (Cemagref, 1982 and updated in OMNIDIA software V.5.5) and a higher probability of being



present in the water quality class 6 or 7 of the IBD (Prygiel and Coste, 2000). These classes are the last two of a group of 7 of increasing water quality (Prygiel and Coste, 2000).

$$MDI(EQR) = \frac{\frac{IPS}{Median\ IPS\ LDC} \times wf_1 + \frac{N^{\circ}Obser.taxa}{Median\ N^{\circ}Pred.taxa} \times wf_2 + \frac{N^{\circ}Obser.sensit.taxa}{Median\ N^{\circ}Pred.sensit.taxa} \times wf_3}{Median\ MDI}$$

Equation 3.2 - Multimetric Diatom Index (MDI) proposed for biological quality assessment of Portuguese lowland streams. IPS='Indice de Polluosensibilité Spécifique'; wf=Weighting factors; N°Obser.taxa=Number of observed taxa; N°Pred.taxa=Number of predicted taxa based on the filters approach; N°Obser.sensit.taxa=Number of observed sensitive taxa; N°Pred.sensit.taxa=Number of predicted sensitive taxa based in the filters approach.

The two multimetric indices were calculated firstly with the current official metric and index reference values and secondly with reference values based on the filters approach. In the latter, reference median values of the diatom metrics number of taxa and number of sensitive taxa (Equation 3.2) and the macroinvertebrate metrics number of families, EPT and IASPT – 2 (Equation 3.1) were obtained from the lists of taxa predicted for each lowland site with the filters approach (see section 3.2.5). Because the macroinvertebrate index was created at the taxonomic level of family, filters' predictions at finer taxonomic levels were amalgamated to family level prior to derivation of metric reference values. The filters approach could not be used to produce reference values for those metrics that require abundance data, *i.e.*, IPS for diatoms in Equation 3.2 and EPTCD for macroinvertebrates in Equation 3.1, because it predicted only presence or absence of taxa. Consequently, median spring values for the nine lowland LDC sites were used instead for those metrics. Some LDC sites had multiple spring samples (range 1-3; total of 12 samples), which were averaged before medians were calculated. The overall reference values for the MDI (denominator in Equation 3.2) and IPTIs (denominator in Equation 3.1) were the median values calculated for the nine lowland LDC sites with metric reference values derived as described above.

Weighting factors (wf) were applied to individual metrics in the new MDI (Equation 3.2), as is done for the IPTI, so that metrics responding strongly to human disturbance would contribute more to the overall score (Hering et al., 2006). The following steps were taken to obtain wf values. First, a Principal Components Analysis (rotated PCA; PRIMER 6 & PERMANOVA) was applied to normalised data for all samples from the lowland streams (all seasons) for environmental variables that express anthropogenic pressure on diatom communities (Table 3.1, footnote d). Second, sample scores on the PCA axes that represented most variation in the data (typically axes 1, 2 and 3) were correlated (Spearman rank correlation coefficient; STATISTICA 7 software, StatSoft Inc.) with corresponding IPS values, number of taxa and number of sensitive taxa. Finally, the average correlation with each

metric was normalised so that the correlations summed to 100%, and the normalised correlations were used as metric wf.

As reference values for the IPS were defined only for spring, a second normalised PCA was performed to verify which of the diatom indices MDI or IPS, better reflected the pressures affecting the lowland streams in that season. Spearman rank correlations (STATISTICA 7 software, StatSoft Inc.) were calculated between diatom index values and PCA axes derived from spring samples only.

For the two PCA Pearson correlation coefficient (STATISTICA 7 software, StatSoft Inc.) between the pressure variables on the first three components and each of the original variables were also performed.

Additionally, new biological quality classes were established for the MDI and IPTIs based on their values for the LDC sites derived with the new reference data. The establishment of class boundaries followed the methods used by Ferreira et al. (2008) and recommended by Hering et al. (2006). All sites were assessed with these two new classification systems as well as the existing ones.

All IPS calculations were done in OMNIDIA software V.5.5 (Lecointe et al., 1993).

### **3.3 Results**

#### **3.3.1 Defining abiotic reference conditions**

The boosted tree model for nitrate was acceptable with  $R^2=0.52$  for linear regression of observed and predicted values for the training data (Figure 3.3) and a training/validation ratio of MSE of 0.81 ( $n=701$ ). All the predictor variables had some importance in the analysis (ranking on a 0-1.00 scale, with higher numbers indicating stronger influence on the response) with values ranging from 0.22 (low mineralisation class) to 1.00 (extensive agriculture). The maximum modelled nitrate value for the lowland streams under reference conditions was  $3.64 \text{ mg NO}_3\cdot\text{L}^{-1}$  whereas the maximum observed value was  $23.9 \text{ mg NO}_3\cdot\text{L}^{-1}$  (Figure 3.4).

We were unable to obtain robust models for DO, phosphates and ammonium because of data limitations such as high detection limits, and consequently some predicted values were not realistic. Therefore, we decided not to use boosted trees to estimate reference values for these variables, and instead used values from Feio et al. (2014a) for Mediterranean rivers in LDC, which generally represented an improvement on measured values for the lowland streams (Table 3.2).

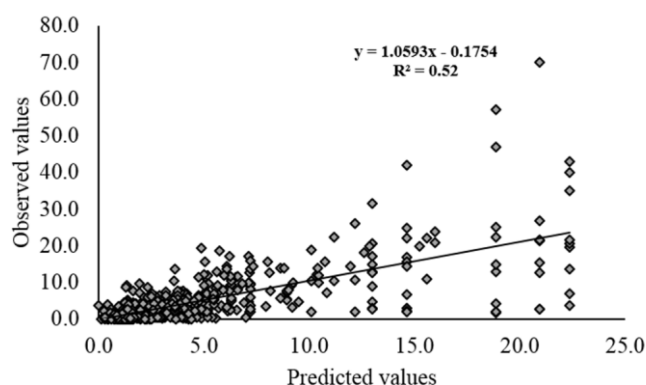


Figure 3.3 - Linear regression of observed nitrate concentrations ( $\text{NO}_3$ ;  $\text{mg}\cdot\text{L}^{-1}$ ) for the entire country and all seasons against actual values predicted by boosted trees.

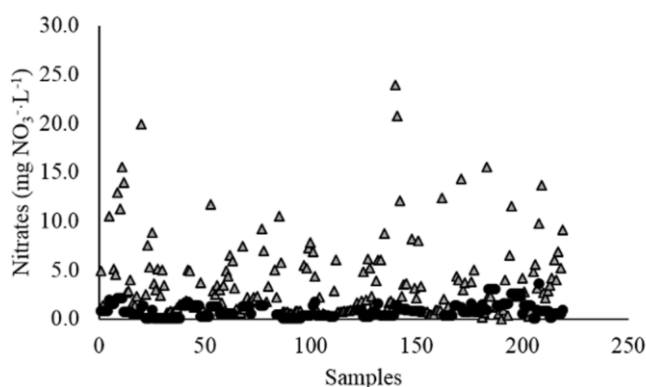


Figure 3.4 - Comparison of observed nitrate concentrations ( $\text{NO}_3$ ;  $\text{mg}\cdot\text{L}^{-1}$ ) for the lowland sites in the training dataset (triangles) and reference values predicted by boosted trees (circles).

### 3.3.2 Predicting reference communities for lowland streams

#### Diatoms

After removing the taxa present in less than 5% of the samples, 115 remained, including 27 sensitive taxa. The median number of taxa *per site* predicted under reference conditions by the environmental filters method was 69 while the median number observed was 27. The median number of sensitive taxa predicted was 11 compared to 4 observed.

The number of taxa predicted to occur in more than 50% of the sites (67) was also much higher than the number of observed (18). Taxa that were predicted to be almost ubiquitous were *Achnanthes minutissimum* (Kützinger) Czarnecki (96%), *Nitzschia palea* (Kützinger) W. Smith (96%), *Navicula gregaria* Donkin (95%) and *Eolimna minima* (Grunow) Lange-Bertalot & W. Schiller (94%). The taxa most frequently observed were *A. minutissimum* (87%), *E.*

*minima* (87%), *N. gregaria* (84%) and *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot (80%) (reported in detail in the Appendix II).

The majority (98%) of the observed taxa were predicted under reference conditions in at least one site. The exception was *Platessa conspicua* (A. Mayer) Lange-Bertalot, which was observed in 9% of the sites but not predicted, primarily because its furthest distance to the source was found to be 1033.7 m. *Melosira varians* C. Agardh was observed at 71% of the sites but was seldom predicted (4%), mainly because its estimated tolerance range for mean annual air temperature was limited between 17.5 and 20.0 °C. Conversely, some taxa were infrequently observed but often predicted, including *Cyclotella meneghiniana* Kützing, *Navicula capitatoradiata* H. Germain, *Nitzschia frustulum* (Kützing) Grunow and *Staurosira venter* (Ehrenberg) Cleve & J. D. Möeller; in 60-66% of the sites, these taxa were predicted but not collected. *Navicula angusta* Grunow was predicted in 4% of the sites but never observed. One of the 115 taxa, *Frustulia erifuga* Lange-Bertalot & Krammer, was the only taxon neither predicted nor observed. This taxon was not predicted because it was found only at a mean annual air temperature value of 12.5 °C which was never recorded in the lowland sites.

The predicted reference diatom communities were statistically different from the observed communities (PERMANOVA: Pseudo-F=13.998, p=0.001) and more homogeneous, with average Bray-Curtis similarity between sites of 62% for the predicted communities and 40% for the observed ones (SIMPER analysis). The MDS stress value (0.13) and PERMANOVA comparisons of sites considered to be in LDC with the remaining sites revealed no significant statistical differences for either observed or predicted communities (Figure 3.5, Table 3.3). However, the observed and predicted communities were significantly different for both LDC and non-LDC sites (Table 3.3).

The filters (Figure 3.2), lithology, mean annual temperature, runoff and alkalinity excluded at least one taxon from the prediction for every site. Distance to source also filtered one or more taxa from 98% of the sites. Among the pressure variables used as filters, those that most frequently excluded taxa were DO (26%), phosphates (22%) and nitrate (22%).

Table 3.3 - PERMANOVA results for pairwise comparisons among observed diatom communities and predicted reference communities. Obs. LDC-observed communities at sites in nominally Least Disturbed Condition; Obs. nLDC-observed communities in non-Least Disturbed Condition sites; Pred. LDC-predicted communities in Least Disturbed Condition sites; Pred. nLDC-predicted communities in non-Least Disturbed Condition sites.

| Comparison |            | t value | p (perm) |
|------------|------------|---------|----------|
| Obs. LDC   | Obs. nLDC  | 0.918   | 0.643    |
| Obs. LDC   | Pred. LDC  | 2.181   | 0.001    |
| Obs. nLDC  | Pred. nLDC | 3.176   | 0.001    |
| Pred. LDC  | Pred. nLDC | 0.901   | 0.513    |

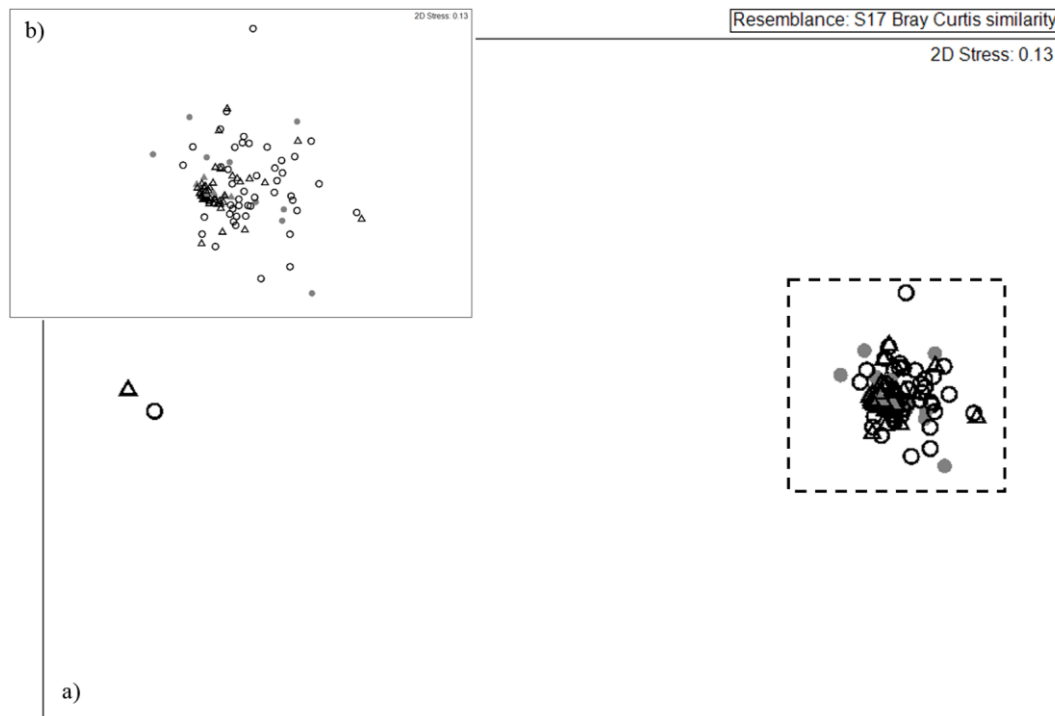


Figure 3.5 - Multidimensional scaling (MDS) ordination of observed (circles) and predicted (triangles) diatom communities from the lowland sites (a) and detailed view of the dashed area (b). The sites considered to be in the least disturbed condition are distinguished by grey shading.

### Macroinvertebrates

After removing the taxa present in less than 5% of the samples, 113 remained, representing 73 families (including classes in the case of Annelida). The median number of families *per site* predicted under reference conditions by the environmental filters method was 53 while the median number observed was 22. Of the 73 families, 66 belonged to the orders Ephemeroptera, Plecoptera and Trichoptera (EPT) that include sensitive taxa. The median number of EPT families predicted was 18 compared to 6 observed.

The number of families predicted to occur in more than 50% of the sites (57) was also much higher than the number of observed families (13). The families most frequently predicted were Physidae (95%), Hydrobiidae (95%), Baetidae (94%), Chironomidae (94%) and Limoniidae (94%), while those most frequently observed were Chironomidae (100%), Oligochaeta (95%), Baetidae (86%), Simuliidae (82%) and Caenidae (74%) (reported in detail in the Appendix III).

The majority (97%) of the predicted macroinvertebrates families were observed in at least one site. Chloroperlidae and Perlidae were exceptions, being predicted in 15% and 27% of sites respectively but never observed. Goeridae and Planariidae were infrequently predicted (13%) but also seldom collected (< 5%).

The MDS illustrates that the predicted and observed communities are separated in the plot (Figure 3.6). Despite the stress value obtained (0.15) the two-dimensional plot can still be viewed as a useful representation of the multidimensional picture as the predicted communities were also statistically different from the observed communities (PERMANOVA: Pseudo-F=13.679,  $p=0.001$ ) and more homogeneous (average inter-site similarity of 69% for predicted and 46% for observed communities) (Figure 3.6). Macroinvertebrate communities observed in LDC sites were also not statistically different from those observed at disturbed sites, but the observed and predicted communities were significantly different for both LDC and non-LDC sites (Table 3.4).

All of the characterisation variables (Table 3.1) that were treated as filters (Figure 3.2) frequently excluded at least one family from the predictions for individual sites (frequencies up to 98%). The pressure variables treated as filters that most frequently excluded families were DO and phosphates (100%). Ammonium and nitrites rarely excluded macroinvertebrates (3%).

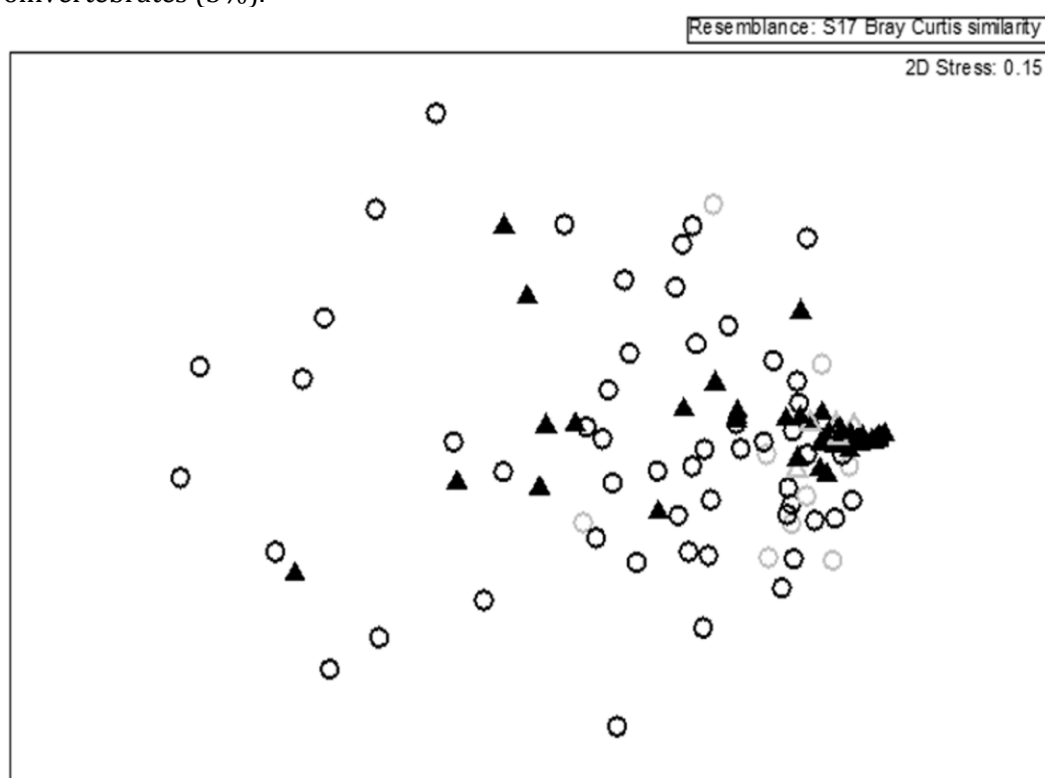


Figure 3.6 - Multidimensional scaling (MDS) ordination of observed (circles) and predicted (triangles) macroinvertebrate communities from the lowland sites. The sites considered to be in the least disturbed condition are distinguished by grey shading.

## Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

Table 3.4 - PERMANOVA results for pairwise comparisons among observed macroinvertebrate communities and predicted reference communities. Obs. LDC-observed communities at sites in nominally Least Disturbed Condition; Obs. nLDC-observed communities in non-Least Disturbed Condition sites; Pred. LDC-predicted communities in Least Disturbed Condition sites; Pred. nLDC-predicted communities in non-Least Disturbed Condition sites.

| Comparison |            | t value | p (perm) |
|------------|------------|---------|----------|
| Obs. LDC   | Obs. nLDC  | 1.386   | 0.056    |
| Obs. LDC   | Pred. LDC  | 2.323   | 0.001    |
| Obs. nLDC  | Pred. nLDC | 3.998   | 0.001    |
| Pred. LDC  | Pred. nLDC | 1.023   | 0.265    |

### 3.3.3 Quality assessment and index development

#### Diatoms

The three most important axes in the PCA of environmental data for all seasons accounted for 44% of total variation, with the first two axes (33%) related mainly to organic contamination and nutrients and the third to hydrological condition, connectivity and pH (Table 3.5). All three diatom metrics were significantly ( $p < 0.05$ ) correlated with PCA axis scores; for example, the IPS value was highly correlated with the first PCA axis while the number of sensitive taxa was correlated with all three axes (Table 3.6). The weighting factors obtained from these correlations were highest for the number of sensitive taxa ( $wf_3$ ) and lowest for the total number of taxa ( $wf_2$ ) (Table 3.7). Median reference IPS and MDI values based on the nine LDC sites are shown in Table 3.7; ranges were 13.4-18.9 for the IPS and 0.70-1.29 for the MDI.

The three first axes of the PCA based on spring data accounted for 45.3% of variation (Table 3.5).

Spearman correlations between the MDI and IPS values and the first axis (spring PCA) were highly negative and significant ( $r_s = 0.54$  and  $r_s = 0.55$  respectively;  $p < 0.05$ ). However, the MDI also had a significant negative correlation with the second axis ( $r_s = 0.28$ ,  $p < 0.05$ ).

Boundaries for quality classes based on the MDI (EQR) calculated for the LDC sites with new reference values (Table 3.8) led to lower classifications of samples than the IPS (Figure 3.7), *i.e.*, the MDI assigned lower quality than the IPS.

#### Macroinvertebrates

The environmental filters approach provided new reference values for all metrics not requiring abundance data, whereas the reference values for  $\text{Log}(\text{Sel. EPTCD}+1)$  and IPTIs were the median observed values for the LDC sites (Table 3.9). IPTIs values at these sites ranged from 0.81 to 1.31.

Boundaries for quality classes based on the IPTIs (EQR) calculated for the LDC sites with new reference values (Table 3.8) were consistently lower than the boundaries currently established for Portugal (INAG, 2009). The IPTIs calculated with the environmental filters approach placed the samples in a lower classes than the IPTIs currently used (Figure 3.8).

Table 3.5 - Pearson correlation coefficient between the pressure variables on the first three components from principal components analysis (PCA) of data for all seasons and spring only and each of the original variables. The three highest coefficients for each PC are in bold font. BOD<sub>5</sub>-biochemical oxygen demand, COD-chemical oxygen demand.

| Pressure variable (units and transformation)                         | All seasons (n=156) |              |               | Spring (n=84) |               |               |
|--|---------------------|--------------|---------------|---------------|---------------|---------------|
|  | PC1                 | PC2          | PC3           | PC1           | PC2           | PC3           |
| Water temperature (°C; square root)                                  | 0.303               | -0.098       | -0.247        | 0.229         | -0.239        | -0.205        |
| Conductivity (µS·cm <sup>-1</sup> ; log)                             | 0.148               | 0.260        | 0.180         | 0.291         | 0.227         | -0.200        |
| pH   | 0.135               | 0.387        | <b>0.512</b>  | 0.348         | -0.152        | 0.102         |
| Dissolved oxygen (% saturation; square root)                         | -0.426              | <b>0.473</b> | 0.024         | -0.469        | 0.125         | 0.110         |
| BOD <sub>5</sub> (mg·L <sup>-1</sup> ; log)                          | 0.322               | 0.030        | 0.144         | -0.021        | 0.395         | <b>-0.576</b> |
| COD (mg·L <sup>-1</sup> ; square root)                               | <b>0.587</b>        | -0.208       | -0.096        | 0.437         | 0.120         | -0.390        |
| Phosphates (mg PO <sub>4</sub> <sup>3-</sup> ·L <sup>-1</sup> ; log) | 0.553               | -0.391       | 0.003         | 0.332         | -0.451        | 0.240         |
| Ammonium (mg N·L <sup>-1</sup> ; log)                                | 0.454               | -0.174       | -0.173        | <b>0.592</b>  | -0.101        | 0.022         |
| Nitrate (mg NO <sub>3</sub> <sup>-</sup> ·L <sup>-1</sup> ; log)     | 0.407               | 0.471        | 0.164         | 0.300         | <b>0.571</b>  | -0.122        |
| Nitrite (mg NO <sub>2</sub> <sup>-</sup> ·L <sup>-1</sup> ; log)     | 0.524               | 0.022        | -0.056        | 0.363         | 0.032         | -0.043        |
| Total P (mg P·L <sup>-1</sup> ; log)                                 | <b>0.611</b>        | -0.328       | -0.059        | 0.327         | <b>-0.541</b> | 0.172         |
| Riparian vegetation <sup>a</sup>                                     | 0.452               | 0.265        | -0.175        | 0.468         | 0.188         | 0.439         |
| Sediment load <sup>a</sup>   | 0.407               | -0.154       | -0.158        | <b>0.581</b>  | -0.078        | -0.033        |
| Hydrological regime <sup>a</sup>                                     | -0.077              | 0.325        | <b>-0.658</b> | -0.169        | 0.400         | <b>0.496</b>  |
| Acidification and toxicity <sup>a</sup>                              | 0.121               | -0.115       | -0.152        | 0.342         | 0.156         | -0.200        |
| Morphological condition <sup>a</sup>                                 | 0.229               | <b>0.473</b> | -0.407        | 0.205         | 0.318         | <b>0.764</b>  |
| Organic contamination and nutrient enrichment <sup>a</sup>           | 0.408               | <b>0.600</b> | -0.224        | 0.394         | <b>0.647</b>  | 0.311         |
| Connectivity <sup>a</sup>  | -0.115              | 0.364        | <b>-0.632</b> | -0.225        | 0.465         | 0.322         |
| Natural and semi-natural areas (%; square root)                      | <b>-0.583</b>       | -0.302       | -0.449        | <b>-0.681</b> | -0.105        | 0.113         |

<sup>a</sup> Variable on a five-class scale described in Table 3.1.

Table 3.6 - Coefficients of Spearman rank correlation between the PCA axis scores (all seasons; Table 3.5) and diatom metrics, used to determine weighting factors (wf). \*– p < 0.05

|                            | IPS    | Nº observed taxa | Nº observed sensitive taxa |
|----------------------------|--------|------------------|----------------------------|
| Nº observed taxa           | -0.48* |                  |                            |
| Nº observed sensitive taxa | 0.28*  | 0.29*            |                            |
| PC1                        | -0.59* | 0.12             | -0.44*                     |



Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|     |       |        |        |
|-----|-------|--------|--------|
| PC2 | -0.09 | -0.26* | -0.42* |
| PC3 | -0.12 | 0.17*  | -0.24* |

Table 3.7 - Median reference values of the MDI and its constituent metrics, and weighting factors (wf), for MDI (EQR) calculation (Equation 3.2).

|   |      |
|---|------|
| Median IPS LDC  | 17.0 |
| Median N <sup>o</sup> predicted taxa <sup>a</sup>           | 69   |
| Median N <sup>o</sup> predicted sensitive taxa <sup>a</sup> | 11   |
| Median MDI reference  | 1.13 |
| wf <sub>1</sub> (IPS)                                       | 0.33 |
| wf <sub>2</sub> (N <sup>o</sup> total taxa)                 | 0.23 |
| wf <sub>3</sub> (N <sup>o</sup> sensitive taxa)             | 0.45 |

<sup>a</sup> Median number obtained through the filters approach, see section 3.3.2.

Table 3.8 - New class boundaries for biological quality assessment of lowland streams using the new diatom index (MDI) and the modified IPTi macroinvertebrate method.

| Boundary      | Diatoms | Macroinvertebrates |
|---------------|---------|--------------------|
| Good/High     | 0.83    | 0.95               |
| Moderate/Good | 0.62    | 0.71               |
| Poor/Moderate | 0.41    | 0.48               |
| Bad/Poor      | 0.21    | 0.24               |

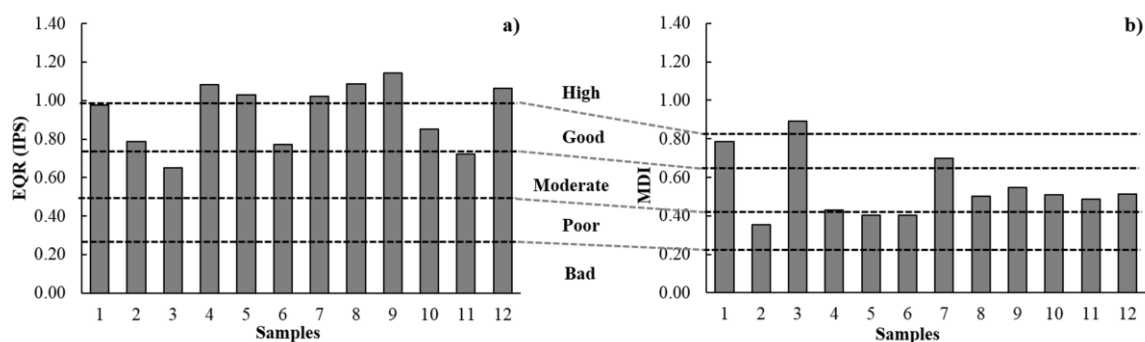


Figure 3.7 - Ecological quality ratios (EQR) for diatom indices based on (a) the presently used IPS and median reference values and (b) the new multimetric diatom index (MDI) and median reference values from the LDC samples. 1-Site L03; 2-Site N0103; 3-Site O92; 4-Site L98; 5-Site L01; 6-Site M06; 7, 8, 9-Site M18; 10, 11-Site M09; 12-V117. Dashed lines indicate quality class boundaries.

Table 3.9 - Metric and index reference values for IPTIs calculation (Equation 3.1).

|  |      |
|--|------|
| N <sup>o</sup> families reference <sup>a</sup> | 52.5 |
| EPT reference <sup>a</sup>                     | 18.0 |
| (IASPT-2) reference <sup>a</sup>               | 3.65 |

|                              |      |
|------------------------------|------|
| Log (Sel. EPTCD+1) reference | 1.16 |
| IPtIs reference              | 1.82 |

<sup>a</sup> Median number obtained through the filters approach, see section 3.3.2.

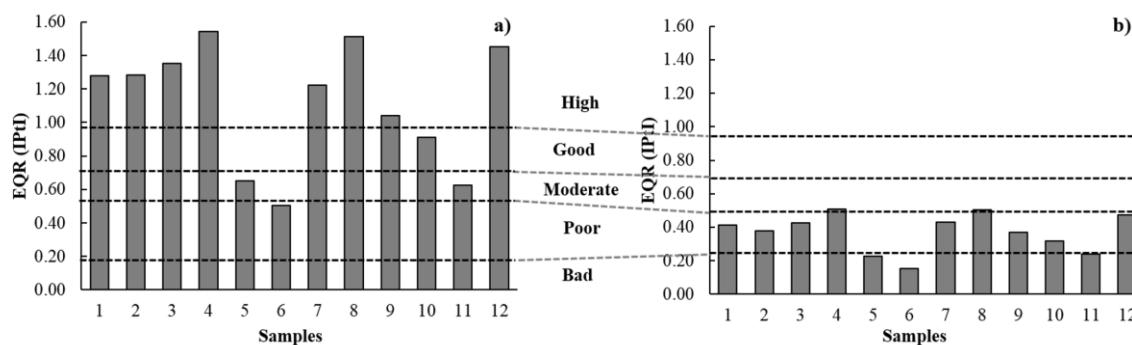


Figure 3.8 - Ecological quality ratios (EQR) for macroinvertebrate indices based on (a) the presently used IPTI median reference values and (b) the new IPTI median reference values from the LDC samples. 1-Site L03; 2-Site N0103; 3-Site O92; 4-Site L98; 5-Site L01; 6-Site M06; 7, 8, 9-Site M18; 10, 11-Site M09; 12-Site V117. Dashed lines indicate quality class quality boundaries.

### 3.4 Discussion

Although several studies have tested alternative ways to establish reference conditions for biological assessment of streams, most have relied entirely on data obtained from reference sites that are not true pristine sites. Even when modelling techniques are applied in determining biological reference condition, reference sites, near-natural or least disturbed sites have been used (Tison et al., 2007; Almeida and Feio, 2012; Feio et al., 2012; Feio and Dolédec, 2012; Feio et al., 2014b; Pardo et al., 2014). Our study demonstrates that the filters' approach with modelling of abiotic reference values enables determination of biological reference conditions for both diatoms and macroinvertebrates with less dependence on reference sites. Our approach, using a combination of modelling and data from LDC sites, exemplifies the third option for determining reference conditions allowed by the WFD.

#### Predicting the lowland reference community: diatoms and macroinvertebrates

The boosted trees successfully modelled reference values of nitrate for the lowland streams but could not be used for other abiotic variables because of high analytical detection limits. Our maximum reference nitrate value ( $3.64 \text{ mg}\cdot\text{L}^{-1}$ ) was high, however was within the range of threshold values for various river types of  $2\text{--}6 \text{ mg}\cdot\text{L}^{-1}$  below which Pardo et al. (2012) considered a site to be 'probably reference'. Feio et al. (2014a) set a lower threshold for Mediterranean areas ( $\leq 1.15 \text{ mg}\cdot\text{L}^{-1}$ ), but for a wider geographic region than the lowland

area considered in the present study. Additionally, the lowland streams as streams with typically moderate to null slope are naturally more sensitive to eutrophication and show lower reference notes (Tison et al., 2007).

Application of the filters approach incorporating boosted trees should have predicted reference data closer to the WFD reference condition for diatoms and macroinvertebrates. For both groups, the predicted communities were statistically different from the observed communities, and the observed communities were less homogeneous, as would be expected with sites having variable levels of change from reference conditions. The median number of predicted taxa was more than double the median number of observed taxa, probably reflecting the elimination of taxa by anthropogenic pressures. However, other factors may also have contributed to the absence of predicted taxa from the collected samples. For diatoms it was assumed that the counting of about 400 valves *per* sample was sufficient to establish community composition (*e.g.*, Prygiel et al., 2002), but ring-testing has shown that to achieve a full picture of a diatom community, subsamples should be collected within a larger transect of the stream site and the slide should be searched *a posteriori* to detect rare taxa that may be missed in the 400 valve count (Besse-Lototskaya et al., 2006). Consequently, rare taxa are absent in the observed communities and present (we considered all taxa predicted) in the predicted communities. Moreover, for diatoms only one type of substrate was sampled, usually rock/stone. Nevertheless, the median ratio of numbers of predicted and observed taxa was very similar for diatoms and macroinvertebrates (2.56 and 2.41, respectively), even though macroinvertebrates were collected from multiple substrata at each site and all individuals in a macroinvertebrate sample were counted (depending of the river these counting's often reached values above 400 individuals).

In some cases, taxa were observed at a site where they were not predicted to occur. This could indicate anthropogenic influences making a stream suitable for taxa that would not naturally occur there. However, it could also have resulted from limitations in estimation of the natural environmental attributes of the sites and environmental tolerance ranges of the taxa (Chessman and Royal, 2004).

Diatoms that were frequently predicted under reference conditions (*e.g.*, *Achnanthes minutissimum*, *Eolimna minima*, *Nitzschia palea*, *Navicula gregaria*, *Planothidium lanceolatum*, *Cyclotella meneghiniana*, *Gomphonema parvulum* (Kützing) Kützing, *Nitzschia inconspicua* Grunow, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot and *Sellaphora seminulum* (Grunow) D. G. Mann are considered cosmopolitan and near ubiquitous in continental waters (Potapova and Charles, 2002). Some of these species have been considered to indicate eutrophic conditions (*e.g.*, *N. inconspicua*, *G. parvulum*, *E. minima*, *N.*

*gregaria*; van Dam et al., 1994) but they may also occur in lower numbers in streams considered to have good water quality (Delgado et al., 2012; Almeida et al., 2014).

The only two diatom species predicted but never collected in the lowland streams (*Navicula angusta* and *Frustulia erifuga*) are sensitive to degradation. *N. angusta* was found in a previous study of Portuguese streams (of the same type as the streams in this study) and showed a preference for low conductivity and acid waters (Almeida and Gil, 2001). *F. erifuga* is a mobile, high-profile species forming a mucous tubule colony (Rimet and Bouchez, 2012). Species with similar morphological traits, such as *Encyonema minutum* (Hilse) D. G. Mann and *Encyonema silesiacum* (Bleisch) D. G. Mann, were frequently collected in the lowland streams, suggesting that chemical factors rather than physical habitat are responsible for the absence of *F. erifuga*.

The macroinvertebrates that were commonly predicted under reference conditions included taxa characteristic of lowland sites as well as taxa requiring habitat conditions that are rarely found there. For example, the families Chloroperlidae and Perlidae that are very sensitive according to the IBMWP scoring system (Alba-Tercedor et al., 2002) were predicted for some sites but never observed. These families require cool, oxygenated waters and gravel or rocky bottoms, which are naturally present in only a few of lowland streams, especially those in the transition to other river types (Tachet et al., 2010). The taxa predicted most frequently included the gastropods Physidae and Hydrobiidae, which were expected because the characteristic bottom sediment at the lowland sites is sand, and families that are nearly ubiquitous in streams and rivers, such as the Chironomidae. However, this family comprises a great diversity of species (estimated at 20,000) with quite variable pollution tolerances (Coffman, 1995; Servia et al., 2004), so it is probable that sensitive taxa among this family could also be expected if a lowest level of resolution was used.

The filters most often responsible for exclusions of diatom and macroinvertebrate taxa from site predictions were mean annual temperature, runoff and variables related to geology (lithology and alkalinity). Such variables have been reported in other studies as influencing diatom and macroinvertebrate distributions (*e.g.*, Potapova and Charles, 2002; Rimet et al., 2004; Tison et al., 2005; Feio et al., 2007; Rimet, 2009; Hawkins et al. 2010; Almeida and Feio, 2012). As expected, the pressure variables treated as filters excluded few taxa when set to predicted values in the absence of anthropogenic influence (reference values). In spite of their importance, some of the filters we applied are not direct determinants of diatom and macroinvertebrate community composition but rather surrogate predictors (Clarke et al., 2003). For example, mean annual air temperature, obtained from cartographic sources, was used here as a surrogate of water temperature and was responsible for the total exclusion of *Frustulia erifuga* and frequent exclusion of *Melosira varians* from the diatom reference lowland communities. Prediction directly from the annual range of water temperature would

have been more desirable, but would have required installing electronic temperature loggers (Clarke et al., 2003). It is also possible that the modelled reference nitrate value is too high (as discussed above), and consequently sometimes wrongly excluded taxa with low upper tolerance limits for nitrate from predictions under reference conditions.

#### Index development and quality assessment

For diatoms, application of the new multimetric index to all sites produced strong correlations with the pressure variables, providing a more comprehensive assessment of biological quality than the IPS (significant correlations with two PCA axes cf. only one axis). This improvement in performance was probably due to the inclusion of the number of sensitive species, as indicated by the high correlation of this metric with the PCA axes.

Despite the limitations we encountered (*e.g.*, in using boosted trees with some environmental variables), our hypothesis was supported. All of the samples from sites considered to be in LDC were rated as poorer biological quality with the reference values derived from the present work than with the existing reference values, indicating that the new reference values created more stringent criteria. This difference was more evident for macroinvertebrates, as almost all the samples considered to be in LDC were assessed as having high biological quality with the old reference values but poor biological quality with the new values. With the old system, the macroinvertebrate index rated many samples in a higher lower quality class than the IPS (EQR) did, but with the new system this difference was reversed.

With the new methods, the assessments based on diatoms and macroinvertebrates became more similar, suggesting that the filters approach accounted for pressures that are presently affecting both diatom and macroinvertebrate communities in the lowland streams. However, differences in assessment between the two communities were evident for some samples, possibly because of differences in the responses of diatoms and macroinvertebrates to particular stressors. For example, sample 10 from site M09 was assessed as moderate by diatom analysis (Figure 3.7) and poor by the macroinvertebrate analysis (Figure 3.8). In this case the macroinvertebrate community may have responded to changes in the riparian vegetation and morphological condition of the site while the diatoms may have reacted to minor changes in water chemistry. Feio et al. (2007) also found the same site could be assessed differently by predictive models applied to diatom and macroinvertebrate communities.

Despite the results obtained by this new method that were, according to our expertise knowledge, closer to the reality of lowland streams and rivers it is useful to remain aware

that all the methodological decision that we made can affect the stream assessment and its true uncertainty (Clarke, 2013).

Stream communities are controlled mainly by physical and chemical factors (Chessman and Royal, 2004; Lange et al., 2011), but biotic interactions such as competition and predation, which were not explored in this work, can also influence the composition of the communities. Therefore, additional filters related to biotic factors may be a useful inclusion in future development of the approach (Chessman and Royal, 2004).

### **3.5 Conclusions**

This study brings new insight to help solve the problem of the lack of acceptable reference sites. Additionally, the new multimetric index for diatoms seems to hold much promise as there was a good correlation between the number of sensitive species and anthropogenic pressures. Although data limitations prevented us from modelling abiotic variables other than nitrate successfully, we believe that such modelling has great future potential. And despite the wealth of available information on the ecological preferences of diatom and macroinvertebrates, their tolerance limits are still far from fully documented. More work is necessary in this respect, including microcosm or mesocosm experiments that enable a high degree of control of environmental factors.

#### **Abbreviations**

|      |   |
|------|---|
| BQE  | Biological Quality Elements                   |
| BT   | Boosted Trees                                 |
| EQR  | Ecological Quality Ratios                     |
| EU   | European Union                                |
| IBD  | <i>Indice Biologique Diatomées</i>            |
| IPS  | <i>Indice de Polluosensibilité Spécifique</i> |
| IPtI | <i>Índice Português de Invertebrados</i>      |
| LDC  | Least Disturbed Condition                     |
| MDI  | Multimetric Diatom Index                      |
| MDS  | non-metric Multidimensional Scaling           |

MSE Mean Squared Errors

PCA Principal Components Analysis

R<sup>2</sup> Square of the correlation coefficient

WFD Water Framework Directive

### Acknowledgements

This study was possible due to the financial support of the Foundation for Science and Technology (Portugal) through the projects AQUAWEB (PTDC/AAC-AMB/105297/2008), FRAMEFFECTIVE (PTDC/AAC-AMB/105411/2008), UID/MAR/04292/2013, UID/GEO/04035/2013 and programmes POPH and QREN (FSE and national funds of MEC) and the Ph.D scholarship SFRH/BD/68973/2010 of the first author. We would like to thank the Portuguese Water Institute (INAG I.P., now Agência Portuguesa do Ambiente) for making data available, to the logistic support at IMAR-CMA, Department of Life Sciences, University of Coimbra, and at GeoBioTec Research Centre and Biology Department of Aveiro, University of Aveiro.

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Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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## **Chapter 4 - From perennial to temporary streams: an extreme drought as a driving force of freshwater communities' traits**

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Published in Marine and Freshwater Research 2015, 66, 469-480.

### **Abstract**

Alterations in trait proportions of diatom and aquatic macroinvertebrate communities were used to evaluate the consequences of the drying of temperate perennial streams due to an uncommon drought event. Four Atlantic-temperate Portuguese streams were sampled on three occasions; spring before the drought, two weeks after the return of water to the streambed, and spring after the drought. The traits from diatom and macroinvertebrate communities from temperate streams were also compared with those from Mediterranean systems to assess possible shifts in traits. The effects from stream drying were long-lasting for macroinvertebrates; however, only the diatom trait proportions shifted towards proportions similar to those occurring in Mediterranean streams. There was a significant reduction of macroinvertebrates with the ability to produce cocoons, to disperse actively by flight and passively disperse in the water and of swimmers following the return of water. A decrease in stalked species immediately after drought was evident in diatom communities. In contrast to temperate communities, Mediterranean ones were characterised by larger and less mobile diatoms, and smaller-bodied macroinvertebrates with shorter life cycles. Additional studies are required to determine the long-term effect of uncharacteristic drought events on biological traits and their ecosystem functions in typically perennial streams.

### **Keywords**

Climatic changes, diatoms, extreme events, macroinvertebrates, Mediterranean, streams, temperate, traits



#### 4.1 Introduction

Climate forecasts predict a doubling in atmospheric carbon dioxide (CO<sub>2</sub>) concentration by the end of this century and a rise in world mean air temperature between 1.8 and 4.0 °C (IPCC, 2007). In central Europe, heat waves are predicted to be more frequent, more intense and longer in duration. Heavy winter precipitation is also predicted to increase in central and northern Europe, and decrease in the south (IPCC, 2007; IPCC, 2013). The southern part of Europe will also suffer a predicted decrease in summer precipitation (Beniston et al., 2007). These changes in precipitation, temperature and consequently evapotranspiration rates will affect volumes of surface water runoff. Model simulations predict a 10-30% decrease in annual runoff in southern Africa, southern Europe and in the western United States by the mid-century (Milly et al., 2005; IPCC, 2007). In contrast, other models predict a decrease in annual runoff in southern Europe that may reach as much as 50% of the current levels (Arnell, 1999). Typically temperate and rainy climate areas (*e.g.*, central Europe) will become more similar to Mediterranean climate areas that are characterised by mild wet winters and hot dry summers (Giorgi and Lionello, 2008), thus suggesting its expansion to northern regions.

In south-western Europe, Portugal was affected by a severe and uncharacteristic drought event from the end of summer 2011 to the winter of 2012. February 2012 was the driest February recorded since 1931, with a total monthly precipitation of 1-10 mm against a long-term mean of 50-150 mm (IPMA, I.P.; <http://www.ipma.pt>, accessed 8 May 2012). The low precipitation recorded during this drought event led to the complete drying of surface water in river channels of some typically Atlantic-temperate watercourses. Drought events with flow cessation are characteristic of Mediterranean climates where streams are physically, chemically and biologically shaped by sequential, predictable and seasonal events of flooding and drying over an annual hydrological cycle (Boix et al., 2010; García-Roger et al., 2011; Delgado et al., 2012). For Atlantic-temperate areas, such flow conditions are uncommon.

Floods and droughts are important hydrologic events in running water ecosystems. Therefore, in-stream biological communities have developed adaptations that range from the avoidance of individual floods or droughts, to life-history strategies that are synchronised with long-term flow patterns (Lytle and Poff, 2004). The traits developed by species in order to enable them to survive, exploit and even depend on disturbance are the result of a long evolutionary period (Lytle and Poff, 2004). In Mediterranean streams during the summer, organisms exhibit strategies such as low metabolic rates, short life spans with an early

maturity, less permeable cuticles and higher water content (Bonada et al., 2007a). That is, the native communities of drought-prone systems are the result of a multihabitat filter process that selects organisms with a set of traits that enable them to either survive the drought (resistance traits) or recolonise and recruit after the drought breaks (resilience traits) (Poff, 1997; Bond et al., 2008). However, the communities of rivers exposed to rapid changes in natural flow conditions (*i.e.*, magnitude, frequency, duration, timing and predictability) are deprived of such an evolutionary period. Changes in the natural flow of temperate streams due to extreme events or climate change may therefore exert stronger effects on the ecological structure and functioning of stream communities (Lake, 2000).

The use of biological traits in the study of aquatic macroinvertebrate communities has increased in the last decade (*e.g.*, Gayraud et al., 2003; Bonada et al., 2007a; Chessman, 2012; Feio and Dolédec, 2012; García-Roger et al., 2013) as they can be related to ecosystem functions (*e.g.*, decomposition, productivity, energy cycling, relationship between different trophic levels) and thus act as potential indicators of a system's integrity (Statzner et al., 2001; Feio and Dolédec, 2012). However, to our knowledge, only the study by Bonada et al. (2007a) was aimed at assessing the trait differences between Mediterranean and temperate regions and only for aquatic macroinvertebrate communities. More recently, García-Roger et al. (2013) assessed trait differences between streams with different types of long-term aquatic regimes, but all within the Mediterranean climate area. Despite the recognition of the importance of traits to predict community composition in phytoplankton ecology (Litchman and Klausmeier, 2008), interest in trait approaches applied to benthic diatoms is relatively recent (Passy, 2007; Stevenson et al., 2008; Berthon et al., 2011; Rimet and Bouchez, 2011) and linked to changes caused by the degradation of stream quality, such as altered nutrient levels, current velocity and sediment regimes (Passy, 2007; Stevenson et al., 2008; Berthon et al., 2011; Rimet and Bouchez, 2011).

In this study, the biological traits of both diatom and macroinvertebrate communities were used to test the hypotheses that a severe drought event, with the complete drying of the stream channel, leads the aquatic communities in the Atlantic-temperate streams to: (1) react to stress by changing their traits and consequently, the traits that confer them an advantage during drought are found in higher proportions, whereas those that constitute a disadvantage during drought are found in lower proportions; and (2) shift trait composition to become more similar to communities found in the Mediterranean streams. Moreover, we test the hypothesis (3) that hydric stress and posterior recovery of primary producers with short life cycles (diatoms) will be faster in comparison to consumers with longer life cycles (macroinvertebrates).

## **4.2 Material and methods**

### **4.2.1 Study sites**

Four small perennial lowland streams, located in the central region of Portugal were sampled between 2011 and 2012: Ribeira de Boialvo (Tb), Rio da Serra (Ts), Ribeira de Ança (Ta) and Rio Alcoa (Tl), which are located in Vouga, Mondego and Ribeiras do Oeste catchments (Figure 4.1). These catchments are within an Atlantic-temperate climate area characterised by mild temperatures, moderate summers and winters, and total annual precipitation above 2,800 mm·year<sup>-1</sup> (Agência Portuguesa do Ambiente, 2007). A similar geology characterised by flatlands of sedimentary origin (as limestone and sand) can be found throughout the study area.

For comparative purposes, four small streams located in a Mediterranean climate area were also selected for this study. Diatom and macroinvertebrate communities of those Mediterranean streams were collected in spring 2004 following the same procedures described for temperate streams. The sampling sites are located in the streams Ribeira do Vidigão (Mv), Ribeira de Oeiras (Mo), Ribeira de Grândola (Mg) and Ribeira da Capelinha (Mc), which belong to the Guadiana, Sado and Mira catchments (Figure 4.1). These temporary streams are located in southern Portugal where the climate is typically Mediterranean, with mild wet winters and hot dry summers, and a mean annual precipitation below 600mm year<sup>-1</sup> (Agência Portuguesa do Ambiente, 2007).

The Mediterranean streams were selected with the aim of having geomorphic and anthropogenic pollution characteristics similar to each other and to the Atlantic-temperate streams. Therefore, the main difference between the Atlantic-temperate and Mediterranean streams (hereinafter referred as T and M) is the climate and, consequently, their natural long-term hydrological regime (perennial and temporary).

### **4.2.2 Climate characterisation of the study period in the Atlantic-temperate area**

Shifts in flow regime and groundwater recharge are determined by changes in temperature, evaporation and precipitation (Chiew, 2006; IPCC, 2012). Atlantic-temperate streams are small lowland water courses and therefore changes in precipitation are rapidly reflected in stream water flow. Mean monthly precipitation in the temperate and Mediterranean streams was obtained using gridded data from a dense network of gauge stations distributed across the study areas (IPMA, I.P.; <http://www.ipma.pt>; Belo-Pereira et al., 2011). The high summer (July, August September) temperatures of 2011 (average ~20 °C) associated with the low precipitation values during the same period led to the complete

drying of some stream channels in a typical Atlantic-temperate area (Figure 4.2a). These low values, similar to those recorded in the summer in the Mediterranean area (Figure 4.2b), remained during autumn (October, November and December) and winter (January, February and March). Only the rainy period recorded in November led to a resumption of flow in the temperate streams (Figure 4.2a).

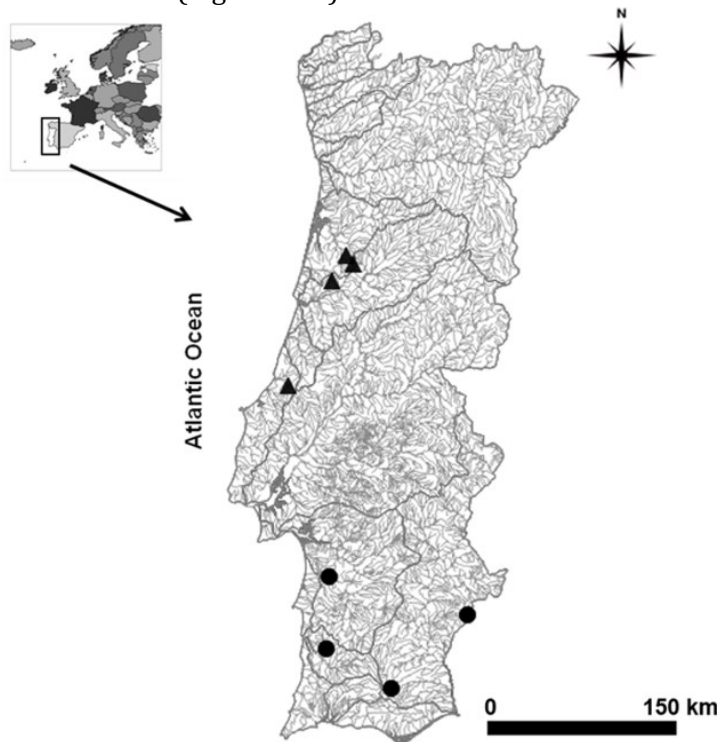


Figure 4.1 - Location of the study sites in continental Portugal in the Atlantic-temperate climate (black triangles) and in the Mediterranean climate (black circles).

#### 4.2.3 Sample collection and processing

The temperate (T) streams were sampled on three occasions: spring 2011 (T1), before the severe and uncharacteristic drought event; autumn 2011 (TAD), 2 weeks after the return of water to the stream channel; and spring 2012 (T2) after the drought period when normal flow conditions were re-established (Figure 4.2a). Hereafter, the use of the term 'drought' refers to the event that caused the complete drying of the streambeds. Sampling for biological communities was always performed when the streams had visible flowing water.

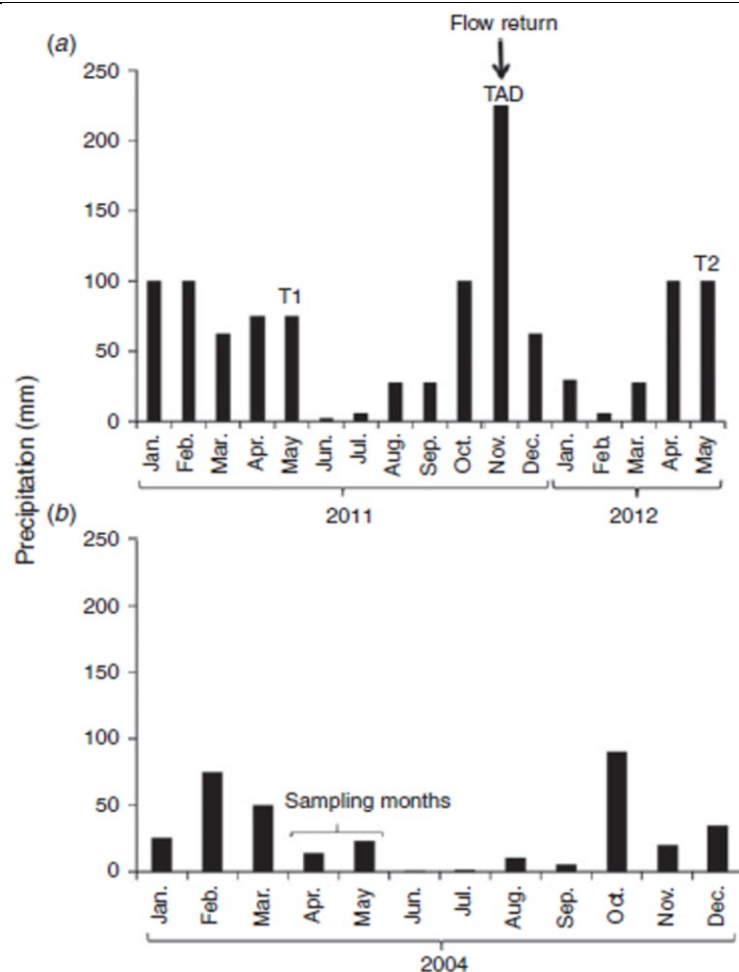


Figure 4.2 - Mean precipitation of the (a) Atlantic-temperate climate and (b) Mediterranean climate sites recorded during the study. Sampling occasions: T1, temperate spring before drought; TAD, temperate first sample after water return; T2, temperate spring after drought. Data from the Portuguese Institute of Sea and Atmosphere (IPMA, I.P.; <http://www.ipma.pt>).

Sampling and treatment of diatoms was performed according to the European standards (European Committee for Standardisation, 2003, 2004, 2006). In each site, hard substrate (rocks or stones) was sampled when present; otherwise, sediment was sampled. Sampling of sediment instead of hard substrate was acceptable in light of a study by Mendes et al. (2012), which validated the use of alternative substrates. For the epilithic biofilm, five random submerged stones (an area of  $\sim 100 \text{ cm}^2$ ) were collected and gently washed with running water. The upper surface was then scraped with a toothbrush. For the epipsammon biofilm (site Ta), a volume of  $\sim 50 \text{ mL}$  of the upper surface of the streambed sediment was collected using a syringe. Samples were preserved with formaldehyde (4%). In order to ensure ecological reliability of the samples, the proportion of living and dead cells was verified under a light microscope. The samples were then oxidised with concentrated nitric acid and potassium dichromate for 24 hours. Thereafter, permanent slides were mounted using

Naphrax® (Brunel Microscopes Ltd, Chippenham, UK). Using a light microscope (100x objective and 1.32 numerical aperture), ~400 diatom valves for each sample were counted and identified to species or infraspecific rank mainly using the floras of Krammer and Lange-Bertalot's (1986, 1988, 1991a, 1991b) and Krammer (2000, 2001, 2009).

Benthic macroinvertebrates were sampled following a multihabitat approach according to the procedures described by the Portuguese Water Institute (INAG I.P.; INAG, 2008). Briefly, each sample was collected by kicking and sweeping approximately 1 m towards upstream with a hand-net (500 µm mesh size; 0.25×0.25 m width) and composed of six composite subsamples corresponding to the most representative habitats (*e.g.*, stones, sand, submerged macrophytes). Macroinvertebrate samples were preserved in formaldehyde (4%) and, after sorting, preserved in ethanol (75%) for later identification and counting. Taxonomic identification, under a stereomicroscope (magnification 60x), was mostly done to genus level with the exception of Chironomidae (subfamily or tribe level).

#### 4.2.4 Biological traits

For this study, we selected diatom and macroinvertebrate biological traits that we predicted would be affected (positively or negatively) by drought. For diatom communities, two biological traits were selected (Table 4.1): life form and biovolume. Within the life-form trait, four categories were considered: mobile, colonial, tube-forming and stalked. The taxa with mobility have the physical ability to select the most suitable habitat. The colonial taxa are in permanent contact with each other and therefore are more capable of surviving with less moisture due to interstitial spaces between the individual cells. The tube-forming life-form is a kind of colonial life-form; however, the diatoms live in a mucilaginous protective case within which they are able to move freely (Berthon et al., 2011; Rimet and Bouchez, 2011, 2012). The stalked taxa have the physical ability to be fixed to the substrate through mucilage stalks, which exposes them more to desiccation. Within the life-form trait, one taxon can be assigned to more than one category. The second trait selected was biovolume, with the categories: small species (5-299 µm<sup>3</sup>) and large species (≥1500 µm<sup>3</sup>). Intermediate biovolume categories were neglected, as we considered that differences caused by the drought would be more evident in small and large species. The higher the differences in the surface-to-volume ratio of the cells, the larger the differences found in the trait proportions of these two categories. The biovolume categories were adapted from a study carried out by Berthon et al. (2011), and the values used were those found in the OMNDIA software (version 5.3) (Lecointe et al., 1993). We predicted that the categories that confer higher resistance or resilience (*e.g.*, mobile, colonial, tube forming and small size) would be favoured by the

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

drought event (Table 4.1). The trait categories that confer lower resistance or resilience (*e.g.*, stalked and large species) would be disadvantaged by the drought (Table 4.1).

Table 4.1 - Predictions and justification for expected behaviour of diatom trait categories in response to an extreme drought event in temperate rivers. Arrows indicate changes in category proportion expected in the temperate samples after drought (TAD and T2) compared with temperate samples before drought (T1) (up arrow, higher proportion; down arrow, lower proportion).

| Trait     | Category                                  |   | Justification   |
|-----------|---|---|---|
| Life form | Mobile                                    | ↑ | Physical capacity to move to the most suitable habitat (refuge use and recolonisation).   |
|           | Colonial                                  | ↑ | Better resistance capacity, higher capacity to live with less moisture due to interstitial spaces between the individual cells.                         |
|           | Tube forming                              | ↑ | Physical capacity to produce a mucilaginous protective substance that confers higher resistance to drought.   |
|           | Stalked                                   | ↓ | Higher cell surface exposed to physical alterations of the habitat induced by the drought (desiccation).  |
| Biovolume | 5-299 $\mu\text{m}^3$ (small species)     | ↑ | Better resilience capacity and higher capacity to live with less moisture. Small species have large local population density, that prevents extinction. |
|           | $\geq 1500 \mu\text{m}^3$ (large species) | ↓ | Higher cell surface exposed reduces their capacity to live with less moisture.  |

For macroinvertebrate communities, seven biological traits were considered here to be more affected by the drought (Table 4.2): maximum body size, life cycle duration, reproductive cycles *per* year, dispersal, locomotion, respiration and resistance forms. Within each trait, several categories were also considered, as shown in Table 4.2. Our predictions for the selected trait categories responses were based on the hypothesis that the categories allowing resilience to disturbances with rapid population growth (*e.g.*, small body size, short life cycles, fast reproductive cycles), behavioural and physiological adaptations to escape and avoid desiccation (*e.g.*, aerial dispersal, aerial respiration, resistance forms against desiccation) would be favoured by the drought event (Bonada et al, 2007a; Dolédec and Statzner, 2008; Feio and Dolédec, 2012; García-Roger et al., 2013). Trait information was derived according to the available biological information databases described in Tachet et al. (2002), for macroinvertebrate genera (and Diptera at family or subfamily level). Only the trait categories with a high affinity (affinity 3 or above, using a fuzzy coding approach, Chevenet et al. 1994) for a given category were considered in order to match the approach used for diatoms, for which affinity scores are not available.

Table 4.2 - Predictions and justification for expected behaviour of macroinvertebrate trait categories in response to an extreme drought event in temperate rivers. Arrows indicate changes in category proportion expected in the temperate samples after drought (TAD and T2) compared with temperate samples before drought (T1) (up arrow, higher proportion; down arrow, lower proportion).

| Trait               | Category              |   | Justification   |
|---------------------|-----------------------|---|---|
| Maximum body size   | <5 mm (small)         | ↑ | Better resilience capacity for smaller sizes.   |
|                     | 20–80 mm (large)      | ↓ |   |
| Life cycle duration | ≤1 year               | ↑ | Shorter cycles improve resilience capacity.   |
| Reproductive cycle  | <1 year <sup>-1</sup> | ↓ | Better resilience capacity with more frequent reproduction.   |
|                     | >1 year <sup>-1</sup> | ↑ |   |
| Dispersal           | Aerial active         | ↑ | Flight enables these animals to be the first to arrive after the flow return.   |
|                     | Aquatic passive       | ↓ | Animals do not drift from above locations due to flow cessation.  |
| Locomotion          | Crawler               | ↑ | Release from action of flow favours crawling.   |
|                     | Swimmer               | ↓ | Flow cessation or bed dryness disfavors swimmers. With lack of water there is no environment for swimmers locomotion. |
| Respiration         | Aerial                | ↑ | Aerial respiration is favoured with aquatic oxygen depletion resulting from dryness.                                  |
|                     | Gills                 | ↑ | Specialised structures are essential to maximise the oxygen uptake in aquatic depletion conditions.                   |
| Resistance forms    | Cocoons               | ↑ | Increased resistance against droughts.  |
|                     | None                  | ↓ | Absence of resistance form against droughts.  |

#### 4.2.5 Statistical analyses

The communities' data consisted of diatom relative abundances  $\geq 1\%$  and macroinvertebrate relative abundances  $> 0.02\%$ . For both groups of organisms, taxa present in only one sample were not considered. Using this approach, we reduced the effect of errors in identification, sample contamination and avoided the effect of very rare taxa that could create a confounding effect in our analyses while preventing the observation of clear multivariate patterns. Abundance data were fourth-root-transformed to down-weight the contributions of abundant taxa (García-Roger et al., 2011). For diatoms, this transformation gives more weight to large species that are often found at low relative abundances in benthic diatom communities, and which can be important for defining diatom assemblages (ter Braak and Verdonschot, 1995; Tison et al., 2005).



The taxa that contributed the most to the Bray-Curtis dissimilarity (up to 80% cumulative contribution) between T1 and TAD, and between T1 and T2, were determined through SIMPER analysis (PRIMER 6 and PERMANOVA+) for both diatom and macroinvertebrate communities. All of the taxa obtained from SIMPER analysis were then assigned to the different trait categories. The relative abundance of each taxon was added to obtain the total proportion for each trait category on each sampling occasion (T1, TAD, T2 and M). The relative abundance of each taxon was always added in total to the trait categories that it was assigned to.

In order to assess differences in groups of samples (T1, TAD, M and T1, T2, M) concerning trait proportions, Multidimensional Scaling Analyses (MDS) were run separately for diatoms and macroinvertebrates, based on a Bray-Curtis similarity matrix (PRIMER 6 and PERMANOVA+; data arcsine square root transformed). Statistical differences between the groups of samples (T1, TAD, T2 and M) were tested with a PERMANOVA pairwise test with unrestricted permutations (PRIMER 6 and PERMANOVA+) for diatoms and macroinvertebrates.

To test our predictions and determine the traits responsible for the differences in communities between the initial status (temperate samples before drought-T1) and after drought (T2 and TAD) two Canonical Analyses of Principal Coordinates (CAP) were run for diatoms and for macroinvertebrates. In addition, to determine the traits responsible for possible differences between the communities in the temperate and Mediterranean streams, we ran two CAP analyses between temperate samples before drought (T1) and Mediterranean (M) samples for both macroinvertebrates and diatoms. The CAP provides a constrained ordination diagram by finding the axis (or axes) in the principal coordinate space that is best for discriminating between the *a priori* groups. Finally, overlaying vectors were added to the CAP ordination showing which traits had the highest correlations with the CAP axes (Pearson correlation > 0.5). The PCA analyses were performed with the PRIMER 6 and PERMANOVA+ software.

For each individual trait category, two one-way ANOVA tests (SigmaPlot 12.0 software; data arcsine square-root-transformed) were carried out to verify statistical significant differences between groups (T1, TAD, M and T1, T2, M). When normality tests and equal variance tests failed, Kruskal-Wallis one-way ANOVA tests (ANOVA on ranks) were used. ANOVAs were followed by pairwise comparisons (Tukey's test) to assess which groups of samples were different.

### 4.3 Results

#### 4.3.1 Trait patterns of diatom communities

##### Effects immediately after the drought

The MDS performed with the diatom trait proportions revealed a segregation of the diatom communities in the Mediterranean streams from those in the temperate streams (T1- before the drought and TAD-2 weeks after the return of water) (Figure 4.3a). However, there was a higher similarity of the Mediterranean (M) communities to those immediately after drought (Bray-Curtis similarity, TAD-M=64% vs. T1-M=60%). In fact, the PERMANOVA pairwise test indicated that there were significant differences only between T1 and Mediterranean samples (T1-M:  $t=4.496$ ,  $p<0.050$ ).

Through the CAP, we verified that species with the trait categories 'stalked', 'mobile', 'colonial' and 'small' were those contributing more to the segregation between T1 and TAD (Figure 4.4a). These species were negatively affected (decreased in abundance) by the drought. Nevertheless, from these trait categories, only the stalked species were significantly different between T1 and TAD. The remaining trait categories only presented statistical differences between temperate and Mediterranean communities (Table 4.3). In general, the temperate streams recorded higher proportions of mobile and small species and lower proportions of large species (Table 4.3).

Table 4.3 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different diatom biological trait categories of communities T1 (temperate spring before drought), TAD (first sample after water return) and M (Mediterranean). Only the trait categories where statistical differences were verified shown. Relationships shown in bold have  $p<0.05$ .

| Trait category                               | Test result | p value  | Relationship between groups                      |
|--|-------------|----------|--|
| Mobile                                       | F = 6.549   | $<0.050$ | T1 = TAD; TAD = M; <b>T1 &gt; M</b> (Tukey test) |
| Stalked                                      | F = 16.465  | $<0.010$ | <b>T1 &gt; TAD = M</b> (Tukey test)              |
| 5-299 $\mu\text{m}^3$<br>(small species)     | F = 8.511   | $<0.050$ | <b>T1 = TAD &gt; M</b> (Tukey test)              |
| $\geq 1500 \mu\text{m}^3$<br>(large species) | F = 14.605  | $<0.050$ | <b>T1 = TAD &lt; M</b> (Tukey test)              |

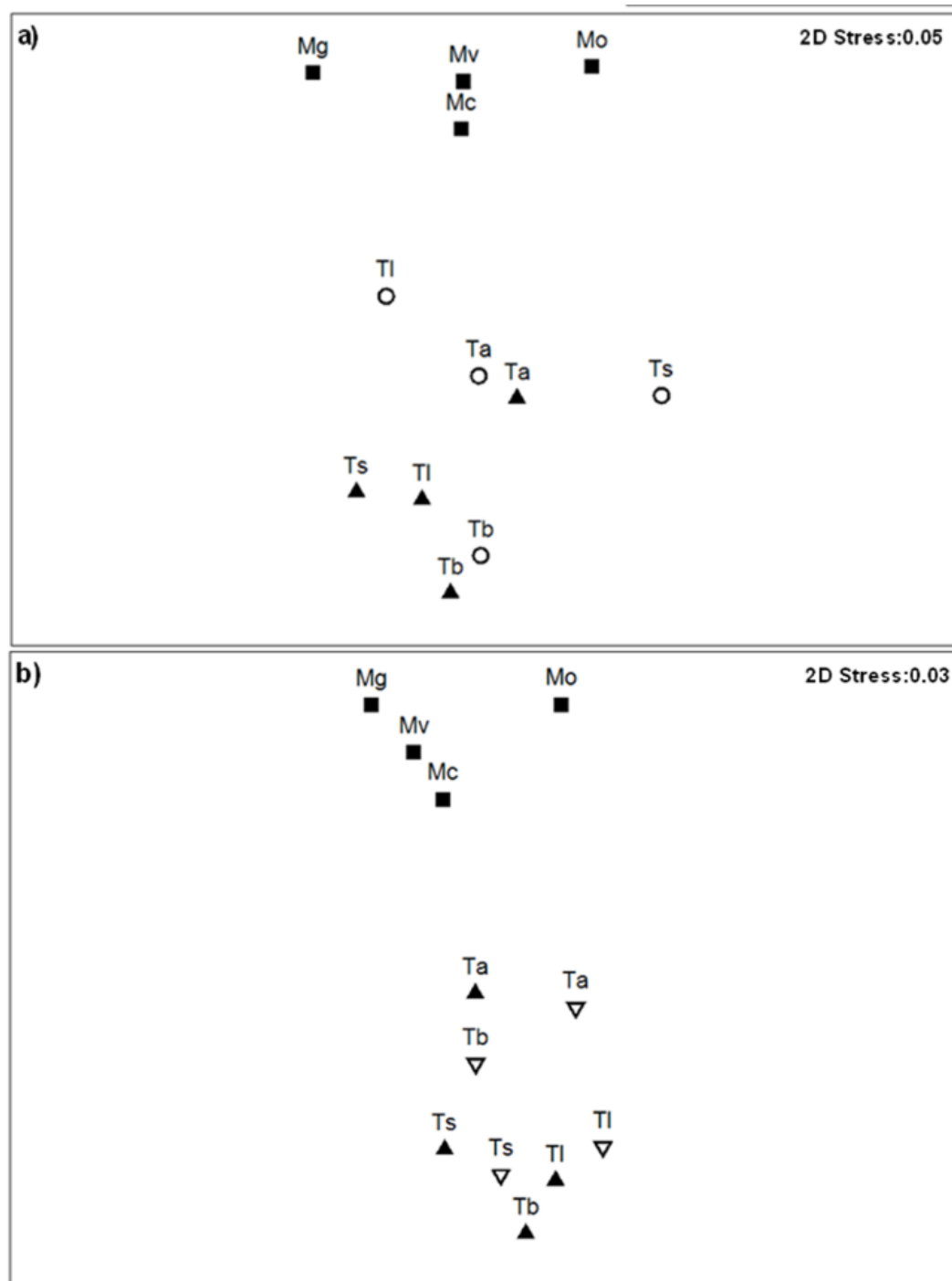


Figure 4.3 - Multidimensional Scaling Analysis (MDS) ordinations of the diatom traits for the groups of samples: (a) temperate spring before drought (T1), first temperate sample after water return (TAD) and Mediterranean spring (M); and (b) temperate spring before drought (T1), temperate spring after drought (T2) and Mediterranean spring (M). T1, black triangle; TAD, white circle; T2, white triangle; M, black square.

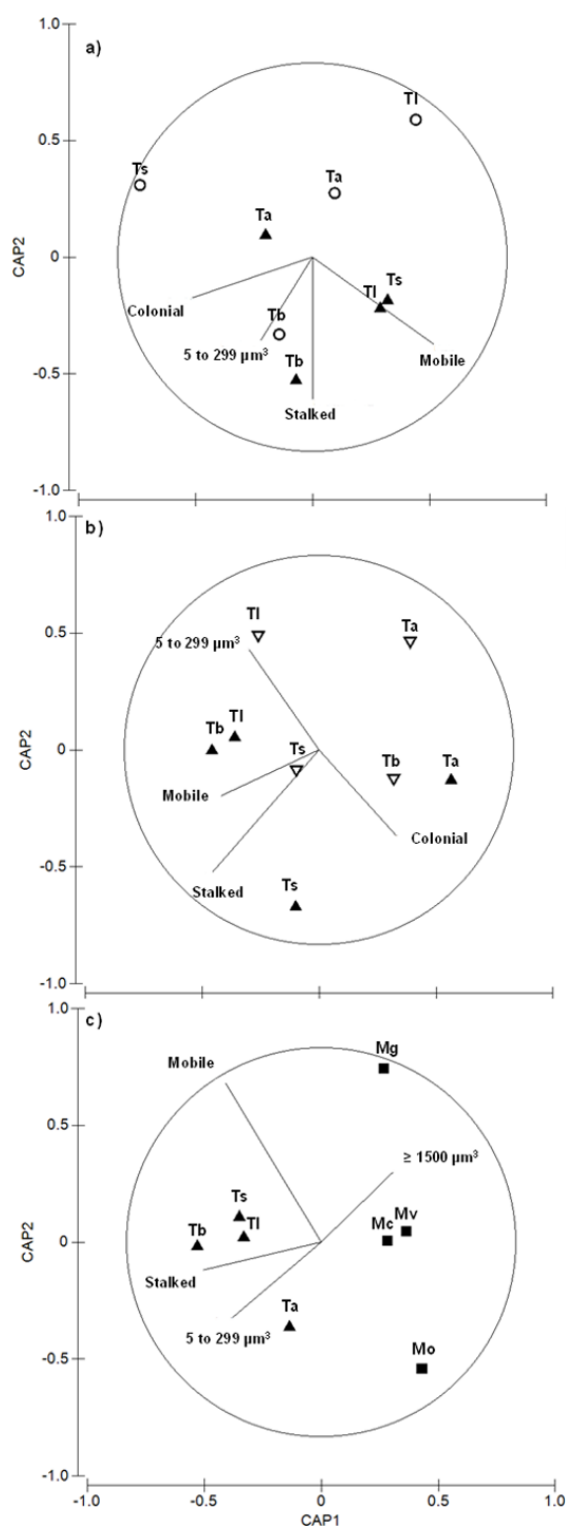


Figure 4.4 - Canonical Analysis of Principal Coordinates (CAP) based on proportions of diatom traits found in: (a) temperate spring before drought (T1) and first temperate sample after water return (TAD); (b) temperate spring before drought (T1) and after drought (T2); and (c) temperate spring before drought (T1) and Mediterranean streams. T1, black triangle; TAD, white circle; T2, white triangle; M, black square.

#### Effects over 1 year

Comparing the two temperate groups of samples (T1-before the drought, and T2-spring after the drought) with the Mediterranean spring, we verified an increase in the similarity of the temperate communities (Figure 4.3b). This increase in the similarity between the temperate samples (T1-TAD=79% vs. T1-T2=84%, Bray-Curtis similarity) suggested a recovery of diatom communities. Additionally, the PERMANOVA pairwise test confirmed significant differences between T1 and M ( $t=4.400$ ,  $p<0.050$ ) and between T2 and M ( $t=4.750$ ,  $p<0.050$ ). Therefore, despite the similarity between T2 and M (63%, Bray-Curtis similarity) being comparable to the one recorded immediately after drought (TAD-M=64%), the spring communities affected by the drought (T2) were already different from the M communities. From TAD to T2, we verified that the temperate communities shifted from being more similar to M to becoming similar to T1.

Similar to the MDS (Figure 4.3b), the CAP analyses also showed a smaller distinction between the temperate spring groups (T1 and T2) in comparison with T1 and TAD (Figure 4.4a, b). No trait category was significantly different among the communities from these groups of samples (T1 and T2) (Table 4.4). Statistical differences were present only between the temperate and Mediterranean communities (Table 4.4). In fact, a CAP analysis performed on the T1 and M samples revealed that there were trait categories with a higher contribution for the differences between the Mediterranean and temperate streams (Figure 4.4c). The Mediterranean communities appeared to be related to higher proportions of larger species and the temperate communities were related to higher proportions of smaller, mobile and stalked species (Table 4.4).

Table 4.4 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different diatom biological trait categories of communities T1 (temperate spring before drought), T2 (temperate spring after drought) and M(Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have  $p<0.05$ .

| Trait category                               | Test result | p value | Relationship between groups        |
|--|-------------|---------|------------------------------------|
| Mobile                                       | F = 8.900   | <0.050  | <b>T1 = T2 &gt; M</b> (Tukey test) |
| Stalked                                      | F = 9.023   | <0.010  | <b>T1 = T2 &gt; M</b> (Tukey test) |
| 5-299 $\mu\text{m}^3$<br>(small species)     | F = 9.187   | <0.010  | <b>T1 = T2 &gt; M</b> (Tukey test) |
| $\geq 1500 \mu\text{m}^3$<br>(large species) | F = 12.544  | <0.010  | <b>T1 = T2 &lt; M</b> (Tukey test) |

### 4.3.2 Trait patterns of macroinvertebrate communities

#### Effects immediately after the drought

The MDS performed with all macroinvertebrate trait proportions from T1, TAD and M (Figure 4.5a) showed segregation of the three groups. This segregation was statistically confirmed by the PERMANOVA pairwise test results (T1-TAD,  $t=2.047$ ,  $p<0.050$ ; T1-M,  $t=1.543$ ,  $p<0.050$ ; TAD-M,  $t=2.116$ ,  $p<0.050$ ). However, TAD samples appeared more separated and presented a lower similarity with the remaining groups of samples (T1-TAD=56%; TAD-M=64%, Bray-Curtis similarity). In fact, TAD similarities were even lower than the similarity found between T1 and M (73%). The proportions of some traits in the after drought communities (TAD) were affected by the drought, but did not shift towards the Mediterranean communities (Figure 4.5a; Table 4.5).

Several traits contributed to the differences found in the temperate communities after drought (Figure 4.6a). There was a decrease in swimmers, animals with shorter life cycles, aerial active and aquatic passive dispersion or cocoons as a resistance form and an increase in animals with extreme sizes (small (<5mm) and large animals (20-80 mm)) and animals breathing through gills. However, from these traits, only the proportion of swimmers, cocoons as a resistance form, aerial active and aquatic passive dispersion were significantly lower after the drought (Table 4.5).

Table 4.5 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different macroinvertebrate trait categories of communities T1 (temperate spring before drought), TAD (temperate first sample after water return) and M (Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have  $p<0.05$ .

| Trait category              | Test result | p value | Relationship between groups                       |
|-----------------------------|-------------|---------|---|
| Aerial active (dispersal)   | F = 10.589  | <0.010  | <b>T1 &gt; TAD</b> ; T1 = M; TAD = M (Tukey test) |
| Aquatic passive (dispersal) | F = 4.513   | <0.050  | <b>T1 &gt; TAD</b> ; T1 = M; TAD = M (Tukey test) |
| Swimmer (locomotion)        | F = 10.951  | <0.010  | <b>T1 = M &gt; TAD</b> (Tukey test)               |
| Cocoons (resistance form)   | H = 8.375   | <0.010  | <b>T1 &gt; TAD</b> ; T1 = M; TAD = M (Tukey test) |



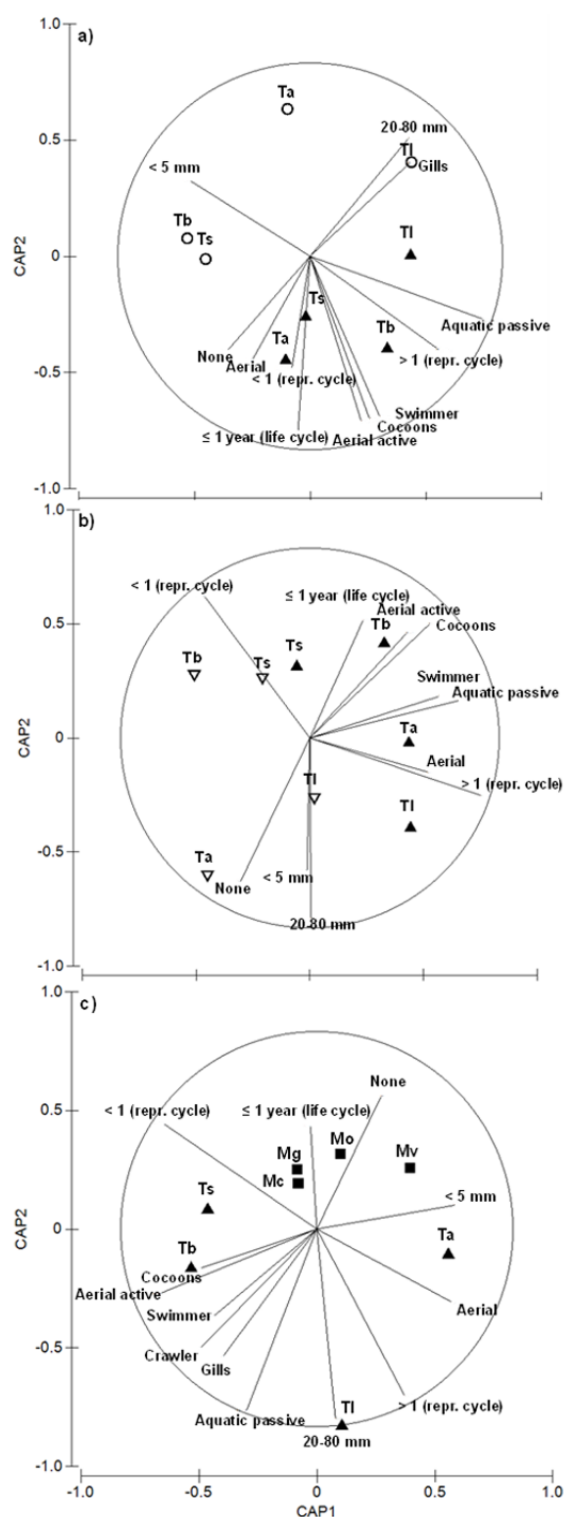


Figure 4.6 – Canonical Analysis of Principal Coordinates (CAP) based on proportions of macroinvertebrate traits found in: (a) temperate spring before drought (T1) and first temperate sample after water return (TAD); (b) temperate spring before drought (T1) and after drought (T2); (c) temperate spring before drought (T1) and Mediterranean streams. T1, black triangle; TAD, white circle; T2, white triangle; M, black square.



#### Effects over 1 year

The comparison of the proportions of macroinvertebrate trait categories found in T1, T2 and M communities revealed a change in the T2 communities towards the initial T1 state (Figure 4.5b). The Bray-Curtis similarity between T1 and T2 (65%) was higher than the similarity between T1 and TAD (56%). The spring samples affected by the drought (T2) also became more similar to the Mediterranean samples (75%). In fact, there were statistical differences only between T1 and M (PERMANOVA pairwise test:  $t=1.577$ ,  $p<0.050$ ).

Despite the shift of T2 communities to the initial state, there were some traits contributing to the segregation between T1 and T2 (Figure 4.6b). From the trait categories affected by the drought in TAD, only the aquatic passive trait was able to recover in T2 to proportions similar to T1 (Table 4.6). The species with the traits 'aerial active', 'swimmers' and 'animals with cocoons' still presented a significantly lower proportion in T2 than before the drought. However, despite the absence of statistical differences within trait categories, it was possible to verify some changes over time. For example, concerning the trait 'small maximum body size' (<5mm), we verified that there was a tendency for the trait proportion from TAD to T2 to become similar to T1 (Figure 4.6a, b).

The CAP contrasting temperate and Mediterranean samples (Figure 4.6c) showed more similarities within Mediterranean samples than within temperate samples of the first spring (T1). The Mediterranean streams had higher proportions of species with no resistance form, shorter life cycles and small sizes, and a reduced proportion in swimmers, animals with aquatic passive dispersion, crawlers, animals breathing with gills, larger animals and cocoons as resistance form. However, individually, we found significant differences only between T1 and M in one trait category (large body size) (Table 4.6).

Table 4.6 - Results of the one-way Analysis of Variance (ANOVA, F) and ANOVA on ranks (Kruskal-Wallis test, H) carried out for the different macroinvertebrate trait categories of communities T1 (temperate spring before drought), T2 (temperate spring after drought) and M (Mediterranean). Only the trait categories where statistical differences were verified are shown. Relationships shown in bold have  $p<0.05$ .

| Trait category              | Test result | p value | Relationship between groups                     |
|-----------------------------|-------------|---------|---|
| 20-80mm (maximum body size) | H = 7.489   | <0.020  | <b>T1 = T2 &gt; M</b> (Tukey test)              |
| Aerial active (dispersal)   | F = 5.214   | <0.050  | <b>T1 &gt; T2</b> ; T1 = M; T2 = M (Tukey test) |
| Swimmer (locomotion)        | F = 7.413   | <0.020  | <b>T1 &gt; T2</b> ; T2 = M; T1 = M (Tukey test) |
| Cocoons (resistance form)   | F = 7.379   | <0.020  | <b>T1 &gt; T2</b> ; T2 = M; T1 = M (Tukey test) |

#### 4.4 Discussion

Macroinvertebrate and diatom communities from typically perennial temperate rivers were affected by the complete drying of the stream channels caused by a drought event, confirming our first hypothesis. This uncharacteristic event led to changes in the trait proportions of the diatom and aquatic macroinvertebrate communities of these streams for at least a 6-month period. However, diatoms and macroinvertebrates had different responses to the drought. In accordance with the second hypothesis, the drought led to a shift in the diatom trait proportions towards proportions similar to those found in Mediterranean communities. However, shortly after the return of water to the channel (TAD) most of the communities' traits did not present significant differences with the temperate communities before the drought (T1). In fact, the diatom communities from temporary rivers are known for their ability to rapidly recolonise a dry river after the water return (Robson et al., 2008). In spite of that, the temperate communities still presented some differences in the following spring (T2) as some traits did not return to their predrought proportions (*i.e.*, 'colonial' and 'stalked') (Figure 4.7). This may reveal more long-lasting changes resulting from the drought but also natural inter-annual variability.

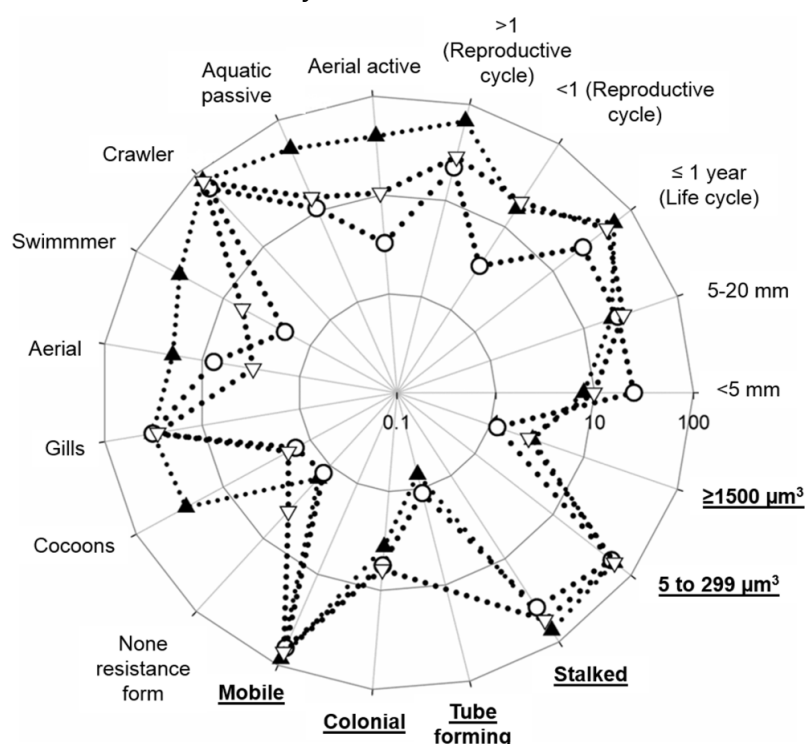


Figure 4.7 - Mean relative abundance of diatom (bold underlined) and macroinvertebrate traits found in the three groups of samples: black triangle, temperate spring before drought (T1); white circles, first temperate sample after water return (TAD); white triangle, temperate spring after drought (T2). Logarithmic scale.

Community attributes for macroinvertebrates just after rewetting were very different from those found in the spring of 2011. However, contrary to our hypothesis, the postdrought communities were different from the Mediterranean ones. This difference could be partially due to natural seasonal patterns of macroinvertebrates (autumn and spring); however, previous studies have already demonstrated the seasonal stability of biological traits in aquatic communities (Bêche et al., 2006). Differences are most probably associated with the drought event itself. Immediately after a stress condition (*i.e.*, drought), it is expected that only the organisms with traits that are able to pass the strong abiotic filters imposed by this stress will be found in the streams (Poff, 1997; Bêche et al., 2006). Indeed, there was a significant decrease in the proportions of some trait categories with the drought event: 'aerial active' and 'aquatic passive dispersion', and 'swimmers' and 'cocoons as a resistance form' for macroinvertebrates. However, no trait category disappeared. The trait 'swimmer' was the only one for which statistical differences were observed between the temperate-after-drought and Mediterranean communities. However, this trait proportion was not statistically different from the Mediterranean and predrought communities.

Despite the confirmation of our first hypothesis, the predictions for the different diatom and macroinvertebrate trait categories were not always confirmed. We expected that a drought event, with flow cessation, would be sufficient for the trait proportions of the communities to change from being adapted to the temperate stream conditions and becoming closer to Mediterranean stream conditions where communities are adapted to droughts; however, that did not occur. We had predicted that the postdrought (both TAD and T2) and Mediterranean samples would have a higher proportion of colonial diatom species. The complex architecture of the colonies (Snoeijs et al., 2002) would allow them a better resistance capacity to the drought. Also, Passy (2002) suggested that increased morphological complexity in colonial diatoms is a possible evolutionary strategy for survival in unpredictable environments. However, we found colonial diatom species such as *Fragilaria vaucheriae* (Kützinger) J. B. Petersen, *Fragilaria capucina* Desmazières and *Melosira varians* C. Agardh in all temperate samples, and in none of the Mediterranean samples. This may have occurred because the colonies from loosely attached mats, which are particularly vulnerable to disturbance (*e.g.*, floods) and may lack traits that enable the retention of interstitial space (Ledger et al., 2008). Therefore, colonial species may have reached high proportions early in the colonisation process after the drought event, but were rapidly replaced by other more competitive species. Therefore, these were not highly represented in the sampled TAD, T2 and M communities.

The diatom species with the ability to move ('mobile' category) have the advantage of being able to select the most suitable habitat (Passy, 2007), finding refuges and thus a faster

recolonising capacity when the basal flow conditions are re-established. Therefore, we expected that species with this trait category would be present in higher proportions in the after drought (TAD and T2) and Mediterranean samples as they would be among the first colonisers. However, our predictions were not supported; in fact, the drought appears to have negatively affected the mobile taxa, and samples from the temperate streams (more stable) tend to have a higher proportion of mobile species than the Mediterranean streams (more unstable). This may be explained by the inability of such taxa to resist to the dislodgement by shear stress imposed by the elevated water currents (Peterson, 1996) of the seasonal events of flooding that are also typical in Mediterranean streams.

A previous study (Virtanen et al., 2011) indicated that diatom taxa with attachment capability ('stalk' category) can resist the water current effects. Here, we predicted that this resistance capacity would be a disadvantage following a drought event due to their inability to seek protection from desiccation. Indeed, this prediction was confirmed as there was a statistically significant reduction of this trait after the drought (TAD). However, in the Mediterranean streams, this trait was found in lower proportions (even though not significantly different from the temperate streams), which may indicate that this is not a relevant adaptation in such systems. We predicted that diatoms with the tube-forming trait category would be able to overcome the cessation of water. The relative abundances of the taxa with this trait category were always very low in the temperate streams (T1 and TAD), and in temperate stream (T2) and Mediterranean streams, these taxa were not present at all. This suggests that our prediction for the trait category 'tube forming' was probably incorrect, as streams typically affected by droughts (Mediterranean streams) did not record taxa with this trait.

The ability of macroinvertebrates to disperse actively by flight was predicted to be an advantage in drought disturbance situations. Individuals with the ability to fly would be able to depart with the drying of the streambed in order to find a suitable habitat, and would consequently be the first colonisers after the water return (*e.g.*, Gasith and Resh, 1999). In fact, the decrease in those animals after the drought may have been due to an active escape to more suitable conditions. However, there was no return of macroinvertebrates with this trait during the study. Another hypothesis is that the macroinvertebrates with aerial dispersion in fact disappeared, as the aerial stage phase was not synchronised with the unpredicted drought period. In this case, it is very difficult to test these predictions without evaluating the phenomenon itself during its occurrence and assessing a larger area surrounding the stream through the capture of flying adults. Regarding aquatic passive dispersion and swimming locomotion, we also confirmed our predictions (Figure 4.7). The proportions of these trait categories were negatively affected by the drought as 'drift' or 'swim' became impracticable with the restricted flow and persistence of the dry bed that acted as a barrier. However, what

we did not expect was that the proportions of taxa with swimming locomotion after drought would be different from those found in the Mediterranean streams. In fact, García-Roger et al. (2013) also verified positive associations between the trait categories 'aquatic passive' and full water 'swimmers' with permanent Mediterranean climate streams and non-significant association with intermittent streams with summer dry channels. Additionally, Bonada et al. (2007b) verified that, in intermittent sites, the organisms were mostly fliers and surface swimmers. For the trait 'cocoons' as a resistance form we verified an unexpected decrease in temperate communities after the drought. It was expected that in a stable environment, such as perennial streams, the production of drought-resistant forms would be unnecessary in comparison with an unstable one, such as temporary streams (García-Roger et al., 2013). This contradiction could again be due to the unpredictability of the drought against the adaptation of communities to predictable changes in flow in Mediterranean streams. However, caution should be applied in the interpretation of the trait category 'cocoons' as a resistance form, as the taxon *Baetis* was the only one contributing to this trait category. Higher proportions were found in T1 samples than in those from after the drought (TAD and T2) and also in Mediterranean samples (M). In fact, these higher proportions of *Baetis* in the predrought temperate streams are in agreement with the results obtained by García-Roger et al. (2011), who found that the family Baetidae was characteristic of perennial streams and also indicative of the riffle mesohabitat of Mediterranean streams.

For both elements of body size category, our predictions were not statistically confirmed. According to Virtanen et al. (2011) there are several mechanisms through which body size and temporal occurrence may be interrelated: (1) the short life cycles and fast reproduction for small organisms may increase their probability to occur at a site because of low extinction risk; (2) small-bodied organisms have the ability to disperse efficiently to another site; (3) small organisms have very large local population density, which prevents extinctions; and (4) the smaller the body size, the more susceptible the species is to environmental physical changes. However, contrary to our prediction, the small diatom species appeared to be more associated with typically more stable environments (T1). This is more evident when we look to the Mediterranean communities. These communities had significantly lower proportions of small diatom species and higher proportions of large species. Therefore, the physical variability (unstable environments) of Mediterranean streams appeared to favour larger diatoms. In contrast, higher proportions of small macroinvertebrates appeared more associated with the hydrologically unstable Mediterranean streams. This result is in accordance with the findings of other authors who stated that small sizes are favoured in more unstable environments (Dolédec and Statzner, 2008; Feio and Dolédec, 2012; García-Roger et al., 2013). Significant differences in the

proportions of macroinvertebrate sizes in temperate streams following the drought event were not evident. However, there was a tendency for the after-drought samples (TAD) to be more highly correlated with both small and large macroinvertebrates. In the samples collected in the spring affected by the drought (T2), this correlation was no longer so evident, suggesting a recovery of the temperate streams with respect to macroinvertebrate sizes. More reproduction cycles *per* unit of time are necessary to mitigate extreme disturbance effects in the resistance of the community (Dolédéc and Statzner, 2008). In agreement with this, individuals that reproduce more slowly ( $<1 \text{ year}^{-1}$ ) were disadvantaged by the drought. Therefore, they were less abundant after rewetting, but the differences were not significant.

Regarding the respiration trait, none of our predictions were confirmed. However, for the trait category 'gills', we found a higher correlation of this trait with some of the after-drought samples (TAD). The drought seems to have caused an increase in the proportions of animals breathing by gills in some of the sites. In contrast, the results obtained by García-Roger et al. (2013), carried out only in Mediterranean climate streams, indicate a positive correlation of this trait category but only with permanent streams. Concerning the trait 'aerial respiration' there was an opposite tendency from that found in the trait 'gills'. The temperate after-drought samples (TAD) correlated less with this trait. As in other cases, this may be related to a poor resistance to drought of other traits of these taxa, which led to its disappearance, even though a certain trait (*e.g.*, aerial respiration) would confer an advantage. Also, the absence of statistically significant differences may be due to a high variability within temperate sites.

The macroinvertebrates with the capacity to crawl were the ones present in higher proportions in all groups of samples (temperate and Mediterranean). We predicted that this trait category would be favoured by the drought. However, our prediction was not confirmed, as the crawlers appeared more associated with the predrought temperate samples than with the Mediterranean samples. This association of the macroinvertebrates with crawler locomotion with temperate streams, rather than with Mediterranean ones, is in accordance with the findings of Bonada et al. (2007a).

The two biological elements of the study (diatoms and macroinvertebrates) appeared to have different responses and recovery times to the drought event, confirming our third hypothesis. Both were affected by the same drought period, yet macroinvertebrate traits recovered more slowly, suggesting that, at least partially, the ecosystem functioning was altered for a longer period. For example, primary production may have been restored rapidly due to the absence of some grazers and scrapers that were not able to resist drought. Therefore, the lack of top-down control may have caused interference in transference of energy and matter across the food chains. In the spring of 2012, some trait categories of the macroinvertebrate communities still occurred in different proportions to those found in the

spring of 2011. Other studies showed that, globally, community post-recovery processes after drought may last for over a few months or extend for much longer periods (*e.g.*, Mosisch, 2001; Death, 2008). However, despite the visibility of some drought effects, ~2 weeks after water resumption (TAD), diatoms in the temperate rivers reached trait proportions close to those of spring 2011. These results indicate the importance of the study of both communities for a more accurate assessment of unexpected drought events, and, in order to detect differences in diatom communities, the sampling should occur less than two weeks after the recovery of flow.

#### **4.5 Conclusions**

Further research is needed to establish a more complete database on diatom traits to be used in similar studies, as information on these traits is scarce compared to those for macroinvertebrates. Moreover, we conclude that traits that confer resistance to unexpected events, such as drought, are not necessarily the same as those that characterise a typically unstable environment where droughts (and floods) are predictable. In these unstable environments, community traits are the result of a long evolutionary process towards adaptation. More studies are needed to fully understand the mechanisms behind functional responses of both microalgae and macroinvertebrates to extreme droughts.

#### **Abbreviations**

|     |   |
|-----|---|
| CAP | Canonical Analyses of Principal Coordinates   |
| M   | Samples from Mediterranean streams  |
| MDS | non-metric Multidimensional Scaling   |
| PCA | Principal Components Analysis   |
| T1  | Samples from Temperate streams collected in spring 2011, before the severe and uncharacteristic drought event                     |
| T2  | Samples from Temperate streams collected in spring 2012, after the drought period when normal flow conditions were re-established |
| TAD | Samples from Temperate streams in autumn 2011, 2 weeks after the return of water to the stream channel                            |

### **Acknowledgement**

This study was made owing to funding by the 'Foundation for Science and Technology', Portugal, through the AQUAWEb project (PTDC/AACAMB/105297/2008), national funds MEC, and the program POPH – QREN and the PhD scholarship SFRH/BD/68973/2010 to C. L. Elias. We acknowledge the Institute of Marine Research (IMAR) and GeoBioTec Research Unit and Biology Department of the University of Aveiro for support.

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## Chapter 5 - Extreme drought effect and recovery patterns in benthic communities of temperate streams

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Published in *Limnetica* 2014, 33, 281-296.

### Abstract

Portugal faced an uncharacteristic hydrological drought in the fall/winter of 2011-2012. Small, typically perennial streams were affected by this extreme event and many dried out. Five of these streams were examined during six sampling events between spring 2011 (pre-drought) and spring 2012 (post-drought) to analyse the effects of this disturbance on the macroinvertebrate and diatom communities. Two weeks after dewatering, macroinvertebrate metrics exhibited accentuated decreases in the total abundance, EPT taxa and the scores of the '*Índice Português de Invertebrados*' but exhibited increases in equitability. The post-drought diatom assemblages showed no significant difference in abundance or evenness in relation to pre-drought conditions, but the diatom quality index ('*Indice de Polluosensibilité Spécifique*' - IPS) decreased. Four weeks after the drought, the invertebrate communities progressively recovered, whereas the diatom metrics were already at pre-drought values, except for the IPS, which improved slowly over time. The benthic communities recovered faster in streams with higher Habitat Quality Assessment scores (HQA). The EPT taxa, *Echinogammarus* spp., *Hydroporus* spp. and *Ancylus fluviatilis*, did not recover to pre-drought values, whereas Lumbricidae and Orthocladinae increased. For diatoms between the pre- and post-drought years, there was a shift from more sensitive to more tolerant taxa (e.g., the disappearance of *Cocconeis euglypta* Ehrenberg and the appearance of *Nitzschia palea* (Kützinger) W. Smith. This study showed that an extreme off-season drought had immediate effects on both of the analysed benthic communities, but the diatoms recovered faster. Over one year, none of the studied benthic communities returned to the same pre-drought conditions, but the differences were stronger for invertebrates. In

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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both cases, the *a priori* habitat condition of streams appeared to control the reaction and recovery patterns of the benthic communities to drought in the studied temperate streams.

### **Keywords**

Diatoms, invertebrates, climate change, dewatering, precipitation

## 5.1 Introduction

The increasing atmospheric concentrations of greenhouse gases and aerosols due to anthropogenic activities have been causing climate change (Forster et al., 2007), which have global implications for all ecosystems. Sea levels are expected to continue rising as the snow and ice extent decrease (IPCC, 2012). In addition, precipitation amounts and patterns are changing, and there are major alterations in the timing of wet and dry seasons (Arnell, 1999a, b). Climate change consequences, such as higher temperatures, more frequent floods and drought, clearly need further attention from both scientists and managers (Bond et al., 2008).

Freshwater ecosystems are particularly vulnerable to climate change (Schindler, 1997; Heino et al., 2009; Whitehead et al., 2009). Shifts in river flow regimes and groundwater recharge are determined by changes in temperature, evaporation, and particularly precipitation (Chiew, 2006; IPCC, 2012). Modifications in river runoff will decrease the recharging of groundwater supplies (Mandal and Zhang, 2012; Thampi and Raneesh, 2012) and will be enhanced by increased evaporation rates. Moreover, this climatic alteration leads to an increase in extreme climate events such as floods and droughts which can be exacerbated by anthropogenic factors such as streambed alterations and deforestation (*e.g.*, Hauer et al., 1997). According to Christensen et al. (2007), diverse future climatic scenarios for Europe predict an increase in annual mean temperatures (more than the global mean) and greater heterogeneity in precipitation patterns; *e.g.*, in the increases in the annual precipitation are forecasted for northern Europe, whereas decreases are forecasted for southern Europe and Mediterranean. In addition, seasonal precipitation is expected to increase in winter and spring and decrease in summer and autumn (Johns et al., 2003; Giorgi et al., 2004).

The northern and central Portuguese coastline and adjacent regions have a temperate Atlantic climate with typically wet winters. Despite these climatic characteristics, the country experienced a severe and uncharacteristic drought in the autumn/winter period of 2011/2012. Throughout the territory, the total monthly precipitation observed in this period was extremely low when compared with the same seasons from previous years. More particularly, in central Portugal, the mean precipitation between October 2011 and March 2012 was of 58.5 mm, compared to 126.6 mm for the same period between 1980 and 2013, according the Portuguese National Information System of Hydric Resources (SNIRH at <http://snirh.pt>). In fact, February 2012 was the driest month since 1931, and the monthly precipitation was only 3.3 mm, which largely contrasts with the 128.6 mm registered for the monthly average in February of previous years (period of 1980 to 2013; SNIRH; <http://snirh.pt>). This absence of precipitation led to a severe dewatering drought event in



some streams of the Portuguese Atlantic humid climate. The intermittence of flow is characteristic of Mediterranean streams where communities have adaptations for desiccation (Steinman and McIntire, 1990; Lake, 2003; Bonada et al., 2006) but constitutes an anomaly in Atlantic temperate areas, where streams are typically perennial.

In previous studies, extreme droughts have caused sharp decreases in the total biomass of aquatic insects (*e.g.*, Walters et al., 2011) and alterations in assemblages (*e.g.*, Thomson et al., 2012), as well as triggered species loss and the collapse of food webs, with important decreases in secondary production (Ledger et al., 2011). Indeed, rapid or unpredicted drying does not provide the necessary amount of time for development of desiccation-resistant structures or physiological adjustments (Stanley et al., 2004). In this case, the ability of biota to recover from drought relies on the environment features (*e.g.*, substratum type; Wright et al., 2003), availability of refugia (*e.g.*, Gasith and Resh, 1999), intensity and/or duration of the hydrological event (*e.g.*, Lake, 2003), and the taxonomic assemblage considered (*e.g.*, Acuña et al., 2005; Ledger et al., 2008; Boinx et al., 2010). Primary producers with short life cycles, such as diatoms, usually recolonise faster after a disturbance when compared with secondary producers (*e.g.*, invertebrates) with longer life cycles (Gasith and Resh, 1999).

Other studies have addressed the impact of extreme climatic events such as droughts in shaping communities of freshwater systems under an intermittent hydrological regime (*e.g.*, Bond et al., 2008; Feio et al., 2010; Marchetti et al., 2011; Thomson et al., 2012). However, little is known about the effects of extreme and uncharacteristic off-season dewatering events (but see Caramujo et al., 2008) in the benthic communities of temperate streams.

This study aims to analyse the effect of an unusual (seasonally and geographically) extreme dewatering drought event in Atlantic temperate streams by comparatively assessing the recover responses of two distinct benthic communities (macroinvertebrates and diatoms) from the progression of dewatering to rewetting.

## **5.2 Methods**

### **5.2.1 Study sites**

Five permanent streams were selected for this study, which are located near the coast of central Portugal (< 40 km from the Atlantic Ocean). This area has an Atlantic-humid temperate climate, with a mean annual precipitation within the period of 1971–2000 ranging from 800 to 1200 mm (Belo-Pereira et al., 2011; IPMA, Portuguese Institute of the Sea and Atmosphere at <http://www.ipma.pt>). These streams have mild temperatures and moderate summers and winters, and all five streams have similar environmental characteristics (size, altitude, geology). These streams, named Ribeira de Boialvo (Boialvo), Rio da Serra (Serra);

Ribeira de Eiras (Eiras); Ribeira de Ança (Ança); and Nascente do Rio Alcoa (Alcoa), are located in Mondego, Vouga and Ribeiras do Oeste catchments in the centre of Portugal (Figure 5.1). This study region is dominated by flatlands with sedimentary rocks (limestone and sand). The altitude of the study sites is relatively low, ranging from 36 (Ança) to 111 meters (Serra). The distance to source varied between 4.2 and 12.0 km. All sites are influenced by mild organic pollution, mainly derived from agriculture activities and housing.

In mid-fall 2011 (late October), all stream sites were found completely dry (without any visible water or remaining pools) because of an unusual climatological drought that occurred throughout 2011. The mean average precipitation registered until October was lower than the historical mean monthly precipitation for the period of 1980-2013, except for two months, May and August (Figure 5.2). In November 2011, a peak of precipitation occurred that allowed a break in the dewatering period in the streams; however, the flow level was still low. Afterwards, the recorded precipitation was still considered low in the following months in comparison to previous years (until March 2012; Figure 5.2), and the flow level was also low. The total monthly precipitation between October 2011 and March 2012 was 50% lower than the precipitation recorded in previous years for the same period (Figure 5.2). It was not until April/May 2012 that the streams appeared to recover to a typical average discharge for that season (based on the observed mean precipitation, which was closer to the historical reference values for the same period).

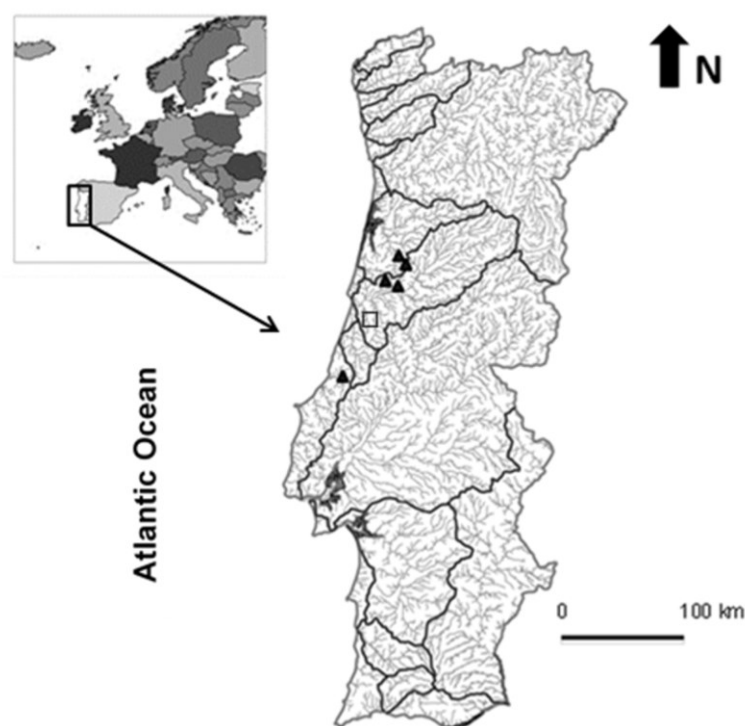


Figure 5.1 - Locations of the study stream sites in Portugal (black triangles) and the precipitation gauge station (square) used in this study.

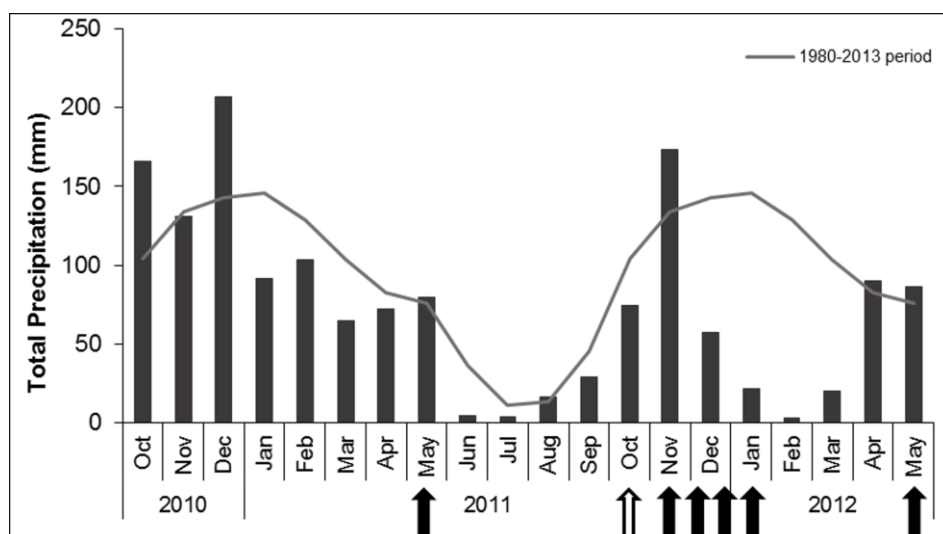


Figure 5.2 - Total monthly precipitation (mm) in the central territory region, Portugal, recorded during the study period (bars) and the historical mean monthly precipitation for the period of 1980-2013 (line). Black arrows represent the sampling events, and the open arrow indicate the time when the streams completely dried out.

### 5.2.2 Sample collection and processing

The streams were first sampled in spring 2011 (Sample 1-s1; May). This sample was considered as the pre-drought condition, as the selected streams were not yet completely dewatered. The post-drought condition was assessed by four biweekly sampling events after rewetting during winter (between November 2011 and January 2012; Sample 2-s2, Sample 3-s3, Sample 4-s4, Sample 5-s5) and then again in spring 2012 (Sample 6-s6; May; Figure 5.2). Sampling was only conducted when the streams had visible water in their streambeds. For each sampling event, measurements of pH, conductivity ( $\mu\text{S}\cdot\text{cm}^{-1}$ ), total dissolved oxygen (DO;  $\text{mg}\cdot\text{L}^{-1}$ ) and current velocity ( $\text{m}\cdot\text{s}^{-1}$ ) were performed *in situ* using field meters (Multiparameter Probe 3430 WTW®; Current Meter 108MKIII VALEPORT). The water samples were collected in spring 2011 and spring 2012 (s1 and s6) for laboratory determination of chemical oxygen demand (COD;  $\text{mg}\cdot\text{L}^{-1}$ ), biochemical oxygen demand (BOD<sub>5</sub>;  $\text{mg}\cdot\text{L}^{-1}$ ), phosphates ( $\text{PO}_4^{3-}$ ;  $\text{mg}\cdot\text{L}^{-1}$ ), total phosphorus (P;  $\text{mg}\cdot\text{L}^{-1}$ ), total nitrogen (N;  $\text{mg}\cdot\text{L}^{-1}$ ), nitrates ( $\text{NO}_3^-$ ;  $\text{mg}\cdot\text{L}^{-1}$ ) and alkalinity ( $\text{CaCO}_3$ ;  $\text{mg}\cdot\text{L}^{-1}$ ). In addition, all stream sites were geomorphologically characterized according to River Habitat Survey methodology (RHS - Environment Agency, 2003) to determine the Habitat Quality Assessment (HQA) scores. The HQA index reflects the overall habitat diversity through the assessment of the flow-types, diversity of channel substratum, in-channel vegetation, and the extent of bank-top trees and near-natural land-use adjacent to the river. The final HQA score results from the sum of the

individual scores attributed to those features; a higher score indicates a site with better habitat condition (36 is the Excellent-Good boundary for the HQA of littoral streams).

Macroinvertebrates were sampled with a hand-net (500  $\mu\text{m}$  mesh size) by kicking and sweeping the benthos, following a multi-habitat approach described in INAG (2008). Each sample was composed of six sub-samples distributed proportionally to the most representative habitats and defined by an area of 1 m  $\times$  0.25 m (hand net side). The samples were preserved in formaldehyde (4%) and conserved in ethanol (90%) after sorting for future identification and counting. Taxonomic identification using a stereomicroscope was typically performed to genus level; however, Chironomidae and Oligochaeta were identified to sub-family or tribe level.

The diatoms were sampled and processed based on European standards (European Committee for Standardisation, 2003, 2004, 2006) and on the recommendations of Kelly et al. (1998). Whenever stones (preferred sampled substrate) were absent or unavailable, stream sediment was sampled as a substitute. A previous study (Mendes et al., 2012) showed that alternative substrates can be used for water quality assessment in the absence of a certain substrate, disregarding substrate variability. The epilithic biofilm was scraped with a toothbrush from the upper surface of submerged stones, comprising an area about 100 cm<sup>2</sup>. For sampling epipsammon/epipelon, a syringe (50 mL) was used to collect streambed sediment. The samples were preserved with formaldehyde (4%) and oxidised using concentrated nitric acid and potassium dichromate for approximately 24 hours for organic matter digestion. The samples were mounted on permanent slides using Naphrax®. Under a light microscope (100  $\times$  objective and 1.32 numerical aperture), up to 400 diatom valves for each sample were counted and identified to species or infra-specific rank mainly using Krammer and Lange-Bertalot's floras (1986, 1988 and 1991a, b).

### 5.2.3 *Community analyses and biological metrics*

To assess differences in the macroinvertebrate and diatom communities between the different sampling events, non-parametric multidimensional scaling analysis (MDS) were run for each biological element based on a Bray-Curtis similarity matrix. We used taxa abundances from sites in spring 2011 pre-drought (s1) and spring 2012 post-drought (s6; diatom data transformed by fourth root). In addition, the ANOSIM global test (analysis of similarities; 126 permutations) was used to test for significant differences between the above groups of samples for both biological elements. ANOSIM is a non-parametric randomization based multivariate test analogous to a standard univariate ANOVA.

To determine the taxa contributing the most to the dissimilarity between pre-drought (May 2011: s1) and post drought conditions (May 2012: s6), a SIMPER analysis (with Bray-

Curtis similarity) was used based on the total abundance of diatoms and invertebrates for all sites at corresponding sampling date. In addition, widely used metrics were applied to evaluate eventual responses to drought. The metric values were represented by the mean ( $\pm$  standard error; SE) from all sites at each sampling event. The differences between sampling occasions were tested for each metric by running PERMANOVA pairwise tests (permutational univariate analysis of variance; based on Euclidean distance matrix; 999 permutations).

For macroinvertebrates, we used the following metrics: total number of individuals, richness (number of taxa), Pielou's evenness, EPT taxa, Coleoptera and Diptera richness (number of Families) and the multimetric index IPTI (*'Índice Português de Invertebrados'*). The IPTI was expressed in an Ecological Quality Ratios (EQR), in which the values represent the relation between the observed biological parameters in a river type and the expected for that same river type in reference conditions, assigning a quality classification as High, Good, Moderate, Poor and Bad, according to the Water Framework Directive (WFD; European Commission, 2000). For diatoms we used the following metrics: richness (number of taxa), Shannon-Wiener diversity index, Pielou's evenness and the IPS index (*'Índice de Poluosensibilidade Específica'*; Cemagref, 1982) expressed in EQR.

For the macroinvertebrates, the metrics and indices were determined using the Amiib@ software ([http://dqa.inag.pt/documenacaoficial\\_PORTUGAL\\_invertebradosbentonicos.html](http://dqa.inag.pt/documenacaoficial_PORTUGAL_invertebradosbentonicos.html)) developed by Instituto da Água, I.P., whereas the OMNIDIA software v. 5.3 (Lecointe et al., 1993) was used for the diatoms. All statistical tests were performed using PRIMER 6 & PERMANOVA+.

## 5.3 Results

### 5.3.1 Abiotic characterisation of the sites

The physical and chemical parameters recorded in the streams studied confirmed that they were all affected by mild organic pollution. The COD values ranged from 2.1 mg/L to 9.8 mg/L, and phosphates values ranged from 0.25 and 0.97 mg  $\text{PO}_4^{3-} \cdot \text{L}^{-1}$  (Table 5.1). The HQA scores varied from 22 to 42, indicating that the Eiras and Alcoa streams had a low habitat quality (lowest scores), whereas the Serra and Boialvo had a high (highest scores), thus revealing an excellent classification according to the Portuguese limits established for the types of rivers under study (INAG, 2009).

Table 5.1 - Mean values ( $\pm$  standard error) of the measured and calculated variables for each study site.

| Parameters   | Ribeira de Boialvo (B) | Ribeira de Eiras (E)   | Rio da Serra (S)      | Nascente do Rio Alcoa (Al) | Ribeira de Ança (An)   |
|--|------------------------|------------------------|-----------------------|----------------------------|------------------------|
| Latitude (y)   | 40.5844                | 40.254                 | 40.4124               | 39.5366                    | 40.2675                |
| Longitude (x)  | -8.3358                | -8.4238                | -8.3486               | -8.9457                    | -8.5153                |
| Distance to source (km)  | 7.1                    | 6.8                    | 6.9                   | 13                         | 12                     |
| Altitude (m)   | 44                     | 38                     | 111                   | 44                         | 36                     |
| Current velocity ( $\text{m}\cdot\text{s}^{-1}$ ) <sup>a</sup>                 | 0.38 ( $\pm 0.09$ )    | 0.41 ( $\pm 0.15$ )    | 0.76 ( $\pm 0.20$ )   | 0.78 ( $\pm 0.16$ )        | 0.04 ( $\pm 0.02$ )    |
| Depth (cm) <sup>a</sup>  | 21.00 ( $\pm 6.42$ )   | 8.80 ( $\pm 3.25$ )    | 21.60 ( $\pm 2.98$ )  | 25.50 ( $\pm 2.35$ )       | 36.33 ( $\pm 7.12$ )   |
| pH <sup>a</sup>  | 6.80 ( $\pm 0.05$ )    | 8.20 ( $\pm 0.14$ )    | 7.35 ( $\pm 0.24$ )   | 6.92 ( $\pm 0.04$ )        | 7.10 ( $\pm 0.08$ )    |
| Oxygen ( $\text{mg}\cdot\text{L}^{-1}$ ) <sup>a</sup>                          | 9.38 ( $\pm 0.36$ )    | 10.10 ( $\pm 0.64$ )   | 11.44 ( $\pm 1.07$ )  | 6.50 ( $\pm 0.18$ )        | 6.18 ( $\pm 0.94$ )    |
| Conductivity ( $\mu\text{S}\cdot\text{cm}^{-1}$ ) <sup>a</sup>                 | 132.72 ( $\pm 8.49$ )  | 275.40 ( $\pm 19.58$ ) | 83.78 ( $\pm 4.26$ )  | 619.33 ( $\pm 32.83$ )     | 697.17 ( $\pm 36.79$ ) |
| Chemical oxygen demand (COD; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup>      | 6.90 ( $\pm 1.42$ )    | 5.97 ( $\pm 0.54$ )    | 7.09 ( $\pm 2.69$ )   | < 2.1                      | 4.08 ( $\pm 3.03$ )    |
| Biochemical oxygen demand (BOD <sub>5</sub> ; $\text{mg}\cdot\text{L}^{-1}$ )  | < 3.3                  | < 3.3                  | < 3.3                 | < 3.3                      | < 3.3                  |
| Phosphates ( $\text{PO}_4^{3-}$ ; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup> | 0.50 ( $\pm 0.25$ )    | 0.45 ( $\pm 0.00$ )    | 0.64 ( $\pm 0.33$ )   | 0.33 ( $\pm 0.03$ )        | 0.46 ( $\pm 0.05$ )    |
| Total P (P; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup>                       | 0.39 ( $\pm 0.07$ )    | 0.49 ( $\pm 0.26$ )    | 0.69 ( $\pm 0.45$ )   | 0.34 ( $\pm 0.09$ )        | 0.46 ( $\pm 0.18$ )    |
| Total N (N; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup>                       | 0.64 ( $\pm 0.23$ )    | 0.23 ( $\pm 0.16$ )    | 0.28 ( $\pm 0.21$ )   | 0.26 ( $\pm 0.20$ )        | 0.13 ( $\pm 0.07$ )    |
| Nitrates ( $\text{NO}_3^-$ ; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup>      | 2.28 ( $\pm 0.58$ )    | 2.01 ( $\pm 0.24$ )    | 0.78 ( $\pm 0.41$ )   | 2.61 ( $\pm 0.38$ )        | 3.03 ( $\pm 0.63$ )    |
| Alcalinity ( $\text{CaCO}_3$ ; $\text{mg}\cdot\text{L}^{-1}$ ) <sup>b</sup>    | 116.35 ( $\pm 96.45$ ) | 71.54 ( $\pm 12.40$ )  | 70.35 ( $\pm 60.75$ ) | 208.85 ( $\pm 16.95$ )     | 282.35 ( $\pm 69.55$ ) |
| Habitat Quality Assessment (HQA score)   | 41                     | 22                     | 42                    | 24                         | 33                     |

<sup>a</sup> all sampling values; <sup>b</sup> spring values.

### 5.3.2 Macroinvertebrate patterns

A total of 114 different macroinvertebrate taxa were identified for all sites throughout the study, comprising a total of 26,631 individuals. Taxa such as *Ancylus fluviatilis* (Planorbidae; Gastropoda), *Baetis* spp. (Baetidae; Ephemeroptera), *Echinogammarus* spp. (Gammaridae; Amphipod), Orthocladiinae (Chironomidae; Diptera), Perlodidae (Plecoptera), *Oulimnius* spp. (Elmidae; Coleoptera) and Simuliidae (Diptera) were among the most abundant (above 1,100 total individuals).

The MDS (stress 0.11) analysis showed a partial segregation between macroinvertebrate communities from pre-drought (s1) and post-drought (s6) conditions; however, for two sites (Boialvo and Serra), the s1 and s6 communities were more similar to each other than in the other cases (Figure 5.3A). The ANOSIM test confirmed differences in macroinvertebrate community composition between the two sampling events: s1 was different from s6 (Global R = 0.30,  $p=0.02$ ), despite the high variability within groups.

The SIMPER analysis revealed 88.3% dissimilarity in the macroinvertebrate community between samples in the pre-drought year (s1) and the post-drought year (s6). This high dissimilarity was mainly due to a decrease in s6 (post-drought) of the mean abundance of taxa such as *Baetis* spp., *Echinogammarus* spp., *Ancylus fluviatilis*, Simuliidae, *Hydroporus* spp. (Dytiscidae; Coleoptera) and Perlodidae and an increase of Leptophebiidae, Oligochaeta, *Dugesia* spp. (Dugesiidae; Turbellaria) and Orthocladiinae (Table 5.2).

When analysing all sampling events, metrics such as the richness (number of taxa), number of EPT taxa, Coleoptera richness and IPTI index followed a similar short-term recovery pattern (Figure 5.4b, d, f, g), *i.e.*, values obtained in spring 2011 (s1) generally decreased after the drought event (s2) and progressively recovered over time along sampling events (s3, s4, s5) until spring 2012 (s6). For the remaining metrics (evenness and Diptera richness), the values increased or did not change immediately after the drought event (s2) and remained generally unchanged over 12 months until s6 (Figure 5.4c, e). However, when evaluating the significant differences among sampling events (PERMANOVA pair-wise test), the total number of individuals in s1 was different from s2 ( $t=3.48$ ;  $p=0.01$ ), s4 ( $t=2.47$ ;  $p=0.03$ ) and s6 ( $t=2.89$ ;  $p=0.01$ ). Moreover, the abundance in s2 was different from s5 ( $t=2.76$ ;  $p=0.01$ ). Further, the total number of macroinvertebrates decreased sharply by 91.5% from s1 to s2 and recovered by 83.7 % from s2 to s5. However, when comparing spring 2011 and spring 2012, the total number of macroinvertebrates decreased by 77.6% (from s1 to s6; Figure 5.4a).

Although we found no significant differences in richness ( $p>0.05$ , PERMANOVA), the richness decreased by 45.3% from s1 to s2 (sampling just after the drought event) but

recovered again in s5 (42.6 %). After one year (s6), the taxa richness was 12.5% lower compared with the pre-drought condition (s1; Figure 5.4b).

Pielou's evenness in s1 was different from s2 ( $t=3.90$ ;  $p=0.016$ ), s3 ( $t=2.86$ ;  $p=0.040$ ) and s6 ( $t=2.42$ ;  $p=0.038$ ) according to the PERMANOVA pair-wise test results. The evenness increased by an average of 30.5% for all sites from s1 to s2. Overall, from s1 to s6, the community evenness presented an increase of 23.7% (Figure 5.4c).

No significant differences were found in the EPT taxa and Coleoptera and Diptera richness ( $p>0.05$ , PERMANOVA pair-wise test) among the sampling events; however, a pattern was detected. In s2, rheophilic taxa such as Ephemeroptera, Plecoptera and Trichoptera decreased abruptly by 77.4% from s1 but progressively recovered over the duration of the sampling events, increasing by 73.1% until s5. After one year, these specific taxa had decreased 32.7% (from spring 2011 to spring 2012; Figure 5.4d). As for Diptera, in the majority of sites, the tendency was to maintain or increase the number of families (mean increased of 16.7%) after one year (Figure 5.4e). In addition, Diptera richness in s2 only decreased by 5 % when compared to s1. In the case of Coleoptera, the number of families decreased by 40.0% in s2 from s1 but recovered progressively by 38.8% until s5. Over one year of sampling, the Coleoptera richness decreased by approximately 10.0% from s1 to s6 (Figure 5.4f).

Finally, regarding the IPTI (macroinvertebrate index) given by the EQR values, the quality status in s1 was different from s2 ( $t=3.48$ ;  $p=0.01$ ), s4 ( $t=2.47$ ;  $p=0.03$ ) and s6 ( $t=2.89$ ;  $p=0.02$ ); this indicates that EQR values after one year (s6) had not recovered their initial status of high ecological quality. In addition, s2 was different from s5 ( $t=2.76$ ;  $p=0.01$ ). The lowest quality status for all streams was observed in s2 (first sampling just after the drought event), with a mean EQR value of 0.49 ( $\pm 0.09$ ), which indicates a moderate quality status (Figure 5.4g).

Table 5.2 - Macroinvertebrate taxa contributing 88.3% to the mean dissimilarity (SIMPER analysis) between s1 and s6 (spring 2011 pre-drought and spring 2012 post-drought, respectively) with their percentage contribution, average dissimilarity and standard deviation (SD). The increase (I) or decrease (D) in the mean abundance of each taxon from s1 to s6 is also shown.

| Taxon                      | Av. Diss. | SD Diss. | Contrib. % | I/D |
|----------------------------|-----------|----------|------------|-----|
| <i>Baetis</i> spp.         | 16.7      | 1.1      | 18.9       | D   |
| <i>Echinogammarus</i> spp. | 12.8      | 0.5      | 14.5       | D   |
| Simuliidae                 | 10.2      | 0.5      | 11.6       | D   |
| <i>Ancylus fluviatilis</i> | 9.1       | 0.5      | 10.3       | D   |
| Leptophlebiidae            | 6.2       | 0.8      | 6.4        | I   |
| Perlodidae                 | 4.8       | 0.5      | 5.2        | D   |
| Hydroporinae               | 3.3       | 0.7      | 3.6        | D   |
| <i>Ephemerella</i> spp.    | 3.3       | 0.7      | 3.6        | D   |



Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|                        |     |     |     |   |
|------------------------|-----|-----|-----|---|
| <i>Oligochaeta</i>     | 2.7 | 0.6 | 3.0 | I |
| <i>Dugesia</i> spp.    | 2.0 | 0.7 | 2.2 | I |
| Orthoclaadiinae        | 2.0 | 0.7 | 2.2 | I |
| <i>Physa</i> spp.      | 2.0 | 0.5 | 2.2 | D |
| Chironominae           | 1.8 | 0.6 | 2.0 | D |
| <i>Oulimnius</i> spp.  | 1.7 | 0.9 | 1.8 | D |
| <i>Ecdyonurus</i> spp. | 1.4 | 0.6 | 1.6 | I |

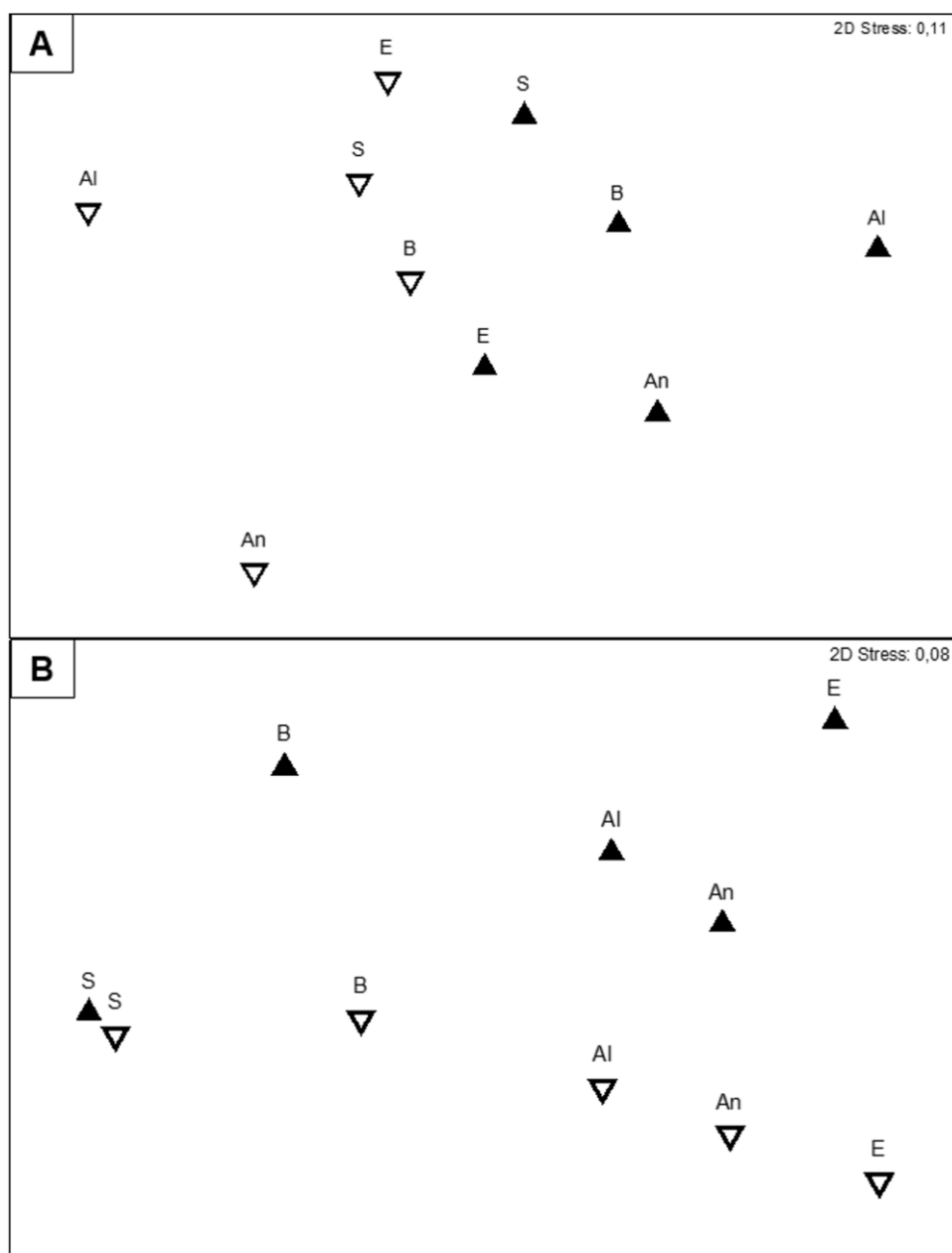


Figure 5.3 - Multidimensional Scaling Analysis ordination for the macroinvertebrate (A) and diatom (B) communities of all study sites sampled in the pre-drought (spring 2011, s1: black triangles) and post-drought (spring 2012, s6: white triangles) conditions. Site codes are also indicated (Al: Alcoa; An: Ança; B: Boialvo; E: Eiras and S: Serra).

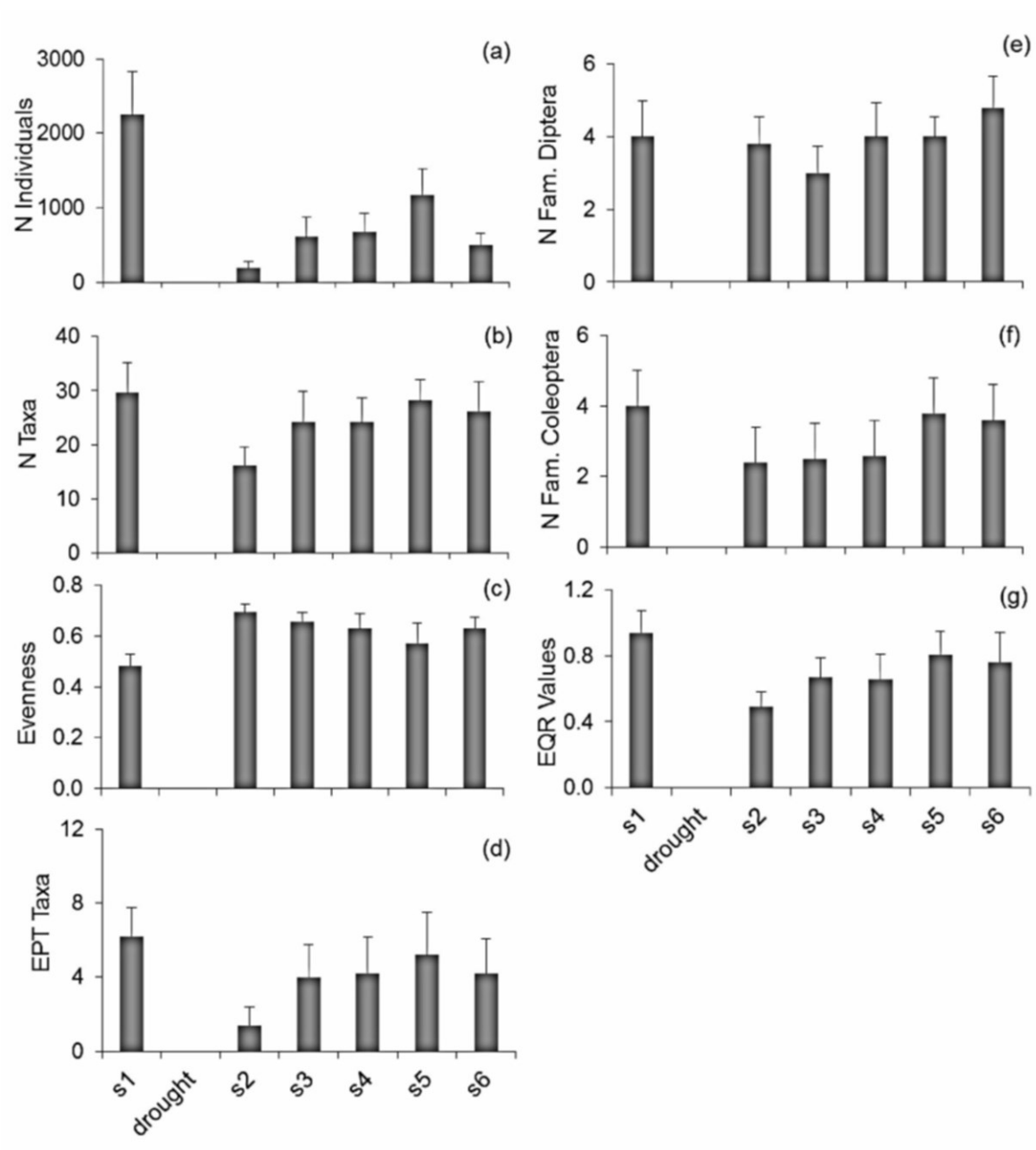


Figure 5.4 - Macroinvertebrate metrics for all study sites (mean  $\pm$  SE) at each sampling event from spring 2011 (s1) until spring 2012 (s6); a) total number of individuals, b) number of taxa (richness), c) Pielou's evenness, d) EPT taxa, e) number of Diptera families (Diptera richness), f) number of Coleoptera families (Coleoptera richness) and g) IPTi index (expressed in Ecological Quality Ratios).

### 5.3.3 *Diatom patterns*

A total of 181 diatom species were identified in all the samples. However, only 66 species presented a relative abundance above or equal to 1% in at least one sample. The

species *Achnantheidium minutissimum* (Kützing) Czarnecki, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, *Achnantheidium* cf. *kranzii* (Lange-Bertalot) Round & Bukhtiyarova and *Karayevia oblongella* (Østrup) Aboal were the most abundant.

The MDS (stress 0.08) showed an apparent segregation between s1 and s6 samples but also a high variability among sites, except for the Serra site (Figure 5.3B). However, no significant differences were confirmed (Global R=0.1,  $p>0.05$ ; ANOSIM). The dissimilarity was 65.4% (SIMPER analysis) between spring 2011 (s1) and spring 2012 (s6), which was mainly due to the disappearance of species in post-drought samples such as *Cocconeis euglypta* Ehrenberg, *Amphora pediculus* (Kützing) Grunow ex A. Schmidt, *Reimeria sinuata* (W. Gregory) Kociolek & Stoermer and *Cocconeis placentula* var. *lineata* (Ehrenberg.) Van Heurck and the appearance of others like *Nitzschia palea* var. *debilis* (Kützing) Grunow and *Fragilaria gracilis* (Østrup). In addition, *Gomphonema rhombicum* Fricke and *Eolimna minima* (Grunow) Lange-Bertalot & W. Schiller were among the species that contributed the most for this dissimilarity by decreasing, whereas species such as *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot, *Navicula veneta* Kützing and *Karayevia oblongella* increased in s6 compared to s1 (Table 5.3).

When analysing the selected metrics in all streams, we found that richness and evenness for the diatom assemblages showed the same recovery trend over the sampling events from s2 to s6 (Figure 5.5a, c), despite there being no significant differences ( $p>0.05$ ; PERMANOVA pair-wise test). For these metrics, an increase of 11.5% (in richness) and 14.3% (in evenness) was observed from s1 to s2; and over one year (from s1 to s6) an increase of 11.5% (richness) and 15.9% (evenness; Figure 5.5c) was also recorded.

When analysing the IPS index scores, the good water quality status observed in s1 was not altered in s2 (before and immediately after the drought event). However, the index values dropped in s3, changing the status to moderate. In s4 and s5, the status recovered to good again. Despite these results, the average of the study sites in spring 2012 did not recover to the pre-drought values of spring 2011 (the quality status changed from good in s1 to moderate in s6; Figure 5.5d).

Table 5.3 - Diatom taxa contributing 65.4% to the mean dissimilarity (SIMPER analysis) between s1 and s6 (spring 2011 pre-drought and spring 2012 post-drought, respectively), with their percentage contribution, average dissimilarity and standard deviation (SD). The increase (I) or decrease (D) in the mean abundance of each taxon from s1 to s6 (spring 2011 pre drought and spring 2012 post-drought) is also shown.

| Taxon   | Av. Diss. | SD Diss. | Contrib. (%) | I/D |
|---|-----------|----------|--------------|-----|
| <i>Nitzschia palea</i> (Kützing) W. Smith                             | 3.3       | 1.2      | 5.0          | I   |
| <i>Planothidium frequentissimum</i> (Lange-Bertalot) Lange-Bertalot   | 3.2       | 1.1      | 4.9          | I   |
| <i>Planothidium lanceolatum</i> (Brébisson ex Kützing) Lange-Bertalot | 3.2       | 1.1      | 4.9          | I   |
| <i>Cocconeis euglypta</i> Ehrenberg                                   | 3.1       | 1.1      | 4.7          | D   |
| <i>Navicula veneta</i> Kützing  | 3.1       | 1.1      | 4.7          | I   |
| <i>Karayevia oblongella</i> (Østrup) Aboal                            | 2.9       | 1.0      | 4.4          | I   |
| <i>Gomphonema rhombicum</i> Fricke                                    | 2.8       | 0.7      | 4.3          | D   |
| <i>Amphora pediculus</i> (Kützing) Grunow ex A. Schmidt               | 2.5       | 1.1      | 3.8          | D   |
| <i>Eolimna minima</i> (Grunow) Lange-Bertalot & W. Schiller           | 2.4       | 1.2      | 3.7          | D   |
| <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer              | 2.4       | 0.8      | 3.6          | D   |
| <i>Nitzschia palea</i> var. <i>debilis</i> (Kützing) Grunow           | 2.3       | 0.8      | 3.5          | I   |
| <i>Fragilaria capucina</i> Desmazières                                | 2.1       | 0.9      | 3.3          | I   |
| <i>Cocconeis placentula</i> Ehrenberg                                 | 2.1       | 0.8      | 3.2          | D   |
| <i>Achnantheidium minutissimum</i> (Kützing) Czarnecki                | 2.1       | 1.5      | 3.2          | D   |
| <i>Fragilaria vaucheriae</i> (Kützing) J. B. Petersen                 | 2.0       | 0.9      | 3.1          | I   |
| <i>Mayamaea atomus</i> var. <i>permitis</i> (Hustedt) Lange-Bertalot  | 1.9       | 1.0      | 3.0          | I   |
| <i>Gomphonema acuminatum</i> Ehrenberg                                | 1.8       | 0.9      | 2.8          | I   |
| <i>Melosira varians</i> C. Agardh                                     | 1.8       | 0.9      | 2.7          | D   |
| <i>Eunotia minor</i> (Kützing) Grunow                                 | 1.8       | 0.9      | 2.7          | D   |
| <i>Gomphonema parvulum</i> (Kützing) Kützing                          | 1.8       | 0.9      | 2.7          | I   |
| <i>Navicula gregaria</i> Donkin                                       | 1.7       | 0.7      | 2.6          | I   |
| <i>Fragilaria gracilis</i> Østrup                                     | 1.7       | 0.8      | 2.6          | I   |
| <i>Ulnaria biceps</i> (Kützing) Compère                               | 1.7       | 0.9      | 2.6          | I   |
| <i>Nitzschia capitellata</i> Hustedt                                  | 1.7       | 0.9      | 2.6          | I   |
| <i>Nitzschia inconspicua</i> Grunow                                   | 1.6       | 0.8      | 2.5          | I   |
| <i>Encyonema minutum</i> (Hilse) D. G. Mann                           | 1.5       | 0.7      | 2.4          | D   |
| <i>Nitzschia fonticola</i> (Grunow) Grunow                            | 1.3       | 0.7      | 2.0          | I   |

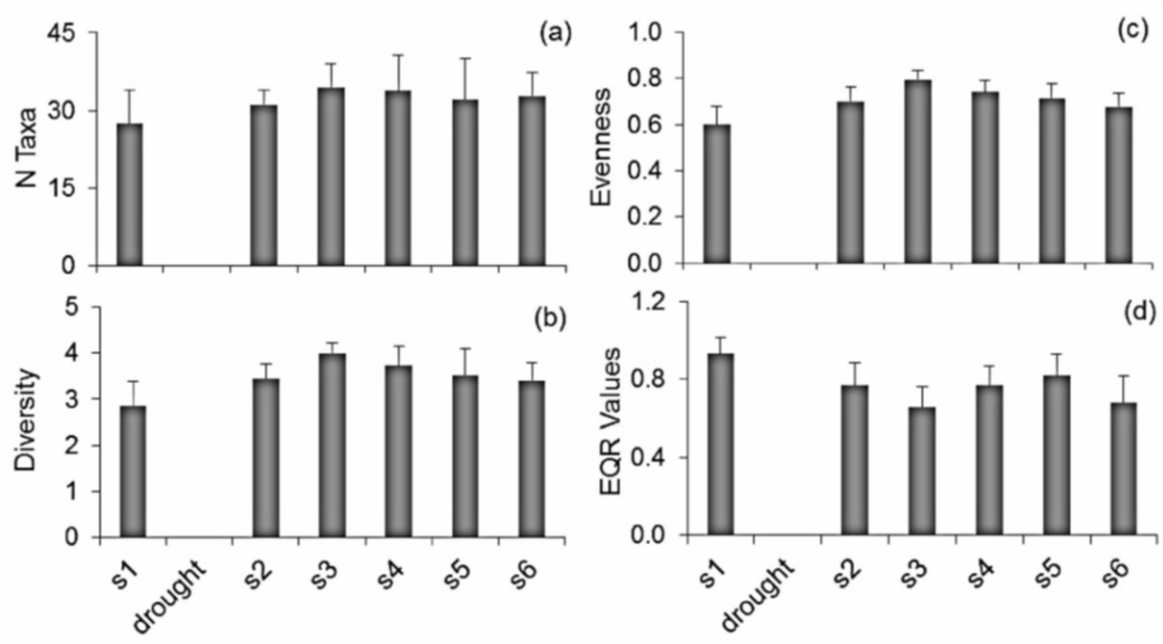


Figure 5.5 - Diatom metrics for all study sites (mean  $\pm$  SE) at each sampling event from spring 2011 (s1) until spring 2012 (s6); a) number of taxa (richness), b) Shannon-Wiener diversity index, c) Pielou's evenness and d) IPS index (expressed in Ecological Quality Ratios).

## 5.4 Discussion

### Diatom and macroinvertebrate community recovery differences

The macroinvertebrates and diatoms differed in their recovery response after dewatering. The macroinvertebrates significantly decreased their abundance when the water first resumed, whereas the diatoms apparently did not differ from the pre-drought-sampled community. In fact, the same pattern prevailed after a year. The diatom biodiversity was not significantly altered over the rewetting progression. This could be because, when compared to macroinvertebrates, diatoms have a rapid life cycle and a consequently rapid ability to colonise a habitat. Oemke and Burton (1986) concluded that a period between 14 days to one month was adequate to complete the colonization of artificial substrates, attaining the equivalent to mature diatom communities, which was quite similar to the recovery time that we found in the present study (approximately two weeks). Despite their short life cycle, diatoms are capable of persisting in refuges during the dry period (Robson et al., 2008). Furthermore, many benthic algal species are able to resist desiccation due to physiological adaptations such as thick cell walls or resistant propagules, facilitating recolonisation (Steinman and McIntire, 1990). Regarding benthic invertebrates, the reduced flow and

decreased water velocity were not beneficial to taxa strongly dependent on flowing water, such as the EPT taxa (Boulton, 2003; Lake, 2003), whereas it had no effect on Diptera or Coleoptera. The latter taxa are most likely able to better tolerate extreme drought because they have desiccation-resistant stages (Coleoptera) or are capable of surviving in moist streambeds (Diptera; Lake, 2003; Boulton and Lake, 2008). Indeed, we found an increase of taxa resistant to drying and typically characteristic of intermittent systems, such as Oligochaeta and several Diptera (Frouz et al., 2003; Dumnicka and Koszalka, 2005).

For both studied benthic elements, the increase in evenness immediately after the dewatering event followed the pattern of the first colonisation stages of empty niches (Begon et al., 1996). In the particular case of diatoms, the dominant species were generally characteristic of oligo- to eutraphentic environments, suggesting that the community shifted from the first colonisation stages to a stage of response from the stress affecting the site (van Dam, 1994).

#### Drought effects on the evaluation of the ecological status

Regarding the biological quality, the IPS index for diatoms was similar over the rewetting progression, but the quality status decreased after one year; this indicates that a shift in species occurred. Indeed, there was a shift from more sensitive species such as *Reimeria sinuata* and *Cocconeis placentula* var. *lineata* in spring 2011 to less sensitive or even tolerant species such as *Nitzschia palea* and *Nitzschia palea* var. *debilis* in spring 2012. Moreover, despite the initial similarity between the studied streams, the drought appeared to induce different environmental (physical and chemical) changes in the streams that led to an irregular response of the diatom communities to the drought. Similarly, Boix et al. (2010) did not find linear or uniform changes in the taxonomic composition or structural physiognomy of diatom communities in streams affected by hydrological alteration.

Contrary to diatoms, stream water quality, given by the macroinvertebrate IPTI index, decreased with the drought event, and although it did not reach the initial reference values after one year, our streams gradually recovered during the winter season. Both communities provide reliable information on water quality (e.g., Alba-Tercedor et al., 2002; Rimet et al., 2005). Diatoms are more sensitive to changes in water chemistry, whereas invertebrates are more susceptible to channel morphological changes and habitat conditions (Passy et al., 2004). Under drought effects, their simultaneous use for bioassessment should be considered, as their responses are different and appear to be complementary (Feio et al., 2007), particularly if considering responses at different temporal scales. In addition, these structural bioassessment measures using taxonomic composition may be complemented by adding trait-based metrics (e.g., body size, life cycle duration, dispersal ability, respiration type), which indirectly provide information on the ecological functioning of streams (Dolédéc

and Statzner, 2010) and could therefore yield accurate insights into the assessment of extreme events such as droughts.

#### The role of habitat features in drought resistance and resilience

The river habitat characteristics appeared to play an important role in the recovery of macroinvertebrate and diatom communities. Our study showed that the initial condition of streams was important in the recovery process, as the communities of streams displaying better environmental conditions (*e.g.*, riparian corridors, HQA) recovered faster and easier from extreme disturbances, which is in agreement with other authors (*e.g.*, Sponseller et al., 2001; Elozegi et al., 2010; Thomson et al., 2012). It is known that benthic communities are strongly influenced by local riparian conditions (Lammert and Allan, 1999; Sponseller et al., 2001; Poole and Berman, 2001; Elias et al., 2012), which might generate a buffer favouring humidity and lower temperatures by shading. Moreover, the heterogeneity of habitats within the channel and the availability, size and the spatial distribution of refugia during drought likely played a crucial role not only in the communities' resistance but also in their resilience (Magoulick and Kobza, 2003; Lake, 2003) to the dewatering event in the streams of this study. By moving to moist leaf litter or to the moisture under rocks and bark, migrating to the hyporheic zone or burrowing into the bed of the water body (Boulton et al., 1992; Clinton et al., 1996; Magoulick and Kobza, 2003), the communities found drought refugia that enabled them to survive and progressively recover.

Under the current climate change scenario and consequent unpredictability of extreme events such as the occurrence of dewatering droughts in small, typically perennial streams, the maintenance of morphological riverine features that enhance habitat quality (*e.g.*, continuous riparian corridors, channel habitat heterogeneity) constitutes a determining factor in the continued resilience of aquatic ecosystems (Lake, 2003).

## 5.5 Conclusion

Our study revealed that isolated, unusual dewatering drought events in temperate streams affected primary and secondary producers despite their differences in reaction and recovery patterns. The ability of diatom assemblages to recover faster than macroinvertebrates after a drought is most likely due to their shorter life cycles, capability to remain in refuges and physiological adaptations to dryness. We also found that the quality of the habitat appears crucial to minimise the impact and accelerate the recovery processes of these key riverine biological communities as soon as water returns to the channel.

With increasing anthropogenic pressures and climatic alterations, we expect biological communities of temperate streams to change through time to comprise more pollution-tolerant taxa and those with a higher resilience to extreme drought events.

### **Abbreviations**

|          |   |
|----------|---|
| EQR      | Ecological Quality Ratios   |
| HQA      | Habitat Quality Assessment  |
| IPS      | <i>Indice de Polluosensibilité Spécifique</i>   |
| IPTI     | <i>Índice Português de Invertebrados</i>  |
| MDS      | non-metric Multidimensional Scaling   |
| RHS      | River Habitat Survey  |
| S1       | Samples collected in spring 2011 (May), before the severe and uncharacteristic drought event, pre-drought condition   |
| S2 to S5 | Samples collected biweekly in winter (between November 2011 and January 2012), after the drought period when normal flow conditions were re-established, post-drought condition |
| S6       | Samples collected in spring 2012 (May), after the drought period when normal flow conditions were re-established, post-drought condition  |
| WFD      | Water Framework Directive   |

### **Acknowledgements**

We would like to thank the Institute of Marine Research (IMAR-CIC) and the GeoBioTec Research Unit and Biology Department of the University of Aveiro for making their facilities available and providing funding, as well as the Fundação para a Ciência e Tecnologia for providing financial support through the AQUAWEB project (PTDC/AACAMB/105297/2008) and a PhD scholarship to the second author (SFRH /BD/68973/2010) financed by the Fundo Nacional do MEC and the program POPH-QREN Tipologia 4.1. We also acknowledge Dr. Amaia Pérez-Bilbao for her contributions in Spanish writing and the anonymous reviewers for their helpful comments and suggestions on the manuscript.

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Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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## **Chapter 6 - Influence of the colonizing substrate on diatom assemblages and implications for bioassessment - a mesocosm experiment**

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Submitted for publication in Aquatic Ecology.

### **ABSTRACT**

Although diatoms are important bioindicators of water quality, their ecological traits are still not well understood. A major issue is that of substrate preferences, which may result in differences in production, and assemblage structure and composition, and which should be taken into account for water quality assessment studies. Thus in this work, the periphyton grown on sand and ceramic tiles in indoor controlled channels were compared to understand if substrate differences lead to differences in: periphyton production (chlorophyll-*a*), chlorophyll-*b* and *c* concentrations, diatom assemblages (diversity-Shannon-Wiener, cell density, taxonomic composition, trait proportions) and water quality assessments (IPS - '*Indice de Polluosensibilité Spécifique*'). A combined inoculum of periphyton from four Portuguese streams was introduced to the running channels (six sand and six tile) and left to colonize for 35 days. Epilithic (tiles) and epipsammic (sand) assemblages were sampled at days 14 and 35. We verified that there were no differences in chlorophyll-*a* concentration over time and between substrates. On both sampling moments the epipsammic assemblages had higher concentration of chlorophyll-*c* and diatom density but without significant differences over time in each substrate. The taxonomic composition was different between substrates and over time. However, these differences were not reflected in water quality assessment. The diversity was also similar between substrates in both sampling moments but it was higher at day 14. Mobile and stalked species were more abundant over the entire study and differed significantly between substrates, with the epipsammic assemblages presenting



Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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higher abundances of both traits. We concluded that the colonizing substrate influences diatom assemblages but not the water quality assessment.

### **Keywords**

Diatoms, chlorophyll, traits, mesocosm, water quality assessment, freshwater

## 6.1 Introduction

Streams are continuously affected by erosion and deposition processes, which along with lithology, slope, current, degree of disturbance, and distance from headwaters result in different sediment sizes (Cattaneo et al., 1997; Rolland et al., 1997). Therefore, there are streams where rocks dominate (large stable substrates), streams that within the same site have large stable and small unstable substrates (*e.g.*, sand), and even streams with fine unstable substrates only (in particular lowland sites). Substrate type, texture, roughness and stability/instability (granulometry) are relevant habitat criteria for the interactions between benthic algae and their substrates (Cattaneo et al., 1997; Hunt and Parry, 1998; Janauer and Dokulil, 2006; Bergey and Cooper, 2015). Substrate size can affect the abundance and composition of the attached algae by providing different degrees of stability to colonizing organisms (Cattaneo et al., 1997). Several studies have addressed the distinct associations of periphyton on different substrates (*e.g.*, rock surface, upper layer of sediment, or aquatic plants) through their biovolume, diversity, algal assemblages and chlorophyll concentration (*e.g.*, Cattaneo et al., 1997; Rolland et al., 1997; Sabater et al., 1998; Potapova and Charles, 2005).

Among algae, diatoms have been selected in most European countries as representative of periphyton (Almeida and Feio, 2012; Kelly et al., 2012; Feio et al., 2014) namely due to their good performance as water quality indicators (Lowe and Pan, 1996; Kelly et al., 1998; Stevenson and Pan, 1999). From all types of substrates found in rivers, most studies focus on epilithon (Winter and Duthie, 2000), as hard surfaces are the preferred substrates used in water quality assessment (Kelly et al., 1998). However, other substrates like submerged macrophytes and sediments are also commonly found and may be dominant in some stream and river sections (Kelly et al., 1998; Elias et al., 2015). Some studies indicate that benthic diatom species present different biological characteristics that enable them to adapt to specific micro-habitats (Krejci and Lowe, 1986; Soininen and Eloranta, 2004). If these natural inter-substrate differences are reflected in water quality assessment metrics, this can potentially mask responses of algal assemblages to stresses associated with human activities and may interfere with water quality assessments based on the knowledge of these responses (Winter and Duthie, 2000; Potapova and Charles, 2005; Bere and Tundisi, 2011; Mendes et al., 2012). However, some studies have tested the effect of different substrates in water quality assessments without finding significant differences (Kitner and Poulíčková, 2003; Potapova and Charles, 2005; Mendes et al., 2012). These studies were conducted under natural conditions where the assemblages are shaped simultaneously by many environmental factors which may lead to confounding effects (Stevenson and Pan, 1999). In

addition the substrate effect is difficult to detect in large-scale, coarse resolution studies, when the role of other factors, such as inter-stream differences in hydrology, physical habitat, and chemistry become more important than the role of substrate (Potapova and Charles, 2005). Even when different diatom assemblages of the same river are compared and differences are verified (Cetin, 2008) it is difficult to ensure that the assemblages have been exposed to the same environmental variations at the same time or even at the same developmental phase. Additionally, it is impossible to avoid contamination between substrates with diatoms migrating between habitats in wadeable streams.

Therefore, the present work was conducted *ex situ* under controlled experimental conditions to investigate: (1) if algae assemblages establishing on new hard (ceramic tiles) and soft (sand) substrates become significantly different concerning chlorophyll-*a* (chl-*a*), *b* (chl-*b*) and *c* (chl-*c*) concentrations, diversity (Shannon-Wiener, *H'*), density (cells·cm<sup>-2</sup>), taxonomic composition and trait proportions; (2) how they evolve over time (up to five weeks of colonization) on the different substrates (3) and if differences in diatom assemblages on the different substrates result in differences in water quality assessment. The water quality of the channels was assessed by using a common autoecological diatom method which is also the Portuguese official index, the '*Indice de Polluosensibilité Spécifique*' (IPS) for monitoring programs, in the context of the Water Framework Directive (INAG, 2009).

## 6.2 Methods

### 6.2.1 Experimental system description

The experimental system was composed of twelve modular mesocosm systems (MMS). Each MMS was composed of one Poly(methyl methacrylate) (PMMA, 8 mm thick) mesocosm channel (150 cm long, 10 cm wide and 12 cm high) with a maximum functional volume of approximately 18 L, connected to a PMMA (8 mm thick) water reservoir (60 cm long, 10 cm wide, and 45 cm high), operating with a maximum functional water volume of approximately 27 L (Figure 6.1).

The MMS operated in recirculated system. Water in the reservoir was pumped through a 25 mm PVC (polyvinyl chloride) inlet pipe system, which allowed flow direction regulation in the mesocosm channel, by a submerged water pump (EHEIM compact+ 3000, Germany) that can operate with a regulated water flow from 200 to 2000 L/h. The water outlet pipe system, from the mesocosm channel to the reservoir, was built with 50 mm PVC pipe, with an adjustable damper placed close to the end of the mesocosm channel, which allowed the regulation of water level.

Each mesocosm channel was illuminated from above with T5 HO 80W tubular fluorescent lamps, Lumilux - 8000 K (Osram, Germany). The distance from the illumination system and the water surface was adjustable in order to control the photosynthetic active radiation (PAR).

### 6.2.2 *Experimental Set-up*

At the beginning of the experiment 25 L of tap water was fed to the water deposit of each channel, after passing through 5  $\mu\text{m}$  wound polypropylene and active carbon filters to remove suspended particulate matter and free chlorine, respectively. The water depth in mesocosm tanks was kept at 5 cm. Water velocity was maintained at approximately 0.05 m/s. The illumination systems were positioned at about 1 m above the channels to provide light to the attached algae ( $\approx 200 \mu\text{mol}/\text{m}^2/\text{s}$ ) with a 12h:12h light-dark cycle. Water temperature was measured by K-type thermocouples (Testo 176T4 data logger) and maintained within 17 °C and 20 °C during the experiment. This was possible with the help of an air conditioning system and a well-isolated experimental room that was able to minimize air temperature variations.

The bottom of all mesocosm channels ( $n=12$ ) was covered with 62 unglazed ceramic tiles-T ( $40 \times 50$  mm in size). In 6 of the channels ( $n=6$ ) the tiles were completely covered with about 1 cm depth of sand bed-S (98%  $\text{SiO}_2$ , 2 mm particles). A two week preliminary study revealed that the tiles did not influence the chemical composition of the circulating water. However, in order to exclude any previously undetected differences due to the tile chemical composition, and as the sand is almost inert ( $\text{SiO}_2$ ), the tiles were kept under the sand bed in the sand channels.

Biofilms samples were collected from four streams in the Portuguese littoral region and mixed in 1 L bottle to be used as inoculum in the channels. These biofilm included epilithic and epipsammic assemblages. Each channel ( $n=12$ ) was seeded with 60 mL inoculum at the beginning of the experiment. Thereafter, 10 L (40%) of water was removed from each channel on a weekly basis and immediately replaced by 10 L of new filtered tap water. The total duration of the experiment was 35 days (d35). The streams were selected to obtain a diverse inoculum community representative of the different streams found in the central-western Portuguese region (Atlantic-temperate climate). This includes a variety of streams with streambeds ranging from rocks to sand, and with a variety of human pressures (from least to highly disturbed) (Feio et al., 2010; Elias et al., 2015).

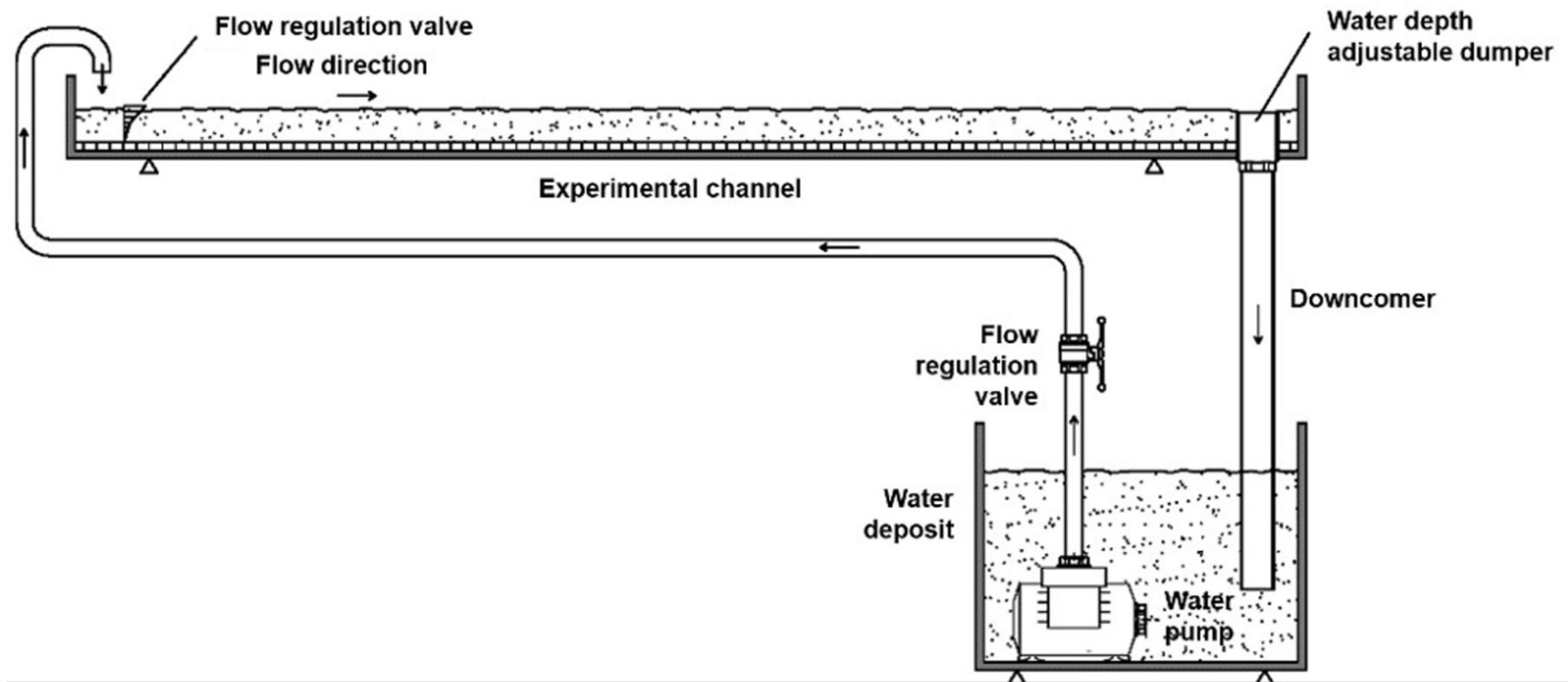


Figure 6.1 - Cross-section of one of the twelve modular mesocosm systems used in the laboratory experiments. See Appendix IV.

### 6.2.3 Water, periphyton sampling and treatment

The inoculum was left to colonize the substrates in the channels for an initial period of seven days, after which water replacement and sampling was initiated. Water samples from the channels and from the tap were collected weekly (d0, d7, d14, d21, d28 and d35) for determination of alkalinity (mg  $\text{HCO}_3^-/\text{L}$ ), nitrate (mg  $\text{NO}_3^-/\text{L}$ ), silica (mg  $\text{Si}/\text{L}$ ), chloride (mg  $\text{Cl}/\text{L}$ ), phosphate (mg  $\text{PO}_4^{3-}/\text{L}$ ) and sulphate (mg  $\text{SO}_4^{2-}/\text{L}$ ). Several (from one to every two days) *in situ* measurements of pH, conductivity ( $\mu\text{S}/\text{cm}$ ), and percentage of total dissolved oxygen were made using a Multiparameter Probe 3430 WTW throughout the experiment. Periphyton samples were randomly retrieved from each channel at two sampling moments: day 14 (d14) and day 35 (d35). For a given sampling moment, periphyton samples were taken from the same position in each channel; however, the sampling position varied with the sampling moment and was always done downstream to upstream to minimize disturbance effects. For each sampling moment four subsamples (four tiles) were collected along the channel in order to embrace all the possible assemblages variability that can exist within the channel. Afterward, these subsamples were all merge in just one sample *per* channel in each sampling moment.

For the epilithic biofilm (tiles), the upper surface of 4 tiles (80.0  $\text{cm}^2$  *per* channel) was scraped with a toothbrush and washed with distilled water into a flask. In epipsammon biofilm (sand) sampling, an area of 57.8  $\text{cm}^2$  of the upper surface of the sand bed was collected into a flask using a syringe. Two distinct samples of each channel were collected at each sampling moment, one for diatom analysis and another for chlorophyll determination (chl-*a*, chl-*b* and chl-*c*). The area contained in the volume treated was always assured so that the results obtained could be expressed *per* unit area of substrate.

For the determination of chlorophylls, the total area sampled (80.0  $\text{cm}^2$  and 57.8  $\text{cm}^2$  for epilithic and epipsammom, respectively) of each channel was used. Following Branco et al. (2010), the samples were repeatedly centrifuged (2000 rpm) for five minutes until all the water was removed and only a pellet remained. Pigments were extracted with 2 mL of acetone (90%v) from the pellet; the extract was protected from light and maintained in the cold. To break the cells, samples on ice were sonicated in 4 cycles of 15 seconds. The extract was stirred for 30 minutes in a refrigerated environment and then centrifuged at 4000 rpm for 10 minutes at 4 °C. Chlorophylls-*a*, *b* and *c* were quantified spectrophotometrically and its concentration (in  $\mu\text{g}\cdot\text{cm}^{-2}$ ) calculated following the procedure of Jeffrey and Humphrey (1975). Chlorophyll-*a* is used as an indicator of primary production as its found in all algae and in Cyanobacteria, chl-*c* is used as indicator of the presence of diatoms, as its one of its

major pigment and chl-*b* will give us an indication of the biomass from other taxonomic algal groups such as Chlorophyta and Euglenophyta.

For diatom assemblage analysis, a subsample of the total area sampled was oxidised with concentrated nitric acid and potassium dichromate for about 24 hours at room temperature. The remaining sample was preserved with formaldehyde (5 to 10% final concentration). Thereafter, a known volume of the oxidised sample was deposited on a coverslip and allowed to dry at room temperature. Permanent slides were mounted using Naphrax®. Using a light microscope (100x objective and 1.32 numerical aperture), all the unbroken diatom valves for each sample were counted and identified to species or infra-specific rank mainly using Krammer and Lange-Bertalot's floras (1986, 1988, 1991a, b) and Krammer (2000, 2001, 2009). From those, diatom cell density was determined and extrapolated to the unit area (cm<sup>2</sup>) of each sample; species diversity (H') and assemblage analysis were also derived. The diatom index IPS (Coste in Cemagref, 1982) was also calculated with the OMINIDIA software (Lecointe et al., 1993).

Permanent slides, with the inoculum of biofilm introduced in the channels, were also mounted and up to 400 diatom valves counted according to the aforementioned description.

Prior to oxidation, microscopic identification of all the samples (from the channels and inoculum) were also carried out to verify if other group of photosynthetic organisms besides diatoms were present and dominating in the samples.

#### 6.2.4 Selection and calculation of biological traits

The biological characteristics that enable species to adapt more easily to specific substrates (here tile vs. sand) is the capacity to resist dislodgement caused to substrates by the stream current and the capacity of moving vertically into or out of the sand depending on water velocity. Thus variations in the diatom biological trait life-form were also investigated in this study by analysing changes in proportions of categories that have high potential to distinguish epipsammic and epilithic diatom assemblages: mobile, planktonic, adnate, and pad (Berthon et al., 2011; Rimet and Bouchez, 2011; Rimet and Bouchez, 2012).

Each species was assigned to all the trait categories that it could display, according to Rimet and Bouchez (2012). For each sample, the total number of valves of all the species presenting a given trait category was counted. To obtain the total number of valves of each trait category in each channel, the average of all the samples from the same treatment was calculated.

### 6.2.5 *Data analysis*

A completely randomized design was used in the experiment. Statistical differences in chlorophyll concentrations, H', diatom cell density, IPS and biological diatom traits resulting from treatment effects were tested with the univariate analysis of variance (equivalent to one-way models) PERMANOVA global tests with unrestricted permutations (Euclidean dissimilarity measure). Additionally, PERMANOVA pairwise tests with unrestricted permutations were performed to assess possible differences between sampling moments (d14 and d35), and substrates (S and T). These two sampling moments were selected as we consider that at the 7<sup>th</sup> day differences between substrates were still not evident, and at least one month has been recommended for sampling the equivalent of the 'mature' community occurring on natural substrates in the stream (Oemke and Burton, 1986; Kelly et al., 1998).

To determine if the treatments lead to differences in the diatom assemblages a Multidimensional Scaling Analyses, MDS (Bray-Curtis dissimilarity measure; data square root transformed) was performed. The statistical differences resulting from the treatment effects were tested with a permutational multivariate analysis of variance (MANOVA equivalent) PERMANOVA global tests (unrestricted permutations). PERMANOVA pairwise tests (unrestricted permutations) were performed to assess at which sampling moments the treatments (two substrates) were different and if the differences across treatments were consistent over time. SIMPER analysis (data square root transformed, Bray-Curtis similarity) was used to determine the most representative taxa (those contributing the most to the average similarity within groups) of the different treatments and sampling moments. In addition, SIMPER analysis (presence/absence data, Bray-Curtis similarity) was used to determine the species that were present in a given substrate and absent from the other at the same sampling moment and that contributed more to the group average dissimilarity.

All the data analyses were performed with PRIMER 6 & PERMANOVA software (PRIMER-E Ltd, Lutton, Plymouth, UK).

## 6.3 **Results**

### 6.3.1 *Physical and chemical parameters*

One of the tile channels was eliminated from the analysis due to difficulties in maintaining the water temperature constant. Therefore, the results presented are from six channels with sand and five with tiles.

The water used throughout the experiment showed similar physical and chemical characteristics in both treatments (see Table 6.1). The nutrient and conductivity values were



relatively high for tap water, *e.g.*, the mean concentration of nitrate found in the channels was 19.5 mg NO<sub>3</sub><sup>-</sup>/L (Table 6.1).

Table 6.1 - Mean ( $\pm$ standard deviation) of the abiotic variables measured in the eleven artificial channels throughout 35 days of the colonization experiment (from day 0 to 35). BDL-Value below detection limit.

|  | Sand             | Tile             |
|--|------------------|------------------|
| Dissolved Oxygen (%)                             | 101.0 $\pm$ 0.9  | 100.4 $\pm$ 0.7  |
| pH   | 8.2 $\pm$ 0.1    | 8.2 $\pm$ 0.1    |
| Conductivity ( $\mu$ S/cm)                       | 509.9 $\pm$ 72.4 | 551.7 $\pm$ 38.0 |
| Alkalinity (mg HCO <sub>3</sub> <sup>-</sup> /L) | 131.6 $\pm$ 13.8 | 137.9 $\pm$ 17.4 |
| Nitrate (mg NO <sub>3</sub> <sup>-</sup> /L)     | 19.5 $\pm$ 4.3   | 21.0 $\pm$ 2.4   |
| Silica (mg Si/L) <sup>a</sup>                    | 0.8 $\pm$ 0.3    | 1.0 $\pm$ 0.4    |
| Chloride (mg Cl <sup>-</sup> /L)                 | 56.8 $\pm$ 13.8  | 61.1 $\pm$ 13.2  |
| Phosphate (mg PO <sub>4</sub> <sup>3-</sup> /L)  | < 1.1 (BDL)      | < 1.1 (BDL)      |
| Sulphate (mg SO <sub>4</sub> <sup>2-</sup> /L)   | 51.1 $\pm$ 12.0  | 54.7 $\pm$ 9.9   |

### 6.3.2 Chlorophylls

The total periphyton biomass, measured as the ubiquitous pigment chlorophyll-*a* (chl-*a*), did not vary over time (d14 vs. d35) nor between substrates (T vs. S) (Pseudo-F=3.10, p(perm)>0.05) (Figure 6.2a). The mean highest chl-*a* concentration (0.134  $\mu$ g/cm<sup>2</sup>) was obtained on sand at day 35 while the mean lowest concentration (0.075  $\mu$ g/cm<sup>2</sup>) was observed on tile at day 14. Chl-*c* concentration showed statistical differences between substrates. In both sampling moments the epipsammic assemblages presented a significantly higher concentration of chl-*c* (Td14 vs. Sd14  $t$ =2.80, p(MC)=0.02; Td35 vs. Sd35  $t$ =2.80, p(MC)=0.03) than the epilithic assemblages. The mean highest chl-*c* value (0.025  $\mu$ g/cm<sup>2</sup>) was obtained on sand at d35 while the mean lowest concentration (0.007  $\mu$ g/cm<sup>2</sup>) was obtained on tile at both d14 and d35. This variable did not vary significantly along time for a given substrate (Td14 vs. Td35  $t$ =0.18, p(MC)=0.92; Sd14 vs. Sd35  $t$ =1.93, p(MC)=0.08) (Figure 6.2b).

The chl-*b* concentration values were inferior to the values of the other chlorophylls. The mean highest chl-*b* concentration (0.009  $\mu$ g/cm<sup>2</sup>) was obtained on the sand at d14. Chl-*b* only varied along time on the epipsammic assemblages (Td14 vs. Td35  $t$ =2.36, p(MC)=0.05; Sd14 vs. Sd35  $t$ =8.84, p(MC)<0.01) (Figure 6.2c). Considering sampling moments the epilithic assemblages never presented significant higher chl-*b* concentrations (Td14 vs. Sd14  $t$ =1.90, p(MC)=0.12; Td35 vs. Sd35  $t$ =2.77, p(MC)=0.08) (Figure 6.2c).

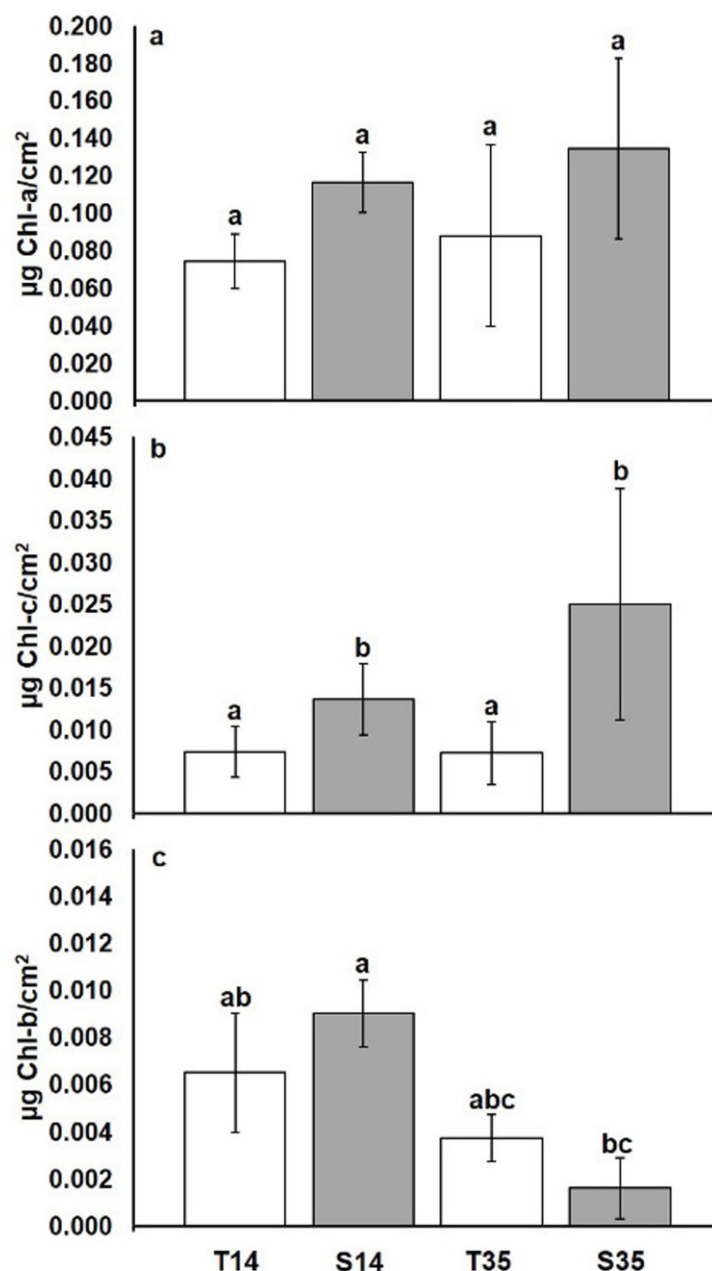


Figure 6.2 - (a) Chlorophyll-*a* (Chl-*a*), (b) chlorophyll-*c* (Chl-*c*) and (c) chlorophyll-*b* (Chl-*b*) concentrations (mean±standard deviation) of the algal assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b, c) do not significantly differ ( $p>0.05$ ; PERMANOVA pairwise test).

### 6.3.3 Periphyton assemblages

The microscopic analyses of the unoxidized samples revealed a clear dominance of diatoms on the periphyton assemblages. The other two groups from which we identified

more individuals were Chlorophyta (e.g., *Scenedesmus*, *Ankistrodesmus*, *Coelastrum* and *Monoraphidium*) and Cyanobacteria (e.g., *Chroococcus*).

The analysis of the inoculum sample used to seed the channels revealed that the most abundant species were *Staurosira venter* (Ehrenberg) Cleve & J. D. Möeller, *Achnantheidium minutissimum* (Kützinger) Czarnecki and *Asterionella formosa* Hassall even though they never exceeded 10% abundance.

In total 174 diatom species were identified during the counting of all the samples collected from the channels. The corresponding total number of valves counted ranged from 568 to 2586. The epipsammic diatom assemblages presented higher cell density in both sampling moments when compared to the epilithic assemblages (Td14 vs. Sd14  $t=2.59$ ,  $p(\text{perm})=0.03$ ; Td35 vs. Sd35  $t=2.39$ ,  $p(\text{perm})=0.04$ ); however, this variable was not different over time within each substrate (Td14 vs. Td35  $t=1.44$ ,  $p(\text{perm})=0.23$ ; Sd14 vs. Sd35  $t=1.76$ ,  $p(\text{perm})=0.11$ ) (Figure 6.3).

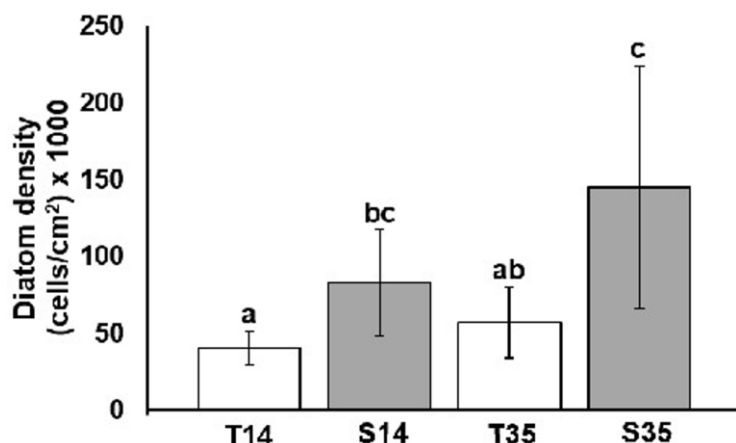


Figure 6.3 - Diatom density (cells/cm<sup>2</sup>) (mean±standard deviation) found in the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b, c) do not significantly differ ( $p>0.05$ ; PERMANOVA pairwise test).

In terms of diatom taxonomic composition we verified a segregation regarding both the sampling moment (Td14 vs. Td35  $t=1.74$ ,  $p(\text{perm})<0.01$ ; Sd14 vs. Sd35  $t=1.68$ ,  $p(\text{perm})<0.01$ ) and substrates (Td14 vs. Sd14  $t=1.68$ ,  $p(\text{perm})<0.01$ ; Td35 vs. Sd35  $t=1.88$ ,  $p(\text{perm})<0.01$ ) (Figure 6.4). For both substrates, the MDS and SIMPER analysis revealed that the assemblages of different channels at day 35 were less similar to each other than at day 14 (within group average similarity: T14=68.8%, S14=71.6%, T35=66.6%, S35=69.5%) (Figure 6.4).

Species analysis (Table 6.2) showed that the species that contributed more to the within group average similarity in both sampling moments and substrates were the same but with different contributing percentages to in-group similarity: *Achnantheidium minutissimum*

(13.6% to 23.9%), *Fragilaria* cf. *parva* (Grunow) A. Tuji & D. M. Williams (6.0% to 11.6%) and *Navicula notha* J. H. Wallace (5.1% to 7.1%) (Table 6.2). The higher contribution of these species to the average similarity was verified at day 35 on both substrates. All these three species were also found in the inoculum: *A. minutissimum* presented an abundance of 9%, the species *F. cf. parva* and *N. notha* presented abundances of 2% and 6%, respectively.

The differences between the substrates at the same sampling moment were due to less abundant taxa and to presence or absence of certain taxa. Comparing both substrates at day 14, the epipsammic assemblages presented less species of the genus *Navicula* contributing to the group similarity (Table 6.2) and a smaller number of species that were only present in the sand assemblages (7 species vs. 18 in the tile) (Table 6.3). Species such as *Adlafia minuscula* var. *muralis* (Grunow) Lange-Bertalot, *Surirella linearis* W. Smith and *Nitzschia sociabilis* Hustedt were only found in the epipsammic assemblages while species such as *Hippodonta capitata* (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski, *Reimeria sinuata* (W. Gregory) Kociolek & Stoermer and *Navicula lanceolata* Ehrenberg were only present in the epilithic assemblages (Table 6.3). However, the diversity was similar between substrates (Td14 vs. Sd14  $t=1.47$ ,  $p(\text{perm})=0.16$ ) with mean values of 2.73 and 2.51, epilithic and epipsammic assemblages respectively (Figure 6.5). At day 35 the species belonging to the genus *Nitzschia* contributed more to the sand group average similarity (Table 6.2). Once more, the epipsammic assemblages presented a smaller number of species that were only present in the sand assemblages and that contributed to the group average dissimilarity (3 species vs. 8 in the tile) (Table 6.3). The species *Diademesmis confervacea* Kützing, *Gomphonema* cf. *affine* Kützing and *Nitzschia acicularis* (Kützing) W. Smith were only found in the epipsammic assemblages while species such as *Nitzschia fonticola* (Grunow) Grunow, *Tryblionella hungarica* (Grunow) Frenguelli and *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot were only present in the epilithic assemblages (Table 6.3). Despite these differences, the diversity was similar between substrates (Td35 vs. Sd35  $t=0.98$ ,  $p(\text{perm})=0.35$ ) with mean values of 1.88 and 1.71 for epilithic and epipsammic assemblages, respectively (Figure 6.5).

The comparison of the same substrate over time revealed that in both cases the number of species contributing to the within group average similarity decreased (up to 80% cumulative contribution; Table 6.2) with *Achnantheidium minutissimum*, *Navicula notha* and *Fragilaria* cf. *parva* becoming most relevant at d35. From day 14 to day 35 there was a significant decrease in the diversity, independently of the substrate (Td14 vs. Td35  $t=5.19$ ,  $p(\text{perm})=0.01$ ; Sd14 vs. Sd35  $t=4.94$ ,  $p(\text{perm})<0.01$ ) (Figure 6.5).

Despite the different multidimensional patterns of the epilithic and epipsammic communities at the same sampling moment, the IPS values among the two substrates had a good agreement (Td14 vs. Sd14  $t=1.32$ ,  $p(\text{MC})=0.23$ ; Td35 vs. Sd35  $t=0.32$ ,  $p(\text{MC})=0.76$ )

(Figure 6.6). There was a significant increase in the IPS values over time in both substrates (Td14 vs. Td35  $t=4.46$ ,  $p(\text{MC})<0.01$ ; Sd14 vs. Sd35  $t=5.07$ ,  $p(\text{MC})<0.01$ ) (Figure 6.6).

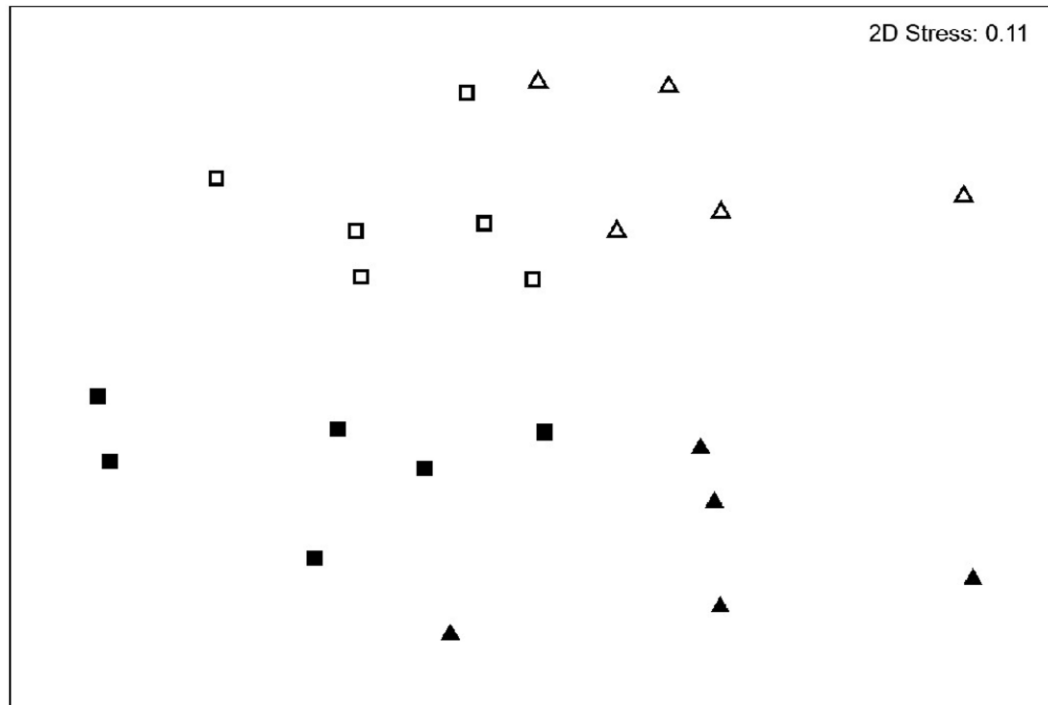


Figure 6.4 - Multidimensional Scaling Analysis (MDS) ordination of diatom assemblages at day 14 (white symbols) and 35 (black symbols) on the tile (triangles) and in the sand (squares).

Table 6.2 - Diatom species that contributed more to the group average similarity (up to 80% of cumulative abundance of the Bray–Curtis similarity) developing on tiles (T) and sand (S) substrates at day 14 and 35 obtained by SIMPER analysis.

| Species  | Species contributive % |       |       |       |
|--|------------------------|-------|-------|-------|
|  | S14                    | T14   | S35   | T35   |
| <i>Achnanthes ricula</i> Hohn & Hellerman                            | 1.63                   | 1.96  | -     | -     |
| <i>Achnanthidium catenatum</i> (Bily & Marvan) Lange-Bertalot        | 3.10                   | 2.66  | 5.43  | 7.89  |
| <i>Achnanthidium minutissimum</i> (Kützing) Czarnecki                | 14.94                  | 13.55 | 23.94 | 22.90 |
| <i>Asterionella formosa</i> Hassall                                  | 2.03                   | 2.12  | -     | -     |
| <i>Aulacoseira granulata</i> (Ehrenberg) Simonsen                    | 1.77                   | 1.86  | -     | 1.75  |
| <i>Cyclotella atomus</i> Hustedt                                     | 2.18                   | 2.95  | 2.01  | -     |
| <i>Encyonema minutum</i> (Hilse) D. G. Mann                          | 2.77                   | 2.26  | 2.30  | -     |
| <i>Eolimna minima</i> (Grunow) Lange-Bertalot & W. Schiller          | 1.66                   | -     | -     | -     |
| <i>Fragilaria</i> aff. <i>pectinalis</i> (O. F. Müller) Lyngbye      | 2.71                   | 2.49  | 4.05  | 4.46  |
| <i>Fragilaria</i> cf. <i>parva</i> (Grunow) A. Tuji & D. M. Williams | 6.01                   | 7.79  | 6.64  | 11.63 |
| <i>Geissleria decussis</i> (Østrup) Lange-Bertalot & Metzeltin       | -                      | 1.46  | 1.94  | 2.55  |
| <i>Gomphonema</i> cf. <i>pseudoaugur</i> Lange-Bertalot              | 1.42                   | -     | -     | -     |
| <i>Gomphonema lagenula</i> Kützing                                   | 2.88                   | 1.57  | 2.77  | -     |

|  |      |      |      |      |
|--|------|------|------|------|
| <i>Gomphonema parvulum</i> (Kützing) Kützing                                       | 1.31 | 1.36 | -    | -    |
| <i>Karayevia oblongella</i> (Østrup) Aboal   | 1.38 | 1.69 | -    | -    |
| <i>Mayamaea atomus</i> var. <i>permitis</i> (Hustedt) Lange-Bertalot               | 2.97 | 1.59 | 2.40 | 2.18 |
| <i>Melosira varians</i> C. Agardh  | -    | 1.39 | -    | -    |
| <i>Navicula cryptocephala</i> Kützing  | 1.89 | 2.15 | 2.35 | 2.97 |
| <i>Navicula gregaria</i> Donkin  | -    | 1.69 | -    | -    |
| <i>Navicula notha</i> J. H. Wallace  | 6.42 | 5.06 | 7.09 | 5.51 |
| <i>Navicula veneta</i> Kützing   | -    | 1.52 | -    | -    |
| <i>Nitzschia acidoclinata</i> Lange-Bertalot                                       | 3.40 | 4.42 | 3.79 | 3.64 |
| <i>Nitzschia agnita</i> Hustedt  | 2.40 | 1.78 | -    | -    |
| <i>Nitzschia</i> cf. <i>laccum</i> Lange-Bertalot                                  | -    | 1.25 | -    | -    |
| <i>Nitzschia</i> cf. <i>palea</i> (Kützing) W. Smith                               | 2.21 | 1.40 | -    | -    |
| <i>Nitzschia palea</i> (Kützing) W. Smith  | 5.50 | 3.77 | 4.43 | 3.13 |
| <i>Nitzschia palea</i> var. <i>debilis</i> (Kützing) Grunow                        | 3.86 | 3.23 | 3.43 | 2.63 |
| <i>Planothidium dau</i> i (Foged) Lange-Bertalot                                   | 2.21 | 2.58 | -    | 2.71 |
| <i>Planothidium frequentissimum</i> (Lange-Bertalot) Lange-Bertalot                | -    | -    | -    | 1.82 |
| <i>Staurosira construens</i> Ehrenberg   | -    | 1.67 | -    | -    |
| <i>Ulnaria delicatissima</i> var. <i>angustissima</i> (Grunow) Aboal & P. C. Silva | 3.49 | 2.42 | 6.08 | 3.10 |

Table 6.3 - Diatom species that contributed more to the group average dissimilarity and that were only present in one of the substrates at the same sampling moment, day 14 and 35 (up to 80% of cumulative abundance of the Bray–Curtis similarity) obtained by SIMPER analysis.

| Day | Sand  | Tile   |
|-----|---|--|
| 14  | <i>Adlafia minuscula</i> var. <i>muralis</i> (Grunow)<br>Lange-Bertalot |  |
|     | <i>Surirella linearis</i> W. Smith                                      |  |
|     | <i>Nitzschia sociabilis</i> Hustedt                                     |  |
|     | <i>Cymbella tumida</i> (Brébisson) Van Heurck                           |  |
|     | <i>Halamphora veneta</i> (Kützing) Levkov                               |  |
|     | <i>Nitzschia perminuta</i> (Grunow) M. Peragallo                        |  |
|     | <i>Achnanthisidium rivulare</i> Potapova & Ponader                      |  |
|     |   | <i>Hippodonta capitata</i> (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski |
|     |   | <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer                     |
|     |   | <i>Navicula lanceolata</i> Ehrenberg   |
|     |   | <i>Placoneis protracta</i> (Grunow) Mereschkowsky                            |
|     |   | <i>Placoneis clementis</i> (Grunow) E. J. Cox                                |
|     |   | <i>Cymbella microcephala</i> Grunow  |
|     |   | <i>Eolimna subminuscula</i> (Manguin) Gerd Moser, Lange-Bertalot & Metzeltin |

|    |   |
|----|---|
|    | <i>Cymbopleura naviculiformis</i> (Auerswald ex Heiberg) Krammer<br><i>Surirella roba</i> Leclercq<br><i>Tryblionella levidensis</i> W. Smith<br><i>Nitzschia paleacea</i> (Grunow) Grunow<br><i>Nitzschia valdestriata</i> Aleem & Hustedt<br><i>Amphora pediculus</i> (Kützing) Grunow ex A. Schmidt<br><i>Eunotia subarcuatooides</i> Alles, Nörpel & Lange-Bertalot<br><i>Eunotia tenella</i> (Grunow) Hustedt<br><i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot<br><i>Navicula germainii</i> Wallace<br><i>Neidium ampliutum</i> (Ehrenberg) Krammer                                 |
| 35 | <i>Diadesmis confervacea</i> Kützing<br><i>Gomphonema cf. affine</i> Kützing<br><i>Nitzschia acicularis</i> (Kützing) W. Smith<br><br><i>Nitzschia fonticola</i> (Grunow) Grunow<br><i>Tryblionella hungarica</i> (Grunow) Frenguelli<br><i>Planothidium lanceolatum</i> (Brébisson ex Kützing) Lange-Bertalot<br><i>Navicula phyllepta</i> Kützing<br><i>Sellaphora pupula</i> (Kützing) Mereschkovsky<br><i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot<br><i>Sellaphora nana</i> (Hustedt) Lange-Bertalot, Cavacini, Tagliaventi & Alfinito<br><i>Pinnularia cf. acoricola</i> Hustedt |

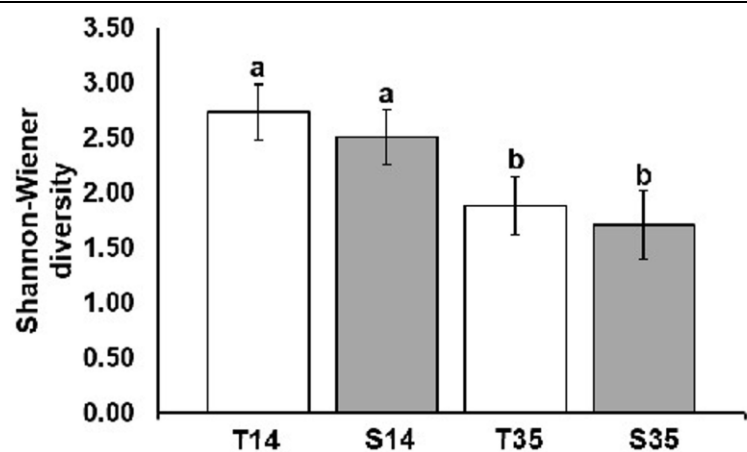


Figure 6.5 - Diatom diversity (H') (mean±standard deviation) found in the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b) do not significantly differ (p>0.05; PERMANOVA pairwise test).

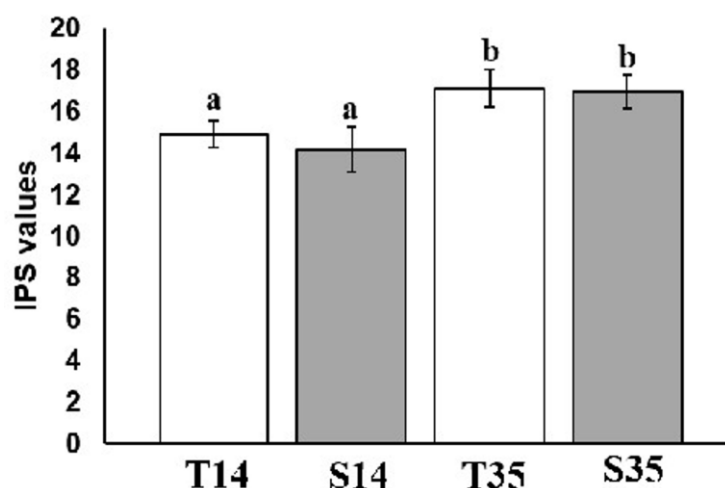


Figure 6.6 - Diatom-based IPS index (mean±standard deviation) obtained from the assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels. Treatment means labelled with the same letter (a, b) do not significantly differ ( $p>0.05$ ; PERMANOVA pairwise test).

#### 6.3.4 Biological traits

In both substrates mobile species were more frequent than planktonic (Figure 6.7a and b). Between substrates, the epipsammic assemblages presented higher number of mobile and planktonic valves in both sampling moments (day 14 and 35) (Figure 6.7a and b). However, the number of mobile and planktonic valves did not change over time in both substrates (Figure 6.7a and b). Concerning the form of attachment (pad, stalked or adnate) a higher abundance of species with the ability to attach to the substrate by stalk was found (Figure 6.7c, d and e).

In both sampling moments (d14 and 35) the sand assemblages presented higher number of stalked species than the tile assemblages (Figure 6.7d). Within sand assemblages there was a significant increase in the number of stalked diatoms from d14 to d35 (Figure 6.7d). Despite the apparent increase in the number of stalked valves in the tile over time, statistical differences were not found (Figure 6.7d). The majority of species that contributed to the stalked category belonged to the genus *Achnantheidium*. The number of diatoms with adnate habit and pads was similar between substrates and over time (Figure 6.7c and e).



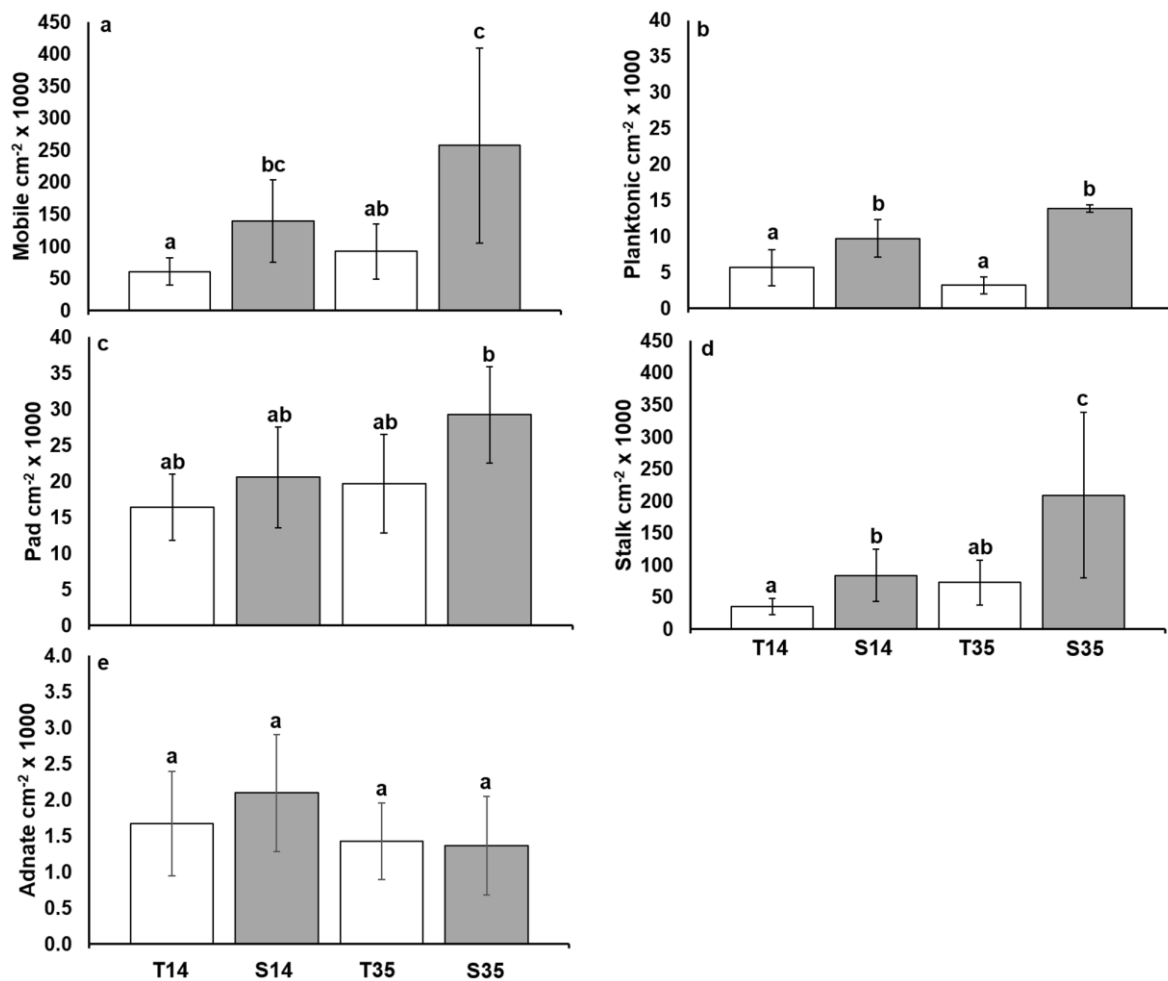


Figure 6.7 - Number of valves *per* cm<sup>2</sup> (mean ± standard deviation) found in the diatom assemblages developing on tiles (T) and sand (S) substrates at day 14 and 35 in the artificial channels with the trait life-form categories: (a) Mobile, (b) Planktonic, (c) Pad, (d) Stalk and (e) Adnate. Treatment means labelled with the same letter (a, b, c) do not significantly differ ( $p > 0.05$ ; PERMANOVA pairwise test).

## 6.4 Discussion

The results of this mesocosm experiment show that the substrate affects diatom assemblage's composition. This is in agreement with other studies that indicated that the composition of diatom assemblages on different substrates was different (Round, 1991; Cattaneo et al., 1997; Potapova and Charles, 2005). Yet, contrary to our findings other studies have not found differences between substrates (Rothfritz et al., 1997; Bere and Tundisi, 2011; Winter and Duthie, 2000). The differences found between the assemblage composition of the two substrates might be due to the species which dominated and were common to both substrates which is probably related to other factors. For example, both *Achnantheidium*

*minutissimum* and *Navicula notha* have high oxygen requirements (polyoxybionte; van Dam et al., 1994) which was a condition satisfied by our experimental design.

We also found a significantly higher number of mobile cells in sand compared with the tile substrates, which is in accordance with other studies (Cattaneo et al., 1997; Potapova and Charles, 2005). Stalked species were also always significantly more abundant in sand compared with the tile, contrary to what we were expecting, as in the sand the majority of the species present will be those which have the necessary traits to tolerate the abrasion of moving grains (Townsend and Gell, 2005) or be able to move (Soininen and Eloranta, 2004) in order to avoid entrapment by the sand grains. However, the species that contributed most to the stalk categories were from the genus *Achnantheidium*, in particular *A. minutissimum*. This species has been found to dominate in highly hydrological disturbed habitats suggesting that it may have resistance to the dislodgement induced by current shear forces (Soininen and Eloranta, 2004).

Contrary to what we were expecting (see also Potapova and Charles, 2005), at the same sampling moment, the sand assemblages were never more diverse than the tile assemblages. It is expected that epilithic diatom assemblages are more stable than epipsammic ones because much less disturbance due to moving substrate particles occurs on firm stony substrates. Therefore, in natural environments the higher diversities found in natural epipsammic assemblages may be also due to the fact that in many occasions the sampled assemblage is not an undisturbed mature one. According to Tuji (2000) when a community is in the last phase of colonization of a substrate and is affected by a disturbance, the resulting community architecture becomes similar to the first phase. Although the epipsammon represents a specialized diatom assemblage that seems well adapted to a variable environment, disturbance probably plays an important role in structuring the assemblage, keeping it in a 'pioneer' state (Miller et al., 1987). So, when we allowed the assemblages to develop during the 35 days without additional disturbances it resulted in similar development states for both substrates and consequently similar diversities. This is in agreement with some studies dealing with differences in diatom assemblages among different substrates, where the role of factors such as hydrology (Soininen and Eloranta, 2004) and pollution (Bere and Tundisi, 2011) were found to overcome that of substrates. In some situations the diversity differences may also be the result of significant differences at the population level often associated with small algal populations that exert little influence on density and diversity, and these differences may be an artefact of a chance encounter of a rare population during algal enumeration (Lowe et al., 1996).

Regarding the colonization process, and according to chl-*c* and diatom density by the fourteenth colonization day, diatom assemblages were already stable, independently of the substrate as there were no significant differences between sampling moments. In agreement,

a study by Oemke and Burton (1986) dealing with diatom colonization dynamics (diatom cell densities) growing on glass slides, showed that the rate of increase of diatom density slowed after 10 or 14 days with the colonization curves reaching an apparent plateau by day 21 or 28. Yet, contrary to diatom density, the last 21 days of colonization of our experiment contributed to changes in the diatom assemblage's composition in both sand and tile channels. These results suggest a decline of less abundant species and the dominance of a small number of species. Oemke and Burton (1986) verified also a gradual decline in diversity after an early peak as a result of an increased dominance of few species. In addition, changes in traits also occurred over time, with a significant increase of the category 'stalked' on tiles from d14 to d35.

Considering the water quality assessment, the IPS values obtained at the same sampling moment did not reflect the differences in epipsammic and epilithic diatom assemblages that were obtained in terms of multivariate patterns. As in other studies, this suggests that hard and soft (sand) substrates can be exchangeable in assessment methods that are based on autoecological methods (Soininen and Könönen, 2004; Potapova and Charles, 2005; Mendes et al., 2012). Apparently, and considering the IPS results, the sand substrate assemblages were not more influenced by the sediment-bound chemicals than the epilithic ones (Kelly et al., 1998).

The significant increase in the IPS values over time in both substrates can be attributed to the dominance of sensitive species at day 35, which is the case of *Achnantheidium minutissimum*, in the IPS index. This index is based on weighted average between the relative abundance and the sensitivity (tolerance) and indicator value of a group selected species. Therefore, the high abundance of a sensitive species, as *A. minutissimum*, may cause such increase. This species has been considered indifferent to nutrient concentrations (van Dam et al., 1994); however a laboratory experiment carried out by Manoylov (2009) suggests that *A. minutissimum* is a good competitor for nutrients when they are in low supply compared with other taxa. Therefore, this adaptation may have allowed *A. minutissimum* to outgrow the other species by the end of the 35 days of colonization.

## 6.5 Conclusions

We verified that both substrates reached an almost maximum production (diatom cell density and chl-*c* concentration) after two weeks of colonization although we did not find any clear patterns among diatom assemblage diversity. The type of colonizing substrate influences diatom assemblages (production, density and composition, traits) but not water quality assessment. Therefore, we can argue that in streams where the preferential substrate

(usually stones or rocks) is not available and sand is the only substrate available this can be used as alternative if the aim is to assess water quality using an autoecological index.

### **Abbreviations**

|               |  |
|---------------|--|
| Chl- <i>a</i> | Chlorophyll- <i>a</i>                                    |
| Chl- <i>b</i> | Chlorophyll- <i>b</i>                                    |
| Chl- <i>c</i> | Chlorophyll- <i>c</i>                                    |
| d14           | Periphyton samples retrieved from the channels at day 14 |
| d35           | Periphyton samples retrieved from the channels at day 35 |
| H'            | Shannon-Wiener diversity index                           |
| IPS           | <i>Indice de Polluosensibilité Spécifique</i>            |
| MDS           | non-metric Multidimensional Scaling                      |
| MMS           | modular mesocosm systems                                 |
| S             | Assemblages growing on sand substrate                    |
| S14           | Epipsammic samples retrieved from the channels at day 14 |
| S35           | Epipsammic samples retrieved from the channels at day 35 |
| M             | Assemblages growing on tile substrate                    |
| M14           | Epilithic samples retrieved from the channels at day 14  |
| M35           | Epilithic samples retrieved from the channels at day 35  |

### **Acknowledgment**

This study was possible due to the financial support of the FOUNDATION FOR SCIENCE AND TECHNOLOGY (Portugal) through the Ph.D. scholarship SFRH/BD/68973/2010 of the first author and through the strategic project UID/MAR/04292/2013 granted to MARE and UID/GEO/04035/2013 granted to GeoBioTec. We thank GeoBioTec Research Centre and Biology Department of Aveiro, University of Aveiro. We thank to the Engineer Acácio Pascoal from the company Gres Panaria, Portugal S.A. - LOVE TILES division for the offer of the ceramic tiles and to the company Water Technologies by all the technical and equipment support.

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## Chapter 7 - Do similar climatic patterns in different continents lead to similar benthic diatom assemblages?

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Submitted for publication in *Hydrobiologia*.

### ABSTRACT

This study aimed to test whether similar macroclimatic patterns induce similar diatom assemblages on different continents, overriding regional environmental differences. We compared diatom assemblages (at species, genus and order taxonomic levels) from 227 rivers and stream sites in regions of Europe (Portugal), North America (USA) and Australia belonging to the Csb Mediterranean climate (updated world Köppen-Geiger climate map). At all levels, the diatom assemblages within each region were significantly more similar to each other than to those of the other regions (Pseudo-Fs: 23.00, 32.25, 28.99;  $p < 0.01$ ). In addition, the Portuguese and USA assemblages were more similar to each other (Bray-Curtis dissimilarity 76.1%) than to the Australian ones (84.4 and 85.0%, respectively, at species level). Among the species that contributed most to similarity within Portuguese and USA rivers were *Achnanthes minutissimum* (4.0-10.9 % contribution) and *Cocconeis placentula* (3.1-6.1% contribution) (SIMPER analyses). The species that contributed the most to similarity within Australian rivers were *Rhoicosphenia abbreviata* (13.0%) and *Amphora pediculus* (6.9%) (SIMPER analyses). Climate did not appear to engender similar assemblages in the different regions, and the inter-continental differences observed were probably related to factors such as biogeography, geology and water chemistry.

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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### **Keywords**

Climate, large-scale, continents, rivers, diatoms, taxonomical levels

## 7.1 Introduction

Climate, the long-term average weather conditions in an area, is one of the main factors controlling the large-scale distribution of species, communities and ecosystems (Lindsay and Bayoh, 2004; Bailey, 2009). As the climatic regime changes (*e.g.*, mean annual temperature), so does the hydrologic cycle (*e.g.*, evapotranspiration) and consequently the variety of plants and animals that can live in a particular region (Bailey, 2009). Several studies have verified that climate can be used to map the distribution of insects (Lindsay and Bayoh, 2004) and plants (Prentice et al., 1992) and can influence migratory frequencies (Dingle et al., 2000) and seed mass (Murray et al., 2004 ).

The Köppen system developed in 1900 by the climatologist Wladimir Köppen (here referred to as Köppen-Geiger classification) is the most widely used climatic classification for geographic purposes (Peel et al., 2007; Bailey, 2009). It was constructed on the basis of five vegetation groups delineated by the botanist De Candolle, which were in turn based on the climate zones of the ancient Greeks (Kottek et al., 2007; Peel et al., 2007). The five groups of Köppen-Geiger climates distinguish five broad plant formations: tropical (A), arid (B), temperate (C), cold zone (D) and polar (E) (Kottek et al., 2007; Peel et al., 2007; Bailey, 2009). The classification has two more levels: a second letter considers precipitation (*e.g.*, Cs for temperate and dry summer, also called Mediterranean) and a third considers the air temperature (*e.g.*, Csb for temperate, dry and warm summer) (Kottek et al., 2007; Peel et al., 2007).

The Mediterranean climate (Cs), although confined to less than 1% of the continental area (or up to 4% depending of the definition: see Gasith and Resh, 1999), is widely spread through almost all continents, occurring for example in the United States of America, countries surrounding the Mediterranean sea, South Africa and Australia (Gasith and Resh, 1999; Kottek et al., 2007; Peel et al., 2007). Streams and rivers in the Csa climate (temperate, dry and hot summer) are physically, chemically and biologically shaped by sequential, predictable and seasonal events of flooding and drying over an annual hydrological cycle, and are those that are usually called Mediterranean streams (Boix et al., 2010; García-Roger et al., 2011; Delgado et al., 2012). On the other hand, the streams and rivers experiencing Csb climate are not typically so affected by these events of flooding and drying as they are mostly perennial streams.

The long-term regime of natural environmental heterogeneity and disturbance patterns may constitute a physical habitat template that constrains the appropriate species attributes for local persistence (Poff and Ward, 1990). Due to the strong influence of Mediterranean climate on several stream characteristics (*e.g.*, hydrological regime, water temperature and

riparian inputs), communities of lotic ecosystems within that climatic region should be more similar to one another than those of other climatic regions (Gasith and Resh, 1999). Indeed, several studies have found similarities in aquatic macroinvertebrate assemblages and their traits among different Csa Mediterranean regions (Bonada et al., 2007; García-Roger et al., 2013) while the influence of Csb climate remains unstudied.

Diatoms typically respond to hydromorphological, chemical, and physical changes in the environment at small spatial scales (*e.g.*, Potapova and Charles, 2003; Almeida and Feio, 2012; Feio et al., 2014). However, these local environmental factors depend on and are influenced by ultimate factors such as climate, geology and land use (Stevenson, 1997). Consequently, climate is an important driver of diatom assemblages, and climatic variables may have a stronger association with stream diatom distributions than local environmental variables alone (Pajunen et al., 2016). Therefore, diatom assemblages from different Csb regions are likely to have similar composition. Although several studies of diatom assemblages have been carried out in Csb regions (Almeida and Gil, 2001; Potapova and Charles, 2003; Philibert et al., 2006; Feio et al., 2007; Feio et al., 2009), they did not specifically assess the influence of climate.

In the present study we hypothesized that climatic similarity alone can lead to consistency in the composition of benthic diatom assemblages, in spite of large-scale (inter-continental) separation and small-scale differences among rivers. We assessed the similarity of diatom assemblage composition from rivers and streams located in different continents (Europe, Australia and North America) but having in common the Csb Mediterranean climate, aiming to verify whether climate indeed shapes diatom assemblage composition. We analysed the similarities among the three regions at species, genus and order levels, in order to test the dependence of compositional similarity on taxonomic resolution.

## **7.2 Methods**

### **7.2.1 Study area and site selection**

The study included 227 river and stream sites in Cbs climatic regions: 139 in Europe - Portugal (PORT), 42 in North America – United States of America (USA) and 46 in Australia (AUS). The PORT sites were all within the northern region of the country, the USA sites were from the western states of California and Oregon, and the AUS sites were all in the coastal region of the state of South Australia. The selection of Cbs regions followed the updated world Köppen-Geiger climate map (Peel et al., 2007) and was done at macroclimatic scale (Bailey, 2009), mesoscale differences being ignored (*e.g.*, high mountains were included as well as lowland areas).

### 7.2.2 Diatom dataset

The PORT diatom dataset was obtained from a broad database (AQUAWEB: [www.aquaweb.pt](http://www.aquaweb.pt)) of diatom records for the entire country from 2004 to 2012. The USA diatom dataset was obtained from the United States Water Quality Portal (<http://waterqualitydata.us/portal/>), which contains diatom counts for benthic river samples collected by the USGS National Water Quality Assessment (NAWQA) Program from 1993 to 2005. The AUS diatom dataset was compiled under the national Monitoring River Health Initiative program from 1995 to 1997. In all regions, sites were sampled more than once and from more than one substratum and season (mostly from summer to autumn); the number of samples *per* site ranged from one to eighteen.

Portuguese sampling and sample treatment followed European standards (European Committee for Standardisation, 2003, 2004, 2006) and Portuguese official protocols (INAG, 2008). Samples were collected mostly from hard substrata. Identification to species or infra-specific rank and enumeration were done with a light microscope (100× objective and 1.32 numerical aperture) and the floras of Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b) and Krammer (2000, 2001, 2009). The USA samples were also collected mostly from hard substrata with the methods described by Porter et al. (1993) and Moulton et al. (2002). Identification and enumeration were done at the Academy of Natural Sciences of Philadelphia with the methods described by Charles et al. (2002). The AUS samples were collected from soft and hard substrata, prepared with the methods described by Battarbee (1986) and mounted on microscope slides with Naphrax®. Identification and enumeration were done with the procedures described in Philibert et al. (2006). The minimum number of valves *per* slide counted varied among countries: 600 in the USA, 400 in PORT and 200 in AUS.

Several steps were undertaken to ensure the comparability of datasets: (1) taxa not identified to species level were eliminated (*e.g.*, *Gomphonema* sp.); (2) some *Achnanthidium* species that we considered to be morphologically similar to *Achnanthidium minutissimum* (Kützing) Czarnecki and that in the past were not separated from its complex were merged as *A. minutissimum* sensu lato (*e.g.*, *Achnanthidium atomoides* Monnier, Lange-Bertalot & Ector and *Achnanthidium caravelense* M. E. Novais & L. Ector); (3) the varieties *Achnanthes subhudsonis* var. *kraeuselii* (Cholnoky) Cholnoky and *Achnanthes subhudsonis* Hustedt were merged at species level as *A. subhudsonis*; (4) the varieties of *Cocconeis placentula* Ehrenberg (*e.g.*, *Cocconeis placentula* var. *lineata* (Ehrenberg) Van Heurck and *Cocconeis placentula* var. *euglypta* (Ehrenberg) Grunow) were all merged in a single species, *C. placentula*; and (5) two additional datasets at higher taxonomic levels (genus and order) were created from the species dataset and the same statistical analyses were performed for the three datasets.

### 7.2.3 Data analyses

Analyses were performed on presence-absence data for each site at the three taxonomic levels (order, genus and species).

Multidimensional scaling (MDS; Bray-Curtis dissimilarity measure) was performed to determine whether diatom assemblages differed among regions. Inter-regional statistical differences were tested with permutational multivariate analysis of variance (PERMANOVA) global tests (unrestricted permutations). PERMANOVA pairwise tests (unrestricted permutations) were performed to assess which regions differed from each other. Similarity percentages analysis (SIMPER; Bray-Curtis similarity) was used to determine the most representative taxa of each region (those contributing the most to the average similarity within groups) and to determine the taxa that contributed the most to Bray-Curtis dissimilarity among regions. These analyses were performed with the PRIMER 6 & PERMANOVA software (PRIMER-E Ltd., Plymouth, UK; Clarke and Gorley, 2006; Anderson et al., 2008).

The frequencies of the five most representative taxa of each region (from SIMPER analyses) were compared among regions through one-way ANOVA tests (SigmaPlot 12.0 software). When normality and equal-variance tests failed, Kruskal–Wallis one-way ANOVA tests (ANOVA on ranks) were used. ANOVAs were followed by pairwise comparisons (Dunn's Method) to assess which regions differed in the percentage of sites where the taxa were found.

A graphical representation of the mean number of species *per* sample was performed to assess whether a variable number of samples *per* site could have contributed to differences among regions. Inter-regional statistical differences were assessed with one-way ANOVA tests (SigmaPlot 12.0 software) as described above.

## 7.3 Results

### 7.3.1 Species-level

The analysis included 634 diatom species. The assemblages of the PORT, USA and AUS regions differed significantly overall (Pseudo-F = 23.00,  $p < 0.01$ ) and in all pairwise comparisons ( $p < 0.01$ ). The USA assemblages were most similar to the PORT ones (average similarity of 23.9%), while the AUS assemblages were the most segregated (average dissimilarity of 85.0% and 84.4% with PORT and USA, respectively) (Figure 7.1). Diatom richness *per* sample was lower in Australian streams than in the other two regions (Figure 7.2).

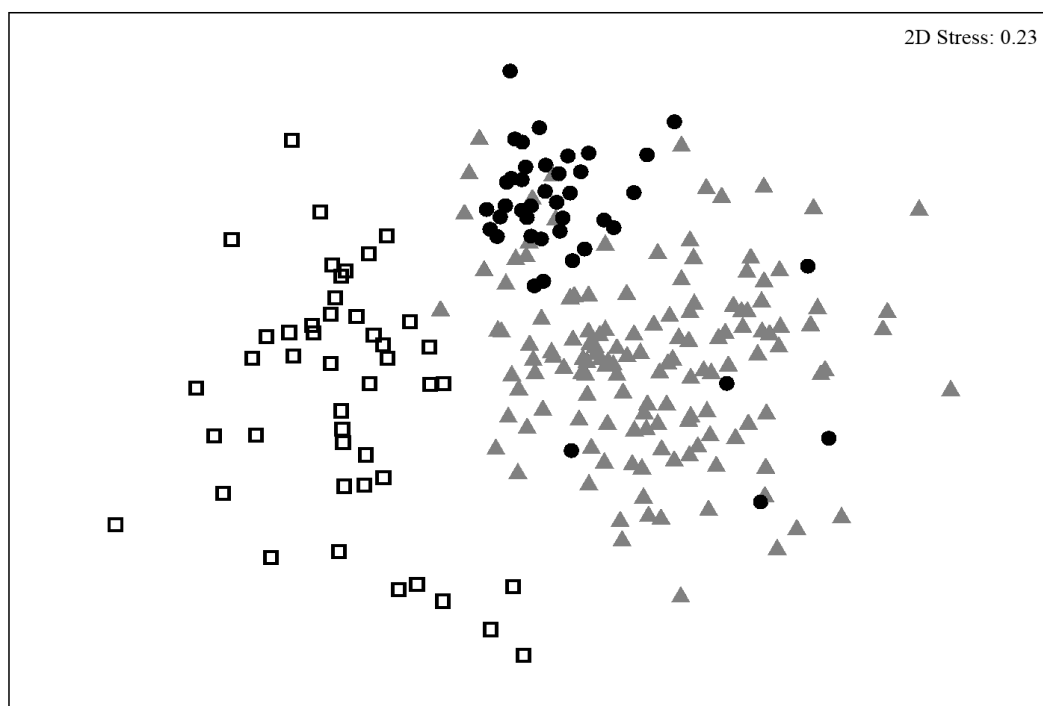


Figure 7.1 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at species level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares).

The species *Achnantheidium minutissimum*, *Gomphonema parvulum* (Kützing) Kützing, *Cocconeis placentula*, *Fragilaria vaucheriae* (Kützing) J. B. Petersen and *Encyonema minutum* (Hilse) D. G. Mann contributed the most to average assemblage similarity within the PORT rivers, with contributions ranging from 5.2 to 10.9% (Table 7.1). *A. minutissimum*, *Eolimna minima* (Grunow) Lange-Bertalot & W. Schiller, *C. placentula*, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot and *G. parvulum* contributed the most to average similarity in the USA (Table 7.1; contributions between 3.0 and 4.0%). These results indicate that these two regions were dominated by circumneutral to alkaliphilous, freshwater species (occurring at < 500 mg Cl<sup>-</sup>/L) and by species with high to low oxygen requirements. In AUS, the species that contributed most to average similarity were *Rhoicosphenia abbreviata* (C. Agardh) Lange-Bertalot, *Amphora pediculus* (Kützing) Grunow ex. A. Schmidt, *C. placentula*, *Nitzschia inconspicua* Grunow and *Tabularia fasciculata* (C. Agardh) D. M. Williams & Round, with percentage contributions ranging from 5.9 to 13.0 (Table 7.1). This list includes alkaliphilous, fresh-brackish (< 500 mg Cl<sup>-</sup>/L) to brackish (1000-5000 mg Cl<sup>-</sup>/L) species and species with fairly high (above 75% saturation) to moderate (above 50% saturation) oxygen requirements. In comparison with the other two regions, the USA had small percentage contributions from all species and thus more species represented in 90% of cumulative contribution (Table 7.1).



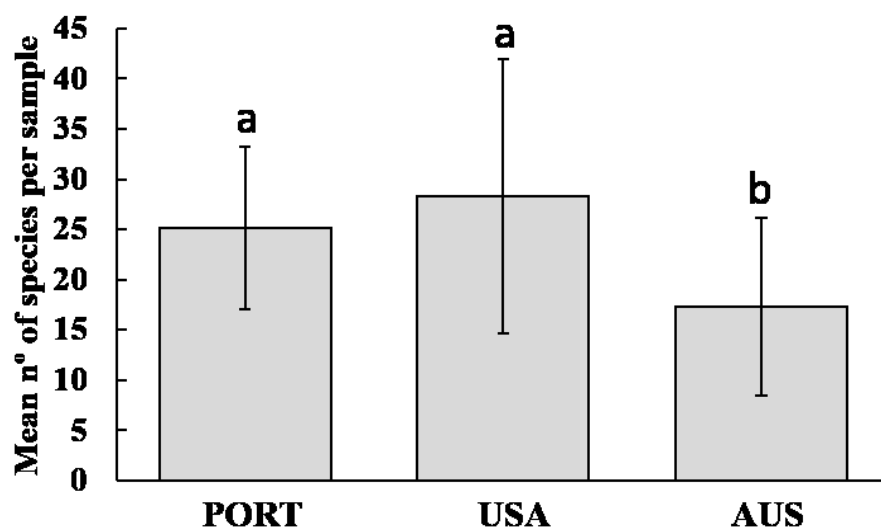


Figure 7.2 - Mean number ( $\pm$ SD) of species *per* sample in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ ).

Of the five species that contributed the most to within-USA similarity, *Eolimna minima* was the only one that was significantly more frequent in the USA (Figure 7.3). None of the five species that contributed most to within-PORT similarity was significantly more frequent in PORT (Figure 7.3), and all were equally frequent, or more frequent (*E. minima*) in the USA. Of the five species that contributed most to within-AUS similarity, only *Tabularia fasciculata* was significantly more frequent in that region. The remainder were all similarly frequent in the AUS and USA regions (Figure 7.3). The species *Rhoicosphenia abbreviata*, *Amphora pediculus* and *Nitzschia inconspicua* were more frequent in the USA and AUS than in PORT. From the set of species contributing most to within-group average similarity it is possible to verify a greater proximity of the USA-AUS and USA-PORT assemblages and a greater distance between the PORT and AUS ones.

The species *Achnantheidium pyrenaicum* (Hustedt) H. Kobayasi, *Fragilaria capucina* Desmazières, *Mayamaea atomus* var. *permitis* (Hustedt) Lange-Bertalot and *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot were found only in PORT and included in those that contributed most to average dissimilarity among regions (Table 7.2). In the USA, the unique species that contributed to average dissimilarity were *Navicula viridulacalcis* Lange-Bertalot, *Gomphonema subclavatum* (Grunow) Grunow and *Sellaphora mutata* (Krasske) Lange-Bertalot. The species *Navicymbula* (=Cymbella) *pusilla* (Grunow) Krammer, *Nitzschia liebethruthii* Rabenhorst, *Nitzschia perspicua* Cholnoky and *Rhopalodia musculus* (Kützing) O. Müller were found only in AUS. The higher average dissimilarity between PORT

and AUS was due to differences in species frequencies and to species that were found only in PORT (Table 7.2). *Rhoicosphenia abbreviata*, *Tabularia fasciculata*, *Amphora pediculus* and *Nitzschia inconspicua* contributed to the dissimilarity between PORT and AUS (up to 18% cumulative) by being more frequent in AUS, while *Fragilaria vaucheriae*, *Encyonema minutum*, *Eolimna minima*, *Planothidium frequentissimum*, *Gomphonema parvulum* and *Nitzschia palea* (Kützing) W. Smith were more frequent in PORT. The dissimilarity between the USA and AUS was also due to differences in species frequencies and to species that were found only in the USA (Table 7.2); the species that contributed most (up to 18% cumulative) were all more frequent in the USA with the exception of *T. fasciculata*. Once again, *E. minima*, *P. frequentissimum*, *E. minutum*, *F. vaucheriae* and *G. parvulum* were among those species. These species were not among those that contributed most to the average dissimilarity between PORT and USA (e.g., *R. abbreviata*, *Sellaphora pupula* (Kützing) Mereschkovsky, *N. inconspicua*, *Melosira varians* C. Agardh).

Table 7.1 - Diatom species that contributed most to average assemblage similarity in PORT-Portugal, USA-United States of America and AUS-Australia (up to 90% of cumulative contribution, Bray–Curtis similarity: SIMPER analysis). The values in bold indicate the species that contributed the most to average similarity within each region.

| Species  | Contribution (%) |            |            |
|--|------------------|------------|------------|
|  | PORT             | USA        | AUS        |
| <i>Achnanthes subhudsonis</i> Hustedt  | 0.5              | 0.7        | -          |
| <i>Achnanthidium minutissimum</i> (Kützing) Czarnecki                          | <b>10.9</b>      | <b>4.0</b> | 4.8        |
| <i>Achnanthidium pyrenaicum</i> (Hustedt) H. Kobayasi                          | 1.2              | -          | -          |
| <i>Achnanthidium subatomoides</i> (Hustedt) O. Monnier, Lange-Bertalot & Ector | 1.8              | 0.3        | -          |
| <i>Amphora copulata</i> (Kützing) Schoeman & R. E. M. Archibald                | -                | 1.1        | -          |
| <i>Amphora pediculus</i> (Kützing) Grunow ex. A. Schmidt                       | 0.5              | 1.3        | <b>6.9</b> |
| <i>Aulacoseira alpigena</i> (Grunow) Krammer                                   | -                | 0.3        | -          |
| <i>Aulacoseira ambigua</i> (Grunow) Simonsen                                   | -                | 0.3        | -          |
| <i>Aulacoseira granulata</i> (Ehrenberg) Simonsen                              | -                | 0.3        | -          |
| <i>Bacillaria paxillifera</i> (O. F. Müller) T. Marsson                        | -                | 0.2        | 4.3        |
| <i>Caloneis bacillum</i> (Grunow) Cleve  | -                | 0.7        | -          |
| <i>Cocconeis placentula</i> Ehrenberg  | <b>6.1</b>       | <b>3.1</b> | <b>6.5</b> |
| <i>Craticula molestiformis</i> (Hustedt) Mayama                                | -                | 0.2        | -          |
| <i>Cyclotella meneghiniana</i> Kützing   | 0.5              | 0.8        | 0.8        |
| <i>Cymbella affinis</i> Kützing  | -                | 0.2        | -          |
| <i>Diadesmis contenta</i> (Grunow) D. G. Mann                                  | -                | 0.5        | -          |
| <i>Diatoma mesodon</i> (Ehrenberg) Kützing                                     | 0.9              | 0.3        | -          |
| <i>Diatoma vulgare</i> Bory  | -                | 0.3        | -          |
| <i>Discotella pseudostelligera</i> (Hustedt) Houk et Klee                      | -                | 0.3        | -          |
| <i>Encyonema minutum</i> (Hilse) D. G. Mann                                    | <b>5.2</b>       | 2.6        | -          |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|  |            |            |     |
|--|------------|------------|-----|
| <i>Encyonema silesiacum</i> (Bleisch) D. G. Mann                             | 0.9        | 0.5        | -   |
| <i>Encyonopsis cesatii</i> (Rabenhorst) Krammer                              | -          | 0.3        | -   |
| <i>Eolimna minima</i> (Grunow) Lange-Bertalot & W. Schiller                  | 4.7        | <b>3.4</b> | -   |
| <i>Epithemia sorex</i> Kützing   | -          | -          | 1.8 |
| <i>Eunotia implicata</i> Nörpel, Lange-Bertalot & Alles                      | -          | 1.2        | -   |
| <i>Eunotia incisa</i> W. Smith ex W. Gregory                                 | -          | 0.9        | -   |
| <i>Eunotia minor</i> (Kützing) Grunow  | 1.6        | -          | -   |
| <i>Eunotia tenella</i> (Grunow) Hustedt                                      | -          | 1.3        | -   |
| <i>Fragilaria capucina</i> Desmazières                                       | 1.5        | -          | -   |
| <i>Fragilaria vaucheriae</i> (Kützing) J. B. Petersen                        | <b>5.8</b> | 2.4        | -   |
| <i>Fragilaria gracilis</i> Østrup  | -          | 0.4        | -   |
| <i>Fragilaria rumpens</i> (Kützing) G. W. F. Carlson                         | 0.7        | 1.3        | 1.5 |
| <i>Frustulia vulgaris</i> (Thwaites) De Toni                                 | -          | 1.1        | -   |
| <i>Frustulia weinholdii</i> Hustedt  | -          | 0.2        | -   |
| <i>Gomphonema angustatum</i> (Kützing) Rabenhorst                            | -          | 0.4        | -   |
| <i>Gomphonema dichotomum</i> Kützing   | -          | 0.3        | -   |
| <i>Gomphonema gracile</i> Ehrenberg  | -          | 0.2        | -   |
| <i>Gomphonema kobayasii</i> Kociolek et Kingston                             | -          | 0.3        | -   |
| <i>Gomphonema parvulum</i> (Kützing) Kützing                                 | <b>7.2</b> | <b>3.0</b> | 2.8 |
| <i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot             | 2.3        | 2.2        | -   |
| <i>Gomphonema rhombicum</i> Fricke   | 1.8        | 0.3        | -   |
| <i>Gomphonema subclavatum</i> (Grunow) Grunow                                | -          | 0.7        | -   |
| <i>Gyrosigma acuminatum</i> (Kützing) Rabenhorst                             | -          | 0.3        | -   |
| <i>Halamphora veneta</i> (Kützing) Levkov                                    | -          | 0.2        | 1.7 |
| <i>Hannaea arcus</i> (Ehrenberg) R. M. Patrick                               | 0.7        | 0.4        | -   |
| <i>Hantzschia amphioxys</i> (Ehrenberg) Grunow                               | -          | 0.2        | -   |
| <i>Hippodonta capitata</i> (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski | -          | 1.3        | 2.3 |
| <i>Karayevia oblongella</i> (Østrup) Aboal                                   | 1.1        | -          | -   |
| <i>Lemnicola hungarica</i> (Grunow) F. E. Round & P. W. Basson               | -          | 0.2        | -   |
| <i>Luticola mutica</i> (Kützing) D. G. Mann                                  | -          | 0.3        | -   |
| <i>Melosira varians</i> C. Agardh  | 0.9        | 2.5        | 3.4 |
| <i>Meridion circulare</i> (Greville) C. Agardh                               | -          | 0.5        | -   |
| <i>Navicula capitatoradiata</i> H. Germain                                   | -          | 0.2        | -   |
| <i>Navicula cryptocephala</i> Kützing  | 3.9        | 2.7        | 1.9 |
| <i>Navicula cryptotenella</i> Lange-Bertalot                                 | 2.3        | 2.4        | -   |
| <i>Navicula escambia</i> (Patrick) Metzeltin & Lange-Bertalot                | -          | 0.4        | -   |
| <i>Navicula germainii</i> Wallace  | -          | 1.1        | 1.2 |
| <i>Navicula gregaria</i> Donkin  | 1.1        | 2.1        | -   |
| <i>Navicula lanceolata</i> Ehrenberg   | -          | 0.5        | -   |
| <i>Navicula menisculus</i> Schumann  | -          | 0.5        | -   |
| <i>Navicula notha</i> J. H. Wallace  | -          | 0.2        | -   |
| <i>Navicula rhynchocephala</i> Kützing                                       | -          | 0.4        | -   |
| <i>Navicula symmetrica</i> Patrick   | -          | 0.4        | -   |

|   |      |            |             |
|---|------|------------|-------------|
| <i>Navicula tenelloides</i> Hustedt                                   | -    | 0.2        | -           |
| <i>Navicula trivialis</i> Lange-Bertalot                              | -    | 1.1        | -           |
| <i>Navicula veneta</i> Kützing  | 1.01 | -          | 5.1         |
| <i>Navicula viridulacalcis</i> Lange-Bertalot                         | -    | 0.3        | -           |
| <i>Nitzschia amphibia</i> Grunow                                      | 0.6  | 1.7        | -           |
| <i>Nitzschia archibaldii</i> Lange-Bertalot                           | -    | 1.6        | -           |
| <i>Nitzschia dissipata</i> (Kützing) Rabenhorst                       | 1.1  | 2.4        | -           |
| <i>Nitzschia dissipata</i> var. <i>media</i> (Hantzsch) Grunow        | -    | 0.3        | -           |
| <i>Nitzschia fonticola</i> (Grunow) Grunow                            | -    | 0.3        | -           |
| <i>Nitzschia frustulum</i> (Kützing) Grunow                           | -    | 1.8        | 1.8         |
| <i>Nitzschia inconspicua</i> Grunow                                   | 0.8  | 2.4        | <b>6.2</b>  |
| <i>Nitzschia lacuum</i> Lange-Bertalot                                | -    | -          | 1.6         |
| <i>Nitzschia liebethruthii</i> Rabenhorst                             | -    | -          | 0.7         |
| <i>Nitzschia linearis</i> var. <i>subtilis</i> (Grunow) Hustedt       | -    | 0.6        | -           |
| <i>Nitzschia linearis</i> var. <i>tenuis</i> (W. Smith) Grunow        | -    | 0.5        | -           |
| <i>Nitzschia microcephala</i> Grunow                                  | -    | -          | 0.5         |
| <i>Nitzschia palea</i> (Kützing) W. Smith                             | 4.5  | 1.2        | 0.9         |
| <i>Nitzschia paleacea</i> (Grunow) Grunow                             | 1.8  | 0.2        | 0.6         |
| <i>Nitzschia recta</i> Hantzsch ex Rabenhorst                         | -    | 0.4        | -           |
| <i>Nitzschia sigma</i> (Kützing) W. Smith                             | -    | -          | 0.8         |
| <i>Pinnularia gibba</i> var. <i>linearis</i> Hustedt                  | -    | 0.2        | -           |
| <i>Pinnularia intermedia</i> (Lagerstedt) Cleve                       | -    | 0.3        | -           |
| <i>Pinnularia subcapitata</i> W. Gregory                              | 0.8  | -          | -           |
| <i>Planothidium delicatulum</i> (Kützing) Round & Bukhtiyarova        | -    | -          | 4.8         |
| <i>Planothidium frequentissimum</i> (Lange-Bertalot) Lange-Bertalot   | 4.4  | <b>3.1</b> | -           |
| <i>Planothidium lanceolatum</i> (Brébisson ex Kützing) Lange-Bertalot | 3.2  | 2.5        | 2.6         |
| <i>Psammothidium helveticum</i> (Hustedt) Bukhtiyarova & Round        | 0.9  | -          | -           |
| <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer              | 2.2  | 1.2        | -           |
| <i>Rhoicosphenia abbreviata</i> (C. Agardh) Lange-Bertalot            | -    | 2.5        | <b>13.0</b> |
| <i>Rhopalodia gibba</i> (Ehrenberg) O. Müller                         | -    | -          | 0.8         |
| <i>Sellaphora mutata</i> (Krasske) Lange-Bertalot                     | -    | 0.5        | -           |
| <i>Sellaphora pupula</i> (Kützing) Mereschkowsky                      | -    | 2.0        | -           |
| <i>Sellaphora seminulum</i> (Grunow) D. G. Mann                       | 1.4  | 1.5        | -           |
| <i>Stauroneis kriegeri</i> R. M. Patrick                              | -    | 0.3        | -           |
| <i>Staurosira brevistriata</i> (Grunow) Grunow                        | -    | 0.3        | -           |
| <i>Staurosira venter</i> (Ehrenberg) Cleve & J. D. Möller             | -    | 0.9        | -           |
| <i>Staurosirella pinnata</i> (Ehrenberg) D. M. Williams & Round       | -    | 0.8        | -           |
| <i>Surirella angusta</i> Kützing                                      | 0.6  | 0.8        | -           |
| <i>Surirella brebissonii</i> Krammer & Lange-Bertalot                 | -    | -          | 1.3         |
| <i>Surirella minuta</i> Brébisson ex Kützing                          | -    | 0.7        | -           |
| <i>Surirella ovalis</i> Brébisson                                     | -    | -          | 0.6         |
| <i>Surirella tenera</i> W. Gregory                                    | -    | 0.2        | -           |
| <i>Tabellaria flocculosa</i> (Roth) Kützing                           | 0.8  | 0.4        | -           |

|   |     |     |            |
|---|-----|-----|------------|
| <i>Tabularia fasciculata</i> (C. Agardh) D. M. Williams & Round | -   | -   | <b>5.9</b> |
| <i>Tryblionella constricta</i> W. Gregory                       | -   | -   | 1.2        |
| <i>Tryblionella hungarica</i> (Grunow) Frenguelli               | -   | -   | 1.0        |
| <i>Ulnaria ulna</i> (Nitzsch) Compère                           | 2.3 | 2.4 | 1.1        |

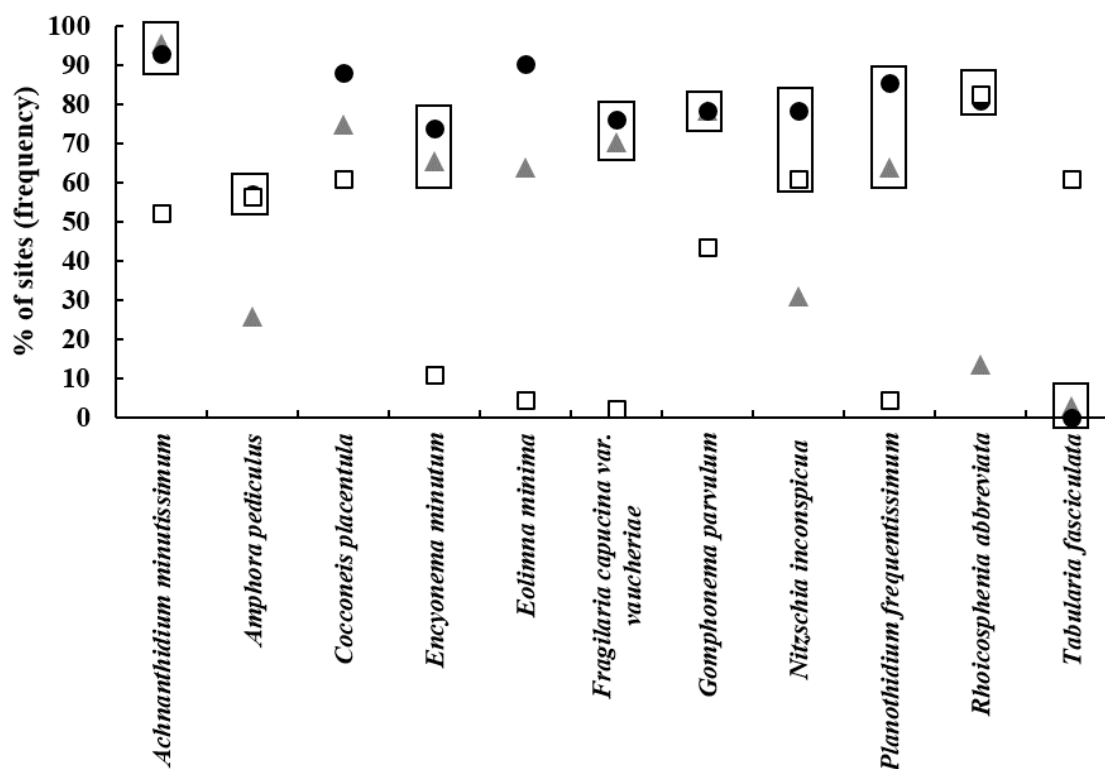


Figure 7.3 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the species that contributed most to within-region average similarity were found (Table 7.1). For each species, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). *Cocconeis placentula* was not significantly different between USA and AUS but the differences were not tested for the other two comparisons (PORT-USA and PORT-AUS).

Table 7.2 - Diatom species that contributed most to average dissimilarity between AUS-Australia, PORT-Portugal and USA-United States of America (up to 80% of cumulative dissimilarity, Bray–Curtis similarity: SIMPER analysis). The region code opposite a species indicates that the species was found only in that region among the two regions being compared.

| Species  | Comparison   |              |             |
|--|--------------|--------------|-------------|
|  | PORT vs. USA | PORT vs. AUS | USA vs. AUS |
| <i>Achnanthes subhudsonis</i> Hustedt  | -            | PORT         | USA         |
| <i>Achnanthidium deflexum</i> (C. W. Reimer) J. C. Kingston                    | USA          | -            | USA         |
| <i>Achnanthidium pyrenaicum</i> (Hustedt) H. Kobayasi                          | PORT         | PORT         | -           |
| <i>Achnanthidium subatomoides</i> (Hustedt) O. Monnier, Lange-Bertalot & Ector | -            | PORT         | USA         |
| <i>Amphora ovalis</i> (Kützing) Kützing  | -            | -            | USA         |
| <i>Asterionella formosa</i> Hassall  | -            | -            | USA         |
| <i>Aulacoseira alpigena</i> (Grunow) Krammer                                   | USA          | -            | USA         |
| <i>Aulacoseira ambigua</i> (Grunow) Simonsen                                   | -            | -            | USA         |
| <i>Aulacoseira granulata</i> (Ehrenberg) Simonsen                              | -            | -            | USA         |
| <i>Aulacoseira italica</i> (Ehrenberg) Simonsen                                | USA          | -            | -           |
| <i>Brachysira microcephala</i> (Grunow) Compère                                | -            | -            | USA         |
| <i>Craticula halophila</i> (Grunow) D. G. Mann                                 | -            | -            | AUS         |
| <i>Cymbella tumida</i> (Brébisson) Van Heurck                                  | -            | -            | USA         |
| <i>Diademesmis contenta</i> (Grunow) D. G. Mann                                | -            | -            | USA         |
| <i>Diatoma mesodon</i> (Ehrenberg) Kützing                                     | -            | PORT         | USA         |
| <i>Diatoma vulgaris</i> Bory   | -            | PORT         | USA         |
| <i>Diploneis oblongella</i> (Nägeli ex Kützing) Cleve-Euler                    | -            | -            | USA         |
| <i>Diploneis puella</i> (Schumann) Cleve                                       | USA          | -            | -           |
| <i>Discotella pseudostelligera</i> (Hustedt) Houk et Klee                      | -            | -            | USA         |
| <i>Discotella stelligera</i> (Cleve & Grunow) Houk & Klee                      | -            | -            | USA         |
| <i>Encyonema lunatum</i> (W. Smith) Van Heurck                                 | USA          | -            | -           |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|   |      |      |     |
|---|------|------|-----|
| <i>Encyonema neogracile</i> Krammer                                   | PORT | -    | -   |
| <i>Encyonema prostratum</i> (Berkeley) Kützing                        | USA  | -    | -   |
| <i>Encyonopsis cesatii</i> (Rabenhorst) Krammer                       | -    | -    | USA |
| <i>Eolimna tantula</i> (Hustedt) Lange-Bertalot                       | USA  | -    | USA |
| <i>Epithemia turgida</i> (Ehrenberg) Kützing                          | USA  | -    | USA |
| <i>Eunotia bilunaris</i> (Ehrenberg) Schaarschmidt                    | -    | PORT | USA |
| <i>Eunotia exigua</i> (Brebisson ex Kützing) Rabenhorst               | -    | PORT | USA |
| <i>Eunotia implicata</i> Nörpel, Lange-Bertalot & Alles               | -    | -    | USA |
| <i>Eunotia incisa</i> W. Smith ex W. Gregory                          | -    | PORT | USA |
| <i>Eunotia minor</i> (Kützing) Grunow                                 | -    | PORT | USA |
| <i>Eunotia monodon</i> Ehrenberg                                      | USA  | -    | USA |
| <i>Eunotia mucophila</i> (Lange-Bertalot, Nörpel-Schempp & Alles)     | -    | PORT | -   |
| <i>Eunotia naegeli</i> Migula   | -    | -    | USA |
| <i>Eunotia perpusilla</i> Grunow                                      | USA  | -    | USA |
| <i>Eunotia tenella</i> (Grunow) Hustedt                               | -    | -    | USA |
| <i>Fistulifera saprophila</i> (Lange-Bertalot & Bonik) Lange-Bertalot | PORT | PORT | -   |
| <i>Fragilaria bicapitata</i> Mayer                                    | USA  | -    | USA |
| <i>Fragilaria bidens</i> Heiberg                                      | PORT | PORT | -   |
| <i>Fragilaria capucina</i> Desmazières                                | PORT | PORT | -   |
| <i>Fragilaria miniscula</i> (Grunow) D. M. Williams & Round           | USA  | -    | USA |
| <i>Fragilaria virescens</i> Ralfs                                     | -    | PORT | -   |
| <i>Frustulia amphipleuroides</i> (Grunow) Cleve-Euler                 | USA  | -    | USA |
| <i>Frustulia erifuga</i> Lange-Bertalot & Krammer                     | -    | PORT | -   |
| <i>Frustulia weinholdii</i> Hustedt                                   | USA  | -    | USA |
| <i>Geissleria decussis</i> (Østrup) Lange-Bertalot & Metzeltin        | -    | -    | USA |
| <i>Gomphonema angustatum</i> (Kützing) Rabenhorst                     | -    | -    | USA |
| <i>Gomphonema angustatum</i> var. <i>intermedia</i> Grunow            | USA  | -    | -   |

|  |      |      |     |
|--|------|------|-----|
| <i>Gomphonema dichotomum</i> Kützing                                 | USA  | -    | USA |
| <i>Gomphonema kobayasii</i> Kociolek et Kingston                     | USA  | -    | USA |
| <i>Gomphonema lagenula</i> Kützing                                   | -    | -    | USA |
| <i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot     | -    | PORT | USA |
| <i>Gomphonema rhombicum</i> Fricke                                   |      | PORT | USA |
| <i>Gomphonema subclavatum</i> (Grunow) Grunow                        | USA  | -    | USA |
| <i>Hannaea arcus</i> (Ehrenberg) R. M. Patrick                       | -    | PORT | USA |
| <i>Karayevia oblongella</i> (Østrup) Aboal                           | PORT | -    | -   |
| <i>Luticola cohnii</i> (Hilse) D. G. Mann                            | -    | -    | USA |
| <i>Luticola goeppertiana</i> (Bleisch in Rabenhorst) D. G. Mann      | -    | PORT | -   |
| <i>Luticola mutica</i> (Kützing) D. G. Mann                          | -    | -    | USA |
| <i>Mayamaea atomus</i> var. <i>permitis</i> (Hustedt) Lange-Bertalot | PORT | PORT | -   |
| <i>Meridion circulare</i> (Greville) C. Agardh                       | -    | -    | USA |
| <i>Meridion circulare</i> var. <i>constrictum</i> (Ralfs) Van Heurck | -    | -    | USA |
| <i>Navicula angusta</i> Grunow                                       | PORT | PORT | -   |
| <i>Navicula arvensis</i> Hustedt                                     | -    | -    | USA |
| <i>Navicula vandamii</i> Schoeman & R. E. M. Archibald               | USA  | -    | USA |
| <i>Navicula concentrica</i> J. R. Carter & Bailey-Watts              | USA  | -    | USA |
| <i>Navicula cryptotenelloides</i> Lange-Bertalot                     | PORT | PORT | -   |
| <i>Navicula escambia</i> (Patrick) Metzeltin & Lange-Bertalot        | USA  | -    | USA |
| <i>Navicula genovefea</i> Fusey                                      | USA  | -    | USA |
| <i>Navicula gregaria</i> Donkin                                      | -    | PORT | USA |
| <i>Navicula heimansioides</i> Lange-Bertalot                         | PORT | PORT | -   |
| <i>Navicula notha</i> J. H. Wallace                                  | USA  | -    | USA |
| <i>Navicula radiosa</i> Kützing                                      | -    | PORT | -   |
| <i>Navicula reichardtiana</i> Lange-Bertalot                         | -    | -    | USA |
| <i>Navicula tripunctata</i> (O. F. Müller) Bory                      | -    | PORT | USA |
| <i>Navicula trivialis</i> Lange-Bertalot                             | -    | -    | USA |



Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|   |      |      |     |
|---|------|------|-----|
| <i>Navicula viridulacalcis</i> Lange-Bertalot                   | USA  | -    | USA |
| <i>Navicymbula pusilla</i> (Grunow) Krammer                     | -    | AUS  | AUS |
| <i>Neidium affine</i> (Ehrenberg) Pfitzer                       | USA  | -    | -   |
| <i>Nitzschia angustatula</i> Lange-Bertalot                     | USA  | -    | USA |
| <i>Nitzschia desertorum</i> Hustedt                             | -    | -    | AUS |
| <i>Nitzschia dissipata</i> var. <i>media</i> (Hantzsch) Grunow  | -    | PORT | USA |
| <i>Nitzschia intermedia</i> Hantzsch                            | -    | -    | USA |
| <i>Nitzschia lacuum</i> Lange-Bertalot                          | -    | -    | AUS |
| <i>Nitzschia liebetruthii</i> Rabenhorst                        | -    | AUS  | AUS |
| <i>Nitzschia linearis</i> var. <i>subtilis</i> (Grunow) Hustedt | -    | -    | USA |
| <i>Nitzschia linearis</i> var. <i>tenuis</i> (W. Smith) Grunow  | -    | -    | USA |
| <i>Nitzschia lorenziana</i> var. <i>subtilis</i> Grunow         | USA  | -    | -   |
| <i>Nitzschia mediastalsis</i> Hohn et Hellermann                | USA  | -    | USA |
| <i>Nitzschia microcephala</i> Grunow                            |      | -    | AUS |
| <i>Nitzschia perminuta</i> (Grunow) M. Peragallo                | PORT | -    | -   |
| <i>Nitzschia perspicua</i> Cholnoky                             | -    | AUS  | AUS |
| <i>Nitzschia sigmoidea</i> (Nitzsch) W. Smith                   | -    | -    | USA |
| <i>Nitzschia similis</i> Hustedt                                | USA  | -    | USA |
| <i>Nitzschia sociabilis</i> Hustedt                             | -    | -    | USA |
| <i>Nupela impexiformis</i> (Lange-Bertalot) Lange-Bertalot      | USA  | -    | USA |
| <i>Nupela lapidosa</i> (Krasske) Lange-Bertalot                 | -    | PORT | -   |
| <i>Pinnularia divergens</i> W. Smith                            | USA  | -    | USA |
| <i>Pinnularia gibba</i> var. <i>linearis</i> Hustedt            |      | -    | USA |
| <i>Pinnularia intermedia</i> (Lagerstedt) Cleve                 | USA  | -    | USA |
| <i>Planothidium haynaldii</i> (Schaarschmidt) Lange-Bertalot    | USA  | -    | USA |
| <i>Platessa conspicua</i> (A. Mayer) Lange-Bertalot             | -    | PORT | USA |
| <i>Psammothidium helveticum</i> (Hustedt) Bukhtiyarova & Round  | -    | PORT | -   |
| <i>Pseudofallacia tenera</i> (Hustedt) Liu, Kociolek & Wang     | USA  | AUS  | -   |

|  |     |      |     |
|--|-----|------|-----|
| <i>Pseudostaurosira parasitica</i> (W. Smith) Morales                            | -   | -    | USA |
| <i>Pseudostaurosira parasitica</i> var. <i>subconstricta</i> (Grunow) E. Morales | -   | -    | USA |
| <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer                         |     | PORT | USA |
| <i>Reimeria sinuata</i> f. <i>antiqua</i> (Grunow) Kociolek et Stoermer          | USA | -    | -   |
| <i>Rhopalodia gibba</i> (Ehrenberg) O. Müller                                    | -   | AUS  | -   |
| <i>Rhopalodia musculus</i> (Kützing) O. Müller                                   | -   | AUS  | AUS |
| <i>Rossithidium lineare</i> (W. Smith) Round & Bukhtiyarova                      | USA | -    | USA |
| <i>Sellaphora mutata</i> (Krasske) Lange-Bertalot                                | USA | -    | USA |
| <i>Sellaphora rectangularis</i> (Gregory) Lange-Bertalot & Metzeltin             | USA | -    | USA |
| <i>Stauroneis kriegeri</i> R. M. Patrick   | -   | -    | USA |
| <i>Stauroneis thermicola</i> (J. B. Peterson) J. W. G. Lund                      | -   | -    | USA |
| <i>Stephanodiscus minutulus</i> (Kützing) Cleve & Möller                         | USA | -    | USA |
| <i>Surirella minuta</i> Brébisson ex Kützing                                     | -   | -    | USA |
| <i>Surirella ovalis</i> Brébisson  | -   | -    | AUS |
| <i>Surirella tenera</i> W. Gregory   | -   | -    | USA |
| <i>Tabellaria fenestrata</i> (Lyngbye) Kützing                                   | -   | -    | USA |
| <i>Tabularia fasciculata</i> (C. Agardh) D. M. Williams & Round                  | -   | -    | AUS |
| <i>Thalassiosira visurgis</i> Hustedt  | USA | -    | USA |

### 7.3.2 Genus-level

The datasets included 98 diatom genera. The assemblages differed significantly among regions at genus level (Pseudo-F = 32.25,  $p < 0.01$ ) and all three regions differed from each other ( $p < 0.01$ ). The AUS assemblages were again most segregated but less so than at species level (average dissimilarity of 63.5% and 62.9% from PORT and USA, respectively) (Figure 7.4). The mean number of genera *per* site in the USA did not differ significantly from the numbers in either PORT or AUS, but PORT had significantly more genera than AUS (Figure 7.5).

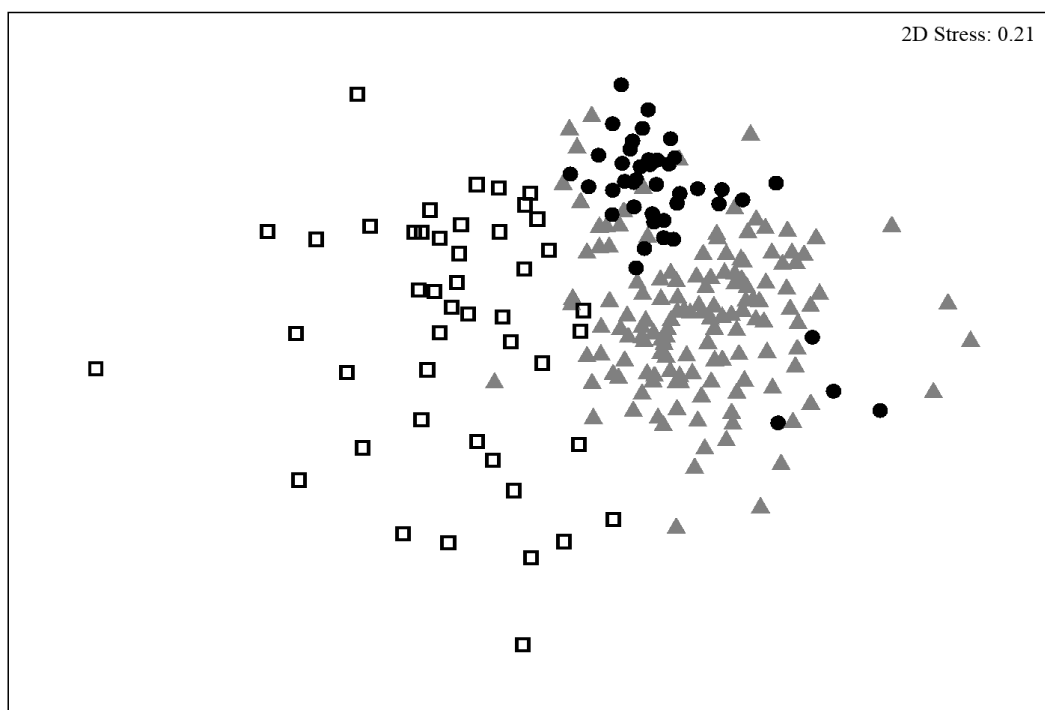


Figure 7.4 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at the genus level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares).

The five genera that contributed most to similarity within Portuguese streams were *Gomphonema*, *Achnantheidium*, *Navicula*, *Nitzschia* and *Fragilaria*, with percentage contributions ranging from 10.4 to 8.9 (Table 7.3). Five of the genera that contributed most to within-USA similarity were also found in PORT with a higher percentage contribution (*Gomphonema*, *Fragilaria*, *Achnantheidium*, *Nitzschia* and *Navicula*). The exceptions were the genera *Encyonema* and *Eolimna* which had similar frequencies in PORT and the USA (Figure 7.6). The five genera that contributed most to within-AUS similarity were *Nitzschia*, *Navicula*, *Rhoicosphenia*, *Planothidium* and *Amphora*, two of which (*Navicula* and *Nitzschia*) were also among those that contributed most to average similarity within other regions. Although

*Rhoicosphenia*, *Planothidium* and *Amphora* were not among the five most genera contributing most to similarity within the USA (or PORT), they were equally frequent in the USA and AUS (Figure 7.6). Frequencies of the selected genera were always significantly different between PORT and AUS, except for *Planothidium*.

The average dissimilarity was lowest between PORT and the USA (50.7 %), with the genera contributing most (up to 18%) to this dissimilarity all more frequent in the USA than in PORT (*e.g.*, *Rhoicosphenia*, *Amphora*, *Melosira*, *Aulacoseira*, *Frustulia* and *Surirella*). Although AUS had comparable average dissimilarity to each of the other two regions, only one genus was present in AUS and absent from PORT, and only one was present in PORT and absent from AUS, whereas eight were absent from AUS and present in the USA (*e.g.*, *Reimeria* and *Stauroneis*) (Table 7.4). The main difference between PORT and AUS (and the USA) was in genera frequencies. There was a higher frequency of genera such as *Rhoicosphenia*, *Amphora*, *Tabularia*, *Bacillaria* and *Tryblionella* in AUS while in PORT there was a higher frequency of *Encyonema*, *Fragilaria*, *Eolimna*, *Gomphonema* and *Ulnaria*.

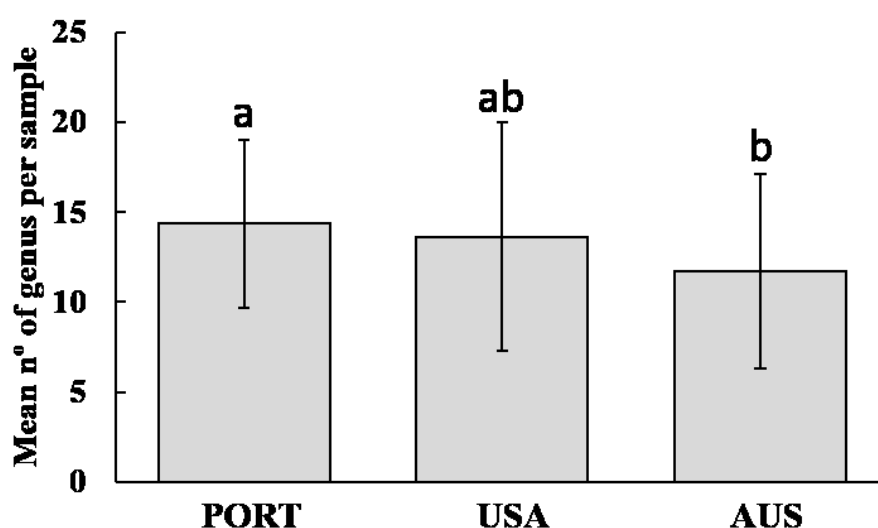


Figure 7.5 - Mean number ( $\pm$ SD) of genera *per sample* in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ ).

Table 7.3 - Diatom genera that contributed most to average similarity in AUS-Australia, PORT-Portugal, and USA-United States of America (up to 90% of cumulative similarity, Bray-Curtis similarity: SIMPER analysis). The values in bold indicate the genera that contributed most to average similarity within each region.

| Species               | Contribution (%) |            |     |
|-----------------------|------------------|------------|-----|
|                       | PORT             | USA        | AUS |
| <i>Achnanthes</i>     | -                | 0.8        | -   |
| <i>Achnantheidium</i> | <b>10.3</b>      | <b>5.1</b> | 4.8 |

Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

|                      |             |            |             |
|----------------------|-------------|------------|-------------|
| <i>Amphora</i>       | -           | 4.1        | <b>7.0</b>  |
| <i>Aulacoseira</i>   | -           | 2.1        | -           |
| <i>Bacillaria</i>    | -           | -          | 3.7         |
| <i>Caloneis</i>      | -           | 1.5        | -           |
| <i>Cocconeis</i>     | 5.8         | 4.1        | 5.7         |
| <i>Cyclotella</i>    | -           | 1.1        | -           |
| <i>Cymbella</i>      | -           | 1.2        | -           |
| <i>Diadlesmis</i>    | -           | 0.8        | -           |
| <i>Diatoma</i>       | 1.3         | 1.0        | -           |
| <i>Discotella</i>    | -           | 0.8        | -           |
| <i>Encyonema</i>     | 6.4         | <b>5.1</b> | -           |
| <i>Eolimna</i>       | 6.3         | <b>5.5</b> | -           |
| <i>Epithemia</i>     | -           | -          | 2.1         |
| <i>Eunotia</i>       | 2.7         | 4.6        | -           |
| <i>Fragilaria</i>    | <b>8.9</b>  | <b>5.2</b> | 1.6         |
| <i>Fragilaria</i>    | -           | 2.2        | -           |
| <i>Gomphonema</i>    | <b>10.4</b> | <b>5.9</b> | 3.7         |
| <i>Halamphora</i>    | -           | -          | 1.9         |
| <i>Hippodonta</i>    | -           | 1.8        | 2.2         |
| <i>Karayevia</i>     | 1.6         | -          | -           |
| <i>Luticola</i>      | -           | 1.0        | -           |
| <i>Melosira</i>      | -           | 3.2        | 3.2         |
| <i>Meridion</i>      | -           | 1.2        | -           |
| <i>Navicula</i>      | <b>10.3</b> | <b>5.1</b> | <b>11.7</b> |
| <i>Nitzschia</i>     | <b>10.1</b> | <b>5.1</b> | <b>12.3</b> |
| <i>Pinnularia</i>    | 1.6         | 1.9        | -           |
| <i>Planothidium</i>  | 7.8         | 4.8        | <b>8.3</b>  |
| <i>Reimeria</i>      | 2.4         | 1.9        | -           |
| <i>Rhoicosphenia</i> | -           | 3.4        | <b>11.5</b> |
| <i>Stauroneis</i>    | -           | 1.6        | -           |
| <i>Staurosira</i>    | -           | 1.6        | -           |
| <i>Staurosirella</i> | -           | 1.1        | -           |
| <i>Surirella</i>     | 1.5         | 2.6        | 1.6         |
| <i>Tabularia</i>     | -           | -          | 5.3         |
| <i>Tryblionella</i>  | -           | -          | 3.3         |
| <i>Ulnaria</i>       | 3.1         | 3.5        | 1.4         |

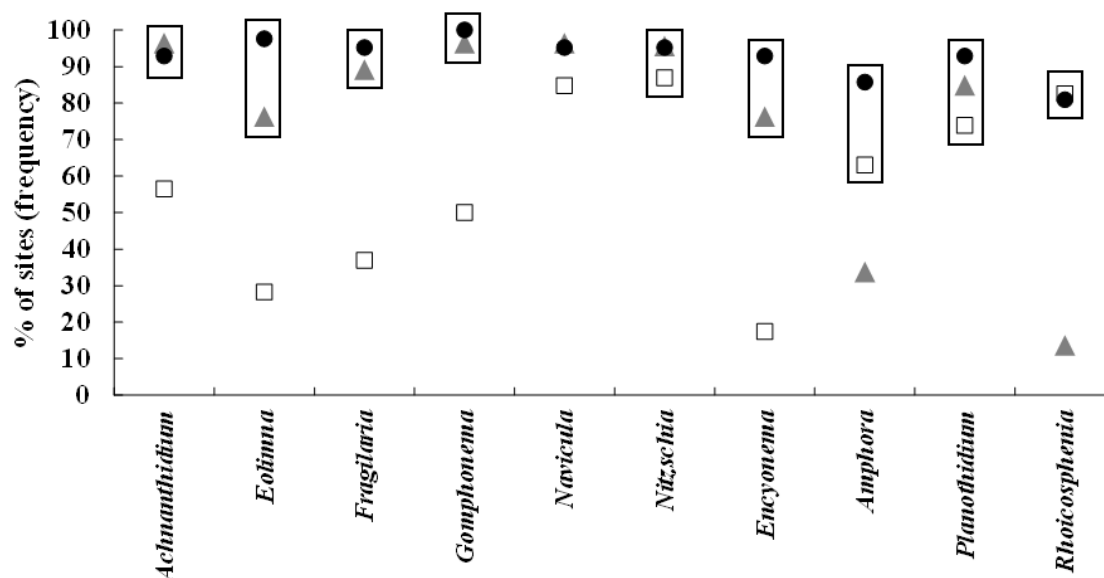


Figure 7.6 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the genera that contributed most to within-region average similarity were found (Table 7.3). For each genus, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). *Navicula* was significantly different between PORT and AUS but the differences were not tested for the other two comparisons (PORT-USA and USA-AUS).

Table 7.4 - Diatom genera that contributed most to average dissimilarity between AUS-Australia, PORT-Portugal and USA-United States of America (up to 80% of cumulative dissimilarity, Bray–Curtis similarity: SIMPER analysis). The region code opposite a genus indicates that the genus was found only in that region among the two regions being compared.

| Genus                | Comparison   |              |             |
|----------------------|--------------|--------------|-------------|
|                      | PORT vs. USA | PORT vs. AUS | USA vs. AUS |
| <i>Diadsmis</i>      | -            | -            | USA         |
| <i>Discotella</i>    | -            | -            | USA         |
| <i>Geissleria</i>    | -            | -            | USA         |
| <i>Luticola</i>      | -            | -            | USA         |
| <i>Meridion</i>      | -            | -            | USA         |
| <i>Neidium</i>       | -            | -            | USA         |
| <i>Psammothidium</i> | -            | PORT         | -           |
| <i>Reimeria</i>      | -            | PORT         | USA         |
| <i>Rhopalodia</i>    | -            | AUS          | -           |
| <i>Stauroneis</i>    | -            | -            | USA         |

### 7.3.3 Order-level

The datasets included 19 diatom orders. At order level the assemblages of the three regions again differed significantly overall (Pseudo-F = 28.99,  $p < 0.01$ ) and in all pairwise comparisons ( $p < 0.01$ ). Once more, the diatom assemblages from Australia were segregated from the others (Figure 7.7). However, in contrast to the other taxonomic levels, the PORT and AUS regions had similar numbers of orders *per sample* (Figure 7.8) whereas the USA had fewer orders *per sample* than PORT.

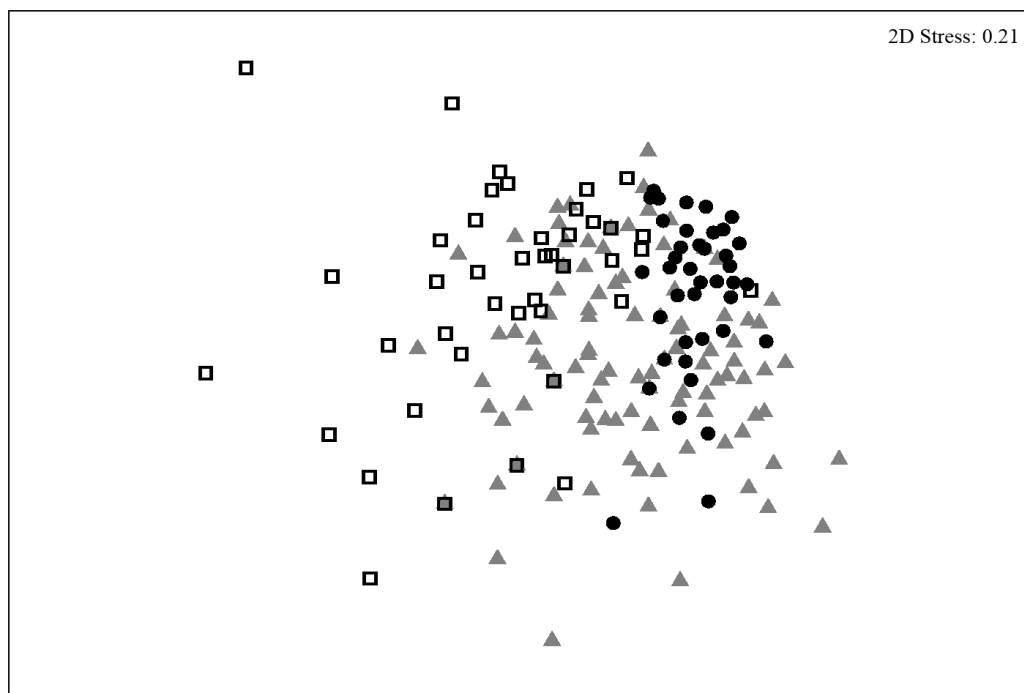


Figure 7.7 - Multidimensional scaling analysis (MDS) of the diatom assemblages, at the order level, of the three regions: Portugal (triangles), United States of America (circles) and Australia (squares).

The orders that contributed the most to assemblage similarity within PORT were Cocconeidales, Naviculales and Cymbellales with percentage contributions up to 16% (Table 7.5). The percentage contributions of the orders found in the USA were fairly uniform and the orders with greatest contributions were the same in PORT and USA (Table 7.5). Therefore, the dissimilarity between PORT and USA was just 27.7%. The orders that contributed the most to similarity within AUS were Cymbellales, Naviculales, Bacillariales, Cocconeidales, and Licmophorales (Table 7.5). The order Licmophorales was among those contributing most to within-region similarity only in AUS, but was always among those contributing to most the dissimilarity among regions. Despite the lack of statistical differences, the PORT assemblages had a lower frequency of Licmophorales (Figure 7.9). The order Fragilariales was the only order with significantly lower frequency in AUS (Figure 7.9).

No order contributed to average dissimilarity by being present in just one region; the inter-regional dissimilarities were due only to differences in frequencies. The orders contributing up to 10% to the dissimilarity between AUS and the other two regions were Thalassiophysales and Tabellariales (AUS-PORT) and Eunotiales and Tabellariales (AUS-USA).

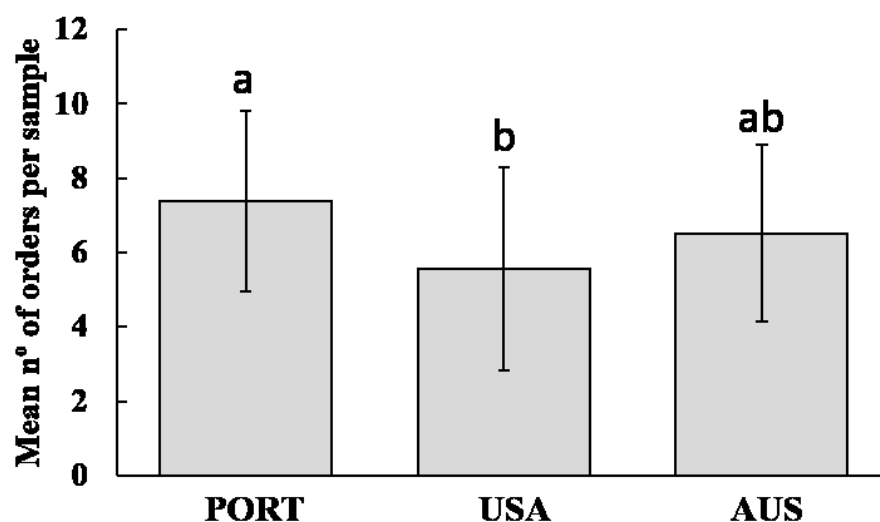


Figure 7.8 - Mean number ( $\pm$ SD) of orders *per sample* found in the three regions (PORT-Portugal, USA-United States of America, AUS-Australia). Regions with the same letter (a, b) do not differ significantly ( $p > 0.05$ ).

Table 7.5 - Diatom orders that contributed most to average similarity in AUS-Australia, PORT-Portugal, and USA-United States of America (up to 90% of cumulative similarity, Bray-Curtis similarity: SIMPER analysis). The values in bold indicate the orders that contributed most to average similarity within each region.

| Order             | Contribution (%) |             |             |
|-------------------|------------------|-------------|-------------|
|                   | POR              | USA         | AUS         |
| Bacillariales     | <b>15.1</b>      | <b>9.4</b>  | <b>15.5</b> |
| Cocconeidales     | <b>16.7</b>      | <b>10.6</b> | <b>15.5</b> |
| Cymbellales       | <b>16.1</b>      | <b>10.6</b> | <b>17.6</b> |
| Eunotiales        | 4.0              | 8.2         | -           |
| Fragilariales     | <b>13.6</b>      | <b>9.5</b>  | 5.7         |
| Licmophorales     | 5.0              | 6.6         | <b>9.5</b>  |
| Melosirales       | -                | 6.1         | -           |
| Naviculales       | <b>16.5</b>      | <b>10.0</b> | <b>16.5</b> |
| Surirellales      | -                | 5.3         | -           |
| Rhopalodiales     | -                | -           | 4.8         |
| Tabellariales     | 5.1              | 6.9         | -           |
| Thalassiophysales | -                | 7.5         | 7.7         |



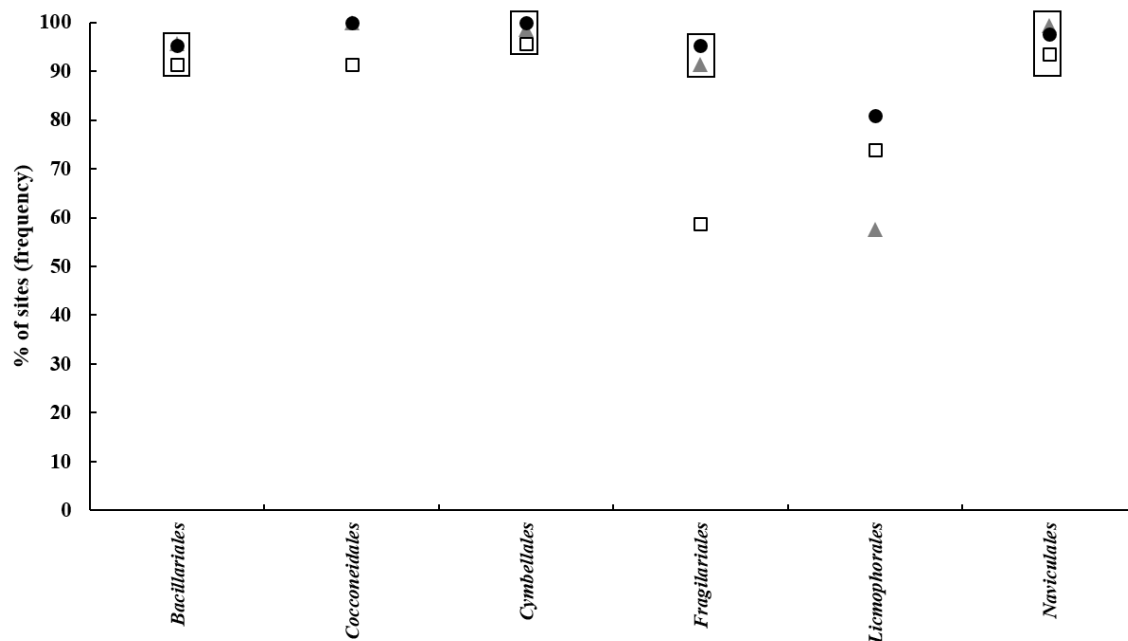


Figure 7.9 - Percentage of sites in Portugal (triangles), United States of America (circles) and Australia (squares) where the orders that contributed most to within-region average similarity were found (Table 7.5). For each order, the regions inside the rectangle are not significantly different ( $p > 0.05$ ) and are significantly different from the region outside the rectangle ( $p < 0.05$ ). *Cocconeidales* was significantly different between POR and AUS but the differences were not tested for the other two comparisons (PORT-USA and USA-AUS). *Licmophorales* was not significantly different between USA and AUS but the differences were not tested for the other two comparisons (PORT-USA and PORT-AUS).

## 7.4 Discussion

We expected similar assemblages from rivers with similar climate (Csb) in a large-scale comparison among regions on different continents. However, significant differences among regions were found even at the lowest level of taxonomic resolution (order). These results may suggest that, as stated by Gaston (2000), no single mechanism adequately explains a given large-scale pattern of biodiversity. Instead, constraints other than climate are likely to have contributed to the inter-regional differences in diatom assemblage composition found at all taxonomic levels, *e.g.*, geology, historical biogeographic processes and hydrology (Stevenson, 1997; Virtanen and Soininen, 2012). Those other constraints were probably large-scale because such marked assemblage differences should not result from constraints acting at the local scale (such as micro-habitats), which would be reflected only in variability

among sites within each region. Also, if micro-scale factors had a strong effect, the samples from the different regions would have been mixed or grouped by similarities in micro-scale conditions, which was not observed.

Diatoms are known to respond strongly to ion concentrations (Potapova and Charles, 2003) and pH (Almeida and Feio, 2012), and these variables are usually related to catchment geology. Therefore, if diatom assemblages of the different regions were structured by geology, regional differences in ionic and pH preferences of the assemblages would be expected. The environmental preferences of the species that contributed most to dissimilarity among regions indicate that the geologies of the Portuguese and USA regions are probably similar and different from the geology of the Australian region. The Portuguese and USA diatom assemblages were dominated by circumneutral (mainly occurring at pH values about 7) to alkaliphilous (mainly occurring at pH > 7) and oligohalobous (occurring at >100 to < 500 mg Cl<sup>-</sup>/L) species, whereas the Australia diatom assemblages included more alkaliphilous (pH >7) and salinity-tolerant species (>100 to 5000 mg Cl<sup>-</sup>/L; van Dam et al., 1994). For example, *Tabularia fasciculata*, a representative taxon of the Australian assemblages, is associated with lowland streams with a pH ranging from 6.8 to 8.6, with relatively high salinities (Blinn and Bailey, 2001) and high conductivities (optimum of 719 µS/cm; Potapova and Charles, 2003). On the other hand, *Rhoicosphenia abbreviata* and *Nitzschia inconspicua* were among the species that contributed most to the dissimilarity between Portugal and the USA, but were equally frequent in USA and Australian assemblages. These species prefer waters with high mineral content, and have conductivity optima around 400 µS/cm (Potapova and Charles, 2003; Potapova et al., 2005; Rimet 2009; Feio et al., 2012). The region of western Victoria (close to the Australian study region) is known for having several lakes with high salinity levels due to saline groundwater and runoff (Gell, 1997).

Despite this evidence of the likely importance of geology, the observed differences in richness and assemblage composition among regions could also be related to biogeographical processes. Diatoms, like other microorganisms, have been considered ubiquitous with rare species not recorded globally simply because sampling effort has been insufficient (Finlay et al., 2002). In contrast to larger organisms such as macroinvertebrates (Heino and Soininen, 2007), diatoms may be easily distributed via wind, currents and animal vectors (*e.g.*, Kristiansen, 1996), resulting in wider distributions than those of metazoans (*e.g.*, Heino and Soininen, 2007; Finlay et al., 2002). For example, in a recent study of assemblage variation of diatoms, bryophytes and macroinvertebrates across the same set of sites, variation was more associated with environmental factors and less associated with geographical distance for diatoms than for the other assemblages (Astorga et al., 2012). However, in recent years, some large-scale studies (Potapova and Charles, 2002; Soininen, 2004; Soininen et al., 2004) have demonstrated that purely spatial factors may also influence diatom distribution patterns

(Virtanen and Soininen, 2012), even at the genus level (Vyverman et al., 2007). Also, the increasing incidence of apparent regional endemism indicates the prevalence of restricted distribution patterns among diatom species (Vyverman et al., 2007; Vanormelingen et al., 2008; Verleyen et al., 2009; Vyverman et al., 2010). Moreover, human-mediated introductions provide a strong indication that geographic dispersal is limited (Vanormelingen et al. 2008). Recognised introduced diatom species include *Achnanthes subhudsonis* in Europe (Coste and Ector, 2000), *Asterionella formosa* in New Zealand (Harper, 2004 in Vanormelingen et al., 2008) and *Didymosphenia geminata* in New Zealand (Blanco and Ector, 2009). According to Coste and Ector (2000), *A. subhudsonis* was initially described in East Africa in 1921 and was first reported in Europe in 1991 (Galicia, NW Spain). Despite being reported in the Southern Hemisphere in 2000 (Coste and Ector, 2000), it is still apparently absent from Australia.

We expected that if dispersal limitation and geographic distance were shaping diatom assemblages, the Australian ones would be the most distinct, because Australia is geographically distant from the other two regions, and this was indeed the case. Moreover, some species were found only in one region, a result suggesting biogeographic patterns. However, the great majority of the species that were absent from a region in this study were found in the same country by other authors (*e.g.*, Blinn and Bailey, 2001; Chessman et al., 2007; Potapova and Charles, 2007). Therefore, these species can be considered ubiquitous, i.e. organisms without biogeography (Soininen, 2007).

According to Bouchard et al. (2004), biogeographical patterns are more distinct at the genus level. In our study, differences among regions were evident even at the order level, with the Australian assemblages being consistently most distinct. However, as the ordinations show, distinctions among regions declined from species to genus to order. This result is expected because a higher taxon will usually have a broader distribution than each of its constituent lower taxa. Our results also show that even small differences among assemblages can be detected at the order level. For example, the order Licmophorales contributed strongly to dissimilarity among regions and similarity within the Australian assemblages despite comprising only the species *Tabularia fasciculata*.

Bouchard et al. (2004) have warned that taxonomic richness should be compared among studies with caution, as richness depends greatly on search effort and sample size. In our study, the databases from each of the three regions comprised a different number of samples *per site*, which could have influenced taxonomic richness. However, Portugal and Australia had a similar number of samples *per site*, yet Portuguese samples had more species (and genera). This result suggests that the lower richness found in Australia was not related to the number of samples. Species richness has also been related to latitude, with tropical zones

supporting more species than temperate ones (Hawkins et al., 2004). However, this issue does not apply to our study because the regions we included are at similar latitudes. Regional inconsistencies in identification could also have contributed to differences at the species level, but the differences remained at genus and order levels where identification problems are unlikely. Finally, differences in richness may have been related to the number of valves counted *per* sample in each region. However, a study by Bate and Newall (2002) comparing valve counts of 100, 200, 300, 400 and 500 found that 200 were sufficient to characterise the diatom assemblage at a site with an efficiency of 80%.

## **7.5 Conclusion**

In summary, our study has demonstrated that streams from different regions with similar climate (Csb) have diatom assemblages with differing taxonomic composition, and that climate cannot be used to map diatom distribution, in contrast to distributions of other organisms (Lindsay and Bayoh, 2004). The clear patterns obtained at all taxonomic levels suggest that regional differences are related to large-scale constraints. In addition, our results confirm that in large-scale studies it is possible to detect statistical differences among diatom assemblages at a high taxonomical rank such as genus or even order.

### **Abbreviations**

|      |                                     |
|------|-------------------------------------|
| AUS  | Australia                           |
| MDS  | non-metric Multidimensional Scaling |
| PORT | Portugal                            |
| USA  | United States of America            |

### **Acknowledgment**

This study was made possible by financial support from the FOUNDATION FOR SCIENCE AND TECHNOLOGY (Portugal) through the strategic project UID/MAR/04292/2013 granted to MARE, UID/GEO/04035/2013 granted to GeoBioTec and the Ph.D. scholarship SFRH/BD/68973/2010 of the first author. We thank the Portuguese Water Institute (INAG I.P., now Agência Portuguesa do Ambiente) for making data available, and IMAR-CMA, Department of Life Sciences, University of Coimbra and GeoBioTec Research Centre and Biology Department of Aveiro, University of Aveiro for logistical support. The Australian samples were collected through the Australian Water Quality Centre in Adelaide, with diatom counts undertaken by Peter Gell and colleagues in Geography at the University of Adelaide.

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Effect of global changes and spatial scale on diatom communities of temperate rivers. Dealing with implications in bioassessment

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## Chapter 8 - Final remarks

'The river is like an organism; it is internally self-adjusting' (Leopold, 1977). It is also resilient and can absorb changes imposed upon it, but not without limit (Leopold, 1977). The limit beyond which a river cannot adjust has been in many situations overcome by man to a point that, even to the casual observer, it is rather obvious that undisturbed conditions can no longer exist (Birk et al., 2012).

Streams and rivers exhibit different environmental characteristics from one region to another as a consequence of differences in climate, geology, hydromorphology, channel substrate, and water chemistry. Even within the small geographic area covered in this work (the Portuguese littoral region), it was possible to differentiate three sub-groups of streams based on differences in mean annual precipitation, mean annual runoff, slope, altitude, size of the drainage area, lithology, and stream substrate (chapter 2). Major anthropogenic alterations were, as expected, related to the riparian vegetation, morphological condition, and sediment load, as the main pressures in the Portuguese littoral region are agriculture and deforestation (chapter 2). Due to this strong anthropogenic influence it was only possible to find sites in Least Disturbed Condition (LDC), which corresponded to only 14.7% of the total samples collected. Nevertheless, the nutrient concentrations obtained for littoral LDC were still high and sites in the larger rivers included in this work were even excluded, due to hydromorphological modifications, specifically in the hydrological regime, connectivity, and/or morphological alterations. From the selected least disturbed sites, two distinct diatom communities (two sub-groups) were found (chapter 2), being differentiated by mean annual runoff, slope, mean annual precipitation, substrate (stone vs. sand), and lithology of the streams.

The two sub-groups of diatom communities that were under similar levels of anthropogenic disturbance yielded considerable differences in the median values of the Portuguese official diatom index (Indice de Polluosensibilité Spécifique - IPS). The sub-group of streams with stony substrate showed much higher IPS median value than the group corresponding to sandy substrate. Therefore, the use of a single IPS benchmark value for both sub-groups to calculate the Ecological Quality Ratio (EQR) values would lead to erroneous classifications. The division by a higher median than the official one, which was based in a mixture of different littoral sites, results in lower EQRs, and therefore potential erroneous

attribution of lower quality to sites with good quality. These Type II errors (false negatives) can have important consequences, as they imply a higher investment than the one needed for streams' restoration (Johnson et al., 2006).

In spite of all efforts to increase the number of LDC sites in the Portuguese littoral region, through higher density of sampling sites and the refinement of the benchmark criteria, the strong anthropogenic pressures prevent an adequate definition of reference conditions for this area based on reference sites. Thus, an alternative procedure to the definition of reference conditions was investigated in chapter 3. To address problems of this type, Chessman and Royal (2004) proposed an alternative approach (filters approach) through which the establishment of biological reference conditions does not rely on reference sites. In fact, this approach, in combination with the modelling of the undisturbed condition of abiotic reference values, enabled us to propose biological reference conditions for the studied region, for both diatoms and macroinvertebrates, with lower dependence on reference sites. Yet, some of the abiotic variables that we considered relevant were not possible to model based on the existing data through boosted trees. The reference maximum nitrate concentration for reference sites was  $3.64 \text{ mg}\cdot\text{L}^{-1}$ , which is within the range of threshold values for various river types ( $2\text{-}6 \text{ mg}\cdot\text{L}^{-1}$ ) below which Pardo et al. (2012) considered a site to be 'probably reference'. Feio et al. (2014) set a lower threshold for Mediterranean areas ( $\leq 1.15 \text{ mg}\cdot\text{L}^{-1}$ ), but it was based on much a wider geographic region than the lowland area considered in this study.

The use of the abiotic reference values (from models and from the literature) in combination with the filter approach allowed the prediction of diatom and macroinvertebrate communities statistically different from those that were observed in the streams during this study (chapter 3). We also verified that the communities observed in the streams were less homogeneous than those predicted (through the filter approach), as would be expected with sites having variable levels of change from reference conditions. Macroinvertebrate communities were also included in parallel in the analysis as they are the most commonly used biotic indicator of streams ecological quality. For both diatoms and macroinvertebrates, the median number of taxa predicted was more than the double of the median number of taxa observed, probably reflecting the long-term elimination of taxa by anthropogenic pressures (chapter 3). In some cases, taxa were observed at a site where they were not predicted to occur which could indicate anthropogenic influences turning a stream suitable for taxa that would not naturally occur there. However, it could also have resulted from limitations in estimating the natural environmental attributes of the sites and the environmental tolerance ranges of the taxa (Chessman and Royal, 2004).

We argue that this approach (combination of modelling and filter approach) is suitable to deal with the implications of global changes on diatoms, and even on macroinvertebrate communities, because, among the filters that were most often responsible for exclusions of diatom and macroinvertebrate taxa, we found mean annual temperature and runoff. These variables were considered to influence both diatom and macroinvertebrate distributions in other studies a (*e.g.*, Potapova and Charles, 2002; Rimet et al., 2004; Tison et al., 2005; Feio et al., 2007; Rimet, 2009; Hawkins et al., 2010; Almeida and Feio, 2012). As expected, the pressure variables treated as filters excluded few taxa when set to normal values in the absence of anthropogenic influence (reference values). That is, when we ‘eliminate’ the effect of global changes from the environment, less taxa are excluded from streams that have natural abiotic characteristics to support those taxa. In spite of their importance, some of our filters are not direct determinants of diatom and macroinvertebrate community composition but rather surrogate predictors (Clarke et al., 2003). For example, mean annual air temperature, as obtained from cartographic sources, was used here as a surrogate of water temperature and was responsible for the total exclusion of *Frustulia erifuga* and frequent exclusion of *Melosira varians* from the diatom reference lowland communities. Direct prediction from the annual range of water temperature would have been more desirable, but water temperature is also highly dependent on the measurement spot and would require temperature loggers to be used continuously (Clarke et al., 2003).

To test the consequences of the predictions through the filter approach to the ecological quality assessment, we developed a new multimetric diatom index (the MDI), following an approach similar to the one used for macroinvertebrates. This new index included the IPS as a metric and also the number of sensitive taxa and total taxa richness (chapter 3). We verified that the MDI produced strong correlations with the pressure variables thus providing a more comprehensive assessment of biological quality than the IPS (significant correlations with two PCA axes vs. only one axis). Such better performance was probably due to the inclusion of the number of sensitive species, as indicated by the high correlation of this metric with the PCA axes. In result of the application of the new reference values, the LDC sites decreased their EQR values, which was expectable. The comparison of the diatom and macroinvertebrate assessment results gave more confidence to our results (chapter 3): the results were similar for both communities and the new MDI resulted in similar water quality assessment than the already developed and highly used Portuguese multimetric index for benthic macroinvertebrates (IPTIs).

Considering the differences found in chapter 2 regarding the IPS median values for sandy and stony streams, it was found important to test the real importance of the colonizing substrate (small-scale) on the diatom communities and its implication in bioassessment (chapter 6) as diatom communities are influenced by different spatial scales (from single

sand grains to across continents) (Biggs, 1996). The results of the mesocosm experiment described in chapter 6 showed that the substrate affects diatom communities by changing its composition and trait proportions. This is in agreement with other studies which indicate differences in the composition of diatom communities among different substrates (Round, 1991; Cattaneo et al., 1997; Potapova and Charles, 2005). Yet, contrary to these findings, other *in situ* studies have not found differences between substrates (Rothfritz et al., 1997; Bere and Tundisi, 2011; Winter and Duthie, 2000). In fact, the differences that we found in the epipsammic and epilithic diatom communities in the streams (chapter 2) were more evident than the ones found in the mesocosm experiment (chapter 6). Furthermore, considering the water quality assessment, the IPS values obtained at the same sampling moment in the channels did not reflect the multivariate differences between the epipsammic and epilithic diatom communities (chapter 6) neither those verified in the streams (chapter 2). However, as in other studies, the results of the mesocosm experiments suggest that hard and soft (sand) substrates can be exchangeable in assessment methods that are based on autoecology (Soininen and Könönen, 2004; Potapova and Charles, 2005; Mendes et al., 2012). In chapter 2 it was stated that due to the small number of LDC sites, particularly for epipsammic diatom communities, we must be cautious in our interpretations. With the results of chapter 6 we verified that in fact under the same physical and chemical conditions the IPS differences between substrates disappeared. Therefore, the differences that were found in the streams may have been due to other differences in the physical or chemical properties of the water that were not detected in the water analyses but were reflected in the diatom communities.

While chapters 2 and 3 focused on the effect of anthropogenic disturbances in aquatic communities, particularly diatoms, chapter 4 and 5 dealt with the effect that can be expected from future extreme events due to climate changes. We verified that traits, community composition and bioassessment classifications of the diatoms and macroinvertebrates were all influenced by the complete drying of stream channels caused by an unusual drought event affecting the Portuguese littoral region during autumn and winter 2011/2012. However, the macroinvertebrates and diatoms differed in their recovery response. The drought led to changes in the trait proportions of the diatom and macroinvertebrate communities for at least a 6-month period (chapter 4). Diatom trait proportions changed towards proportions similar to those found in Mediterranean communities. However, shortly after the return of water to the channel, diatom communities' traits were similar to those observed before the drought (chapter 4). The post-drought macroinvertebrate communities were however very different from those found before the drought but were also different from the Mediterranean ones (chapter 4). These changes in macroinvertebrate communities should

lead to an alteration of the ecosystem functioning for a relatively long period. For example, primary production may have been higher than normal due to the absence of some grazers and scrapers that were not able to resist to drought, as the primary producers recovered faster. Therefore, the lack of top-down control may have interfered in transference of energy and matter across the food chains.

The influence of the drought in the water quality was more evident with the macroinvertebrate assessment as the IPS index for diatoms was similar over the rewetting progression. The stream water quality, given by the macroinvertebrate IPTI index, decreased with the drought event, and although it did not attain the pre-drought values within one year, the streams gradually recovered during the winter season. Both communities provide reliable information on water quality (*e.g.*, Alba-Tercedor et al., 2002; Rimet et al., 2005). Diatoms are more sensitive to changes in water chemistry, whereas macroinvertebrates are more susceptible to channel morphological changes and habitat conditions (Passy et al., 2004). Under drought effects, their simultaneous use for bioassessment should be considered, as their responses are different and appear to be complementary (Feio et al., 2007), particularly if one considers responses at different time scales.

Both chapters 4 and 5 suggest that the predicted drought events due to climatic changes influence diatom and macroinvertebrate communities. With increasing anthropogenic pressures and climatic alterations, we expect biological communities of temperate streams to change through time towards pollution-tolerant taxa with higher resilience to extreme drought events. Also, the river habitat characteristics appeared to play an important role in the recovery of macroinvertebrate and diatom communities. This study showed that the initial condition of streams was important in the recovery process, as the communities of streams showing better environmental conditions (*e.g.*, riparian corridors, Habitat Quality Assessment) recovered faster and easier from extreme disturbances, which is in agreement with other studies (*e.g.*, Sponseller et al., 2001; Elozegi et al., 2010; Thomson et al., 2012).

In chapter 6 we showed that there are variations in diatom communities in a small spatial scale (substrate). Yet, large spatial scales have not been sufficiently explored for diatom communities. Thus, in chapter 7 we explored the plausible inter-continental (Europe, America and Australia) influence on the diatom communities. Based on the collected data we also found that diatom communities are different between regions with the same (temperate) climate, suggesting that diatom assemblages are also influenced at the large-scale (chapter 7). Initially we expected similar communities from rivers with similar climate (Csb) at a large-scale level of comparison (inter-continents). However, significant differences between regions were found even at the highest level of taxonomical resolution (order). So, other than climate constraints should have contributed to the differences found at all taxonomical levels in benthic algal communities structure (*e.g.*, geology, historical biogeographic processes and



hydrology; Stevenson, 1997; Virtanen and Soininen, 2012). The differences could be due to other large-scale factors because such marked dissimilarities should not result from direct constraints acting at the local and regional scale (as micro-habitats), which are only reflected in the variability between sites within each region. Also, if micro-scale factors had a strong effect, the samples from the different continents would be mixed or grouped by similarities in micro-scale conditions, but this was not revealed by the collected data.

#### Future work

How to deal with the implications of global changes and spatial scale in bioassessment using diatom communities of temperate rivers has not yet been completely understood. Although the effects of these factors on diatom communities and its implication in bioassessment were not fully addressed in the present work, our results can be combined in different ways to achieve a much better insight over it. The experimental facilities developed during this work can be used and/or further developed in the future to investigate a number of issues that has emerged. A selection of proposals for future work is:

Issues based on the experimental facilities (artificial channels):

- investigate the influence of temperature increase, based in the global warming predictions, on the water quality assessment based on diatom communities;
- investigate the combined effects of the temperature and other important factors whose effects on the diatom communities can be potentiated by the temperature increase (*e.g.*, temperature/light conditions, temperature/consumers) on the diatom communities;

Issues based on data obtained for this thesis:

- investigate if the continental differences that were found at all taxonomical level are reflected in the water quality assessment. If these differences are not reflect in the water quality assessment streams and rivers from different continents but under reference conditions can be used as reference sites for other continents were references sites do no longer exist.

#### Abbreviations

|      |   |
|------|---|
| EQR  | Ecological Quality Ratios                     |
| IPS  | <i>Indice de Polluosensibilité Spécifique</i> |
| IPtI | <i>Índice Português de Invertebrados</i>      |
| LDC  | Least Disturbed Condition                     |

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|-----|-------------------------------|
| MDI | Multimetric Diatom Index      |
| PCA | Principal Components Analysis |

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**Appendix I – Description of the lithology categories found  
in the Portuguese lowland sites – Chapter 3**



Description of the lithology categories found in the Portuguese lowland sites (n=218 samples from 62 sites).

| Lithology categories | Description  |
|----------------------|--|
| 1                    | Sands, sand dunes, gravels, rolled stones, little consolidated sandstones, clays                                       |
| 2                    | Glacial deposits   |
| 3                    | Limestones, dolomitic limestones, marly limestones, marls  |
| 4                    | Plateau gravel, Beira Baixa arkoses, sandstones, limestones  |
| 5                    | Conglomerates, sandstones, limestones, dolomitic limestones, marly limestones, marls, carbonaceous schist's, schist's  |
| 6                    | Sandstones, more or less marly limestones, sands, gravels, claysconglomerates, limestones, dolomitic limestones, marls |
| 7                    | Red stoneware, conglomerates, marls, limestones generally dolomitic  |
| 8                    | Metavolcanics  |
| 9                    | Carbonate rocks  |
| 10                   | Quartzites   |
| 11                   | Schists and related rocks  |
| 12                   | Basaltic mantles and tuffs, basalts  |
| 13                   | "Teschenitos" (alkaline rocks)   |
| 14                   | Dolerite   |
| 15                   | Andesite   |
| 16                   | Aplite-pegmatite   |
| 17                   | Granites and related rocks   |





**Appendix II – Results of the filters approach, diatoms –  
Chapter 3**



Frequencies (%) with which the filters approach predicted the occurrence of diatoms at 55 sites in spring, compared with frequencies of collection. PC-taxon was predicted and collected, Pc-taxon predicted but not collected, pC-taxon not predicted but collected, pc-taxon neither predicted nor collected.

| Taxon  | PC | Pc | pC | pc |
|--|----|----|----|----|
| <i>Achnantheidium minutissimum</i> (Kützing) Czarnecki                             | 85 | 11 | 2  | 2  |
| <i>Achnantheidium pyrenaicum</i> (Hustedt) H. Kobayasi                             | 7  | 44 | 2  | 47 |
| <i>Achnantheidium rivulare</i> Potapova & Ponader                                  | 11 | 7  | 2  | 80 |
| <i>Achnantheidium subatomoides</i> (Hustedt) O. Monnier,<br>Lange-Bertalot & Ector | 9  | 38 | 0  | 53 |
| <i>Amphora inariensis</i> Krammer  | 11 | 45 | 0  | 44 |
| <i>Amphora lybica</i> Ehrenberg  | 20 | 42 | 0  | 38 |
| <i>Amphora pediculus</i> (Kützing) Grunow ex A. Schmidt                            | 56 | 33 | 0  | 11 |
| <i>Aulacoseira granulata</i> (Ehrenberg) Simonsen                                  | 5  | 47 | 2  | 45 |
| <i>Bacillaria paxillifera</i> (O. F. Müller) T. Marsson                            | 9  | 27 | 4  | 60 |
| <i>Caloneis bacillum</i> (Grunow) Cleve  | 20 | 29 | 4  | 47 |
| <i>Cocconeis euglypta</i> Ehrenberg  | 65 | 25 | 2  | 7  |
| <i>Cocconeis pediculus</i> Ehrenberg   | 31 | 45 | 0  | 24 |
| <i>Cocconeis placentula</i> var. <i>lineata</i> (Ehrenberg)<br>Van Heurck          | 55 | 31 | 0  | 15 |
| <i>Cocconeis placentula</i> Ehrenberg  | 25 | 27 | 15 | 33 |
| <i>Cocconeis pseudolineata</i> (Geitler) Lange-Bertalot                            | 11 | 31 | 4  | 55 |
| <i>Craticula halophila</i> (Grunow) D. G. Mann                                     | 9  | 35 | 2  | 55 |
| <i>Ctenophora pulchella</i> (Ralfs ex Kützing) D. M. Williams & Round              | 4  | 16 | 0  | 80 |
| <i>Cyclotella meneghiniana</i> Kützing   | 27 | 65 | 0  | 7  |
| <i>Cymbella affinis</i> Kützing  | 5  | 18 | 0  | 76 |
| <i>Cymbella tumida</i> (Brébisson) Van Heurck                                      | 4  | 25 | 0  | 71 |
| <i>Diadsmis contenta</i> (Grunow) D. G. Mann                                       | 11 | 27 | 5  | 56 |
| <i>Diatoma mesodon</i> (Ehrenberg) Kützing   | 2  | 9  | 0  | 89 |
| <i>Diatoma vulgare</i> Bory  | 9  | 47 | 4  | 40 |
| <i>Diploneis oblonga</i> (Naegeli) Cleve-Euler                                     | 11 | 29 | 0  | 60 |
| <i>Encyonema minutum</i> (Hilse) D. G. Mann  | 36 | 35 | 4  | 25 |
| <i>Encyonema neogracile</i> Krammer  | 2  | 7  | 0  | 91 |
| <i>Encyonema silesiacum</i> (Bleisch) D. G. Mann                                   | 0  | 47 | 2  | 51 |
| <i>Eolimna minima</i> (Grunow) Lange-Bertalot                                      | 85 | 9  | 2  | 4  |
| <i>Eolimna subminuscula</i> (Manguin) Gerd Moser Lange-Bertalot<br>& Metzeltin     | 40 | 45 | 0  | 15 |
| <i>Eunotia bilunaris</i> (Ehrenberg) Schaarschmidt                                 | 4  | 22 | 2  | 73 |
| <i>Eunotia minor</i> (Kützing) Grunow  | 16 | 47 | 2  | 35 |
| <i>Fistulifera saprophila</i> (Lange-Bertalot & Bonik) Lange-Bertalot              | 15 | 35 | 0  | 51 |
| <i>Fragilaria bidens</i> Heiberg   | 2  | 18 | 0  | 80 |
| <i>Fragilaria capitellata</i> (Grunow) J. B. Peterson                              | 4  | 20 | 0  | 76 |
| <i>Fragilaria capucina</i> Desmazières   | 15 | 45 | 5  | 35 |

## Appendix II

|  |    |    |    |     |
|--|----|----|----|-----|
| <i>Fragilaria crotonensis</i> Kitton   | 7  | 18 | 2  | 73  |
| <i>Fragilaria gracilis</i> Østrup  | 25 | 47 | 4  | 24  |
| <i>Fragilaria rumpens</i> (Kützing) G. W. F. Carlson                         | 9  | 16 | 5  | 69  |
| <i>Fragilaria vaucheriae</i> (Kützing) J. B. Peterson                        | 31 | 56 | 4  | 9   |
| <i>Frustulia erifuga</i> Lange-Bertalot & Krammer                            | 0  | 0  | 0  | 100 |
| <i>Frustulia vulgaris</i> (Thwaites) De Toni                                 | 9  | 55 | 2  | 35  |
| <i>Geissleria decussis</i> (Østrup) Lange-Bertalot & Metzeltin               | 7  | 42 | 4  | 47  |
| <i>Gomphonema acuminatum</i> Ehrenberg                                       | 5  | 38 | 0  | 56  |
| <i>Gomphonema exilissimum</i> (Grunow) Lange-Bertalot & E. Reichardt         | 9  | 38 | 0  | 53  |
| <i>Gomphonema gracile</i> Ehrenberg  | 5  | 51 | 0  | 44  |
| <i>Gomphonema micropus</i> Kützing   | 2  | 36 | 0  | 62  |
| <i>Gomphonema minutum</i> (C. Agardh) C. Agardh                              | 27 | 51 | 0  | 22  |
| <i>Gomphonema olivaceum</i> (Hornemann) Brébisson                            | 16 | 36 | 0  | 47  |
| <i>Gomphonema parvulum</i> (Kützing) Kützing                                 | 71 | 22 | 2  | 5   |
| <i>Gomphonema pumilum</i> (Grunow) E. Reichardt & Lange-Bertalot             | 45 | 45 | 0  | 9   |
| <i>Gomphonema rhombicum</i> Fricke   | 22 | 45 | 0  | 33  |
| <i>Gomphonema truncatum</i> Ehrenberg  | 2  | 18 | 2  | 78  |
| <i>Halamphora veneta</i> (Kützing) Levkov                                    | 11 | 13 | 4  | 73  |
| <i>Hannaea arcus</i> (Ehrenberg) R. M. Patrick                               | 4  | 4  | 4  | 89  |
| <i>Hippodonta capitata</i> (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski | 29 | 31 | 0  | 40  |
| <i>Karayevia oblongella</i> (Østrup) Aboal                                   | 36 | 42 | 2  | 20  |
| <i>Lemnicola hungarica</i> (Grunow) F. E. Round & P. W. Basson               | 5  | 27 | 5  | 62  |
| <i>Luticola goeppertiana</i> (Bleisch in Rabenhorst) D. G. Mann              | 13 | 40 | 4  | 44  |
| <i>Mayamaea atomus</i> var. <i>permitis</i> (Hustedt) Lange-Bertalot         | 60 | 29 | 0  | 11  |
| <i>Melosira varians</i> C. Agardh  | 2  | 2  | 69 | 27  |
| <i>Meridion circulare</i> var. <i>constrictum</i> (Ralfs) Van Heurck         | 2  | 9  | 2  | 87  |
| <i>Navicula angusta</i> Grunow   | 0  | 4  | 0  | 96  |
| <i>Navicula antonii</i> Lange-Bertalot                                       | 25 | 31 | 0  | 44  |
| <i>Navicula capitatoradiata</i> H. Germain                                   | 11 | 62 | 0  | 27  |
| <i>Navicula cryptocephala</i> Kützing  | 53 | 40 | 2  | 5   |
| <i>Navicula cryptotenella</i> Lange-Bertalot                                 | 55 | 33 | 0  | 13  |
| <i>Navicula cryptotenelloides</i> Lange-Bertalot                             | 11 | 58 | 0  | 31  |
| <i>Navicula gregaria</i> Donkin  | 84 | 11 | 0  | 5   |
| <i>Navicula heimansioides</i> Lange-Bertalot                                 | 9  | 49 | 2  | 40  |
| <i>Navicula lanceolata</i> Ehrenberg   | 31 | 24 | 7  | 38  |
| <i>Navicula radiosa</i> Kützing  | 5  | 27 | 2  | 65  |
| <i>Navicula reichardtiana</i> Lange-Bertalot                                 | 24 | 22 | 4  | 51  |
| <i>Navicula rhynchocephala</i> Kützing                                       | 15 | 49 | 5  | 31  |
| <i>Navicula tripunctata</i> (O. F. Müller) Bory                              | 40 | 31 | 0  | 29  |
| <i>Navicula veneta</i> Kützing   | 40 | 51 | 0  | 9   |
| <i>Nitzschia amphibia</i> Grunow   | 33 | 49 | 0  | 18  |
| <i>Nitzschia capitellata</i> Hustedt   | 18 | 47 | 4  | 31  |
| <i>Nitzschia dissipata</i> (Kützing) Grunow var. <i>dissipata</i>            | 56 | 31 | 4  | 9   |

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|   |    |    |   |    |
|---|----|----|---|----|
| <i>Nitzschia dissipata</i> var. <i>media</i> (Hantzsch) Grunow        | 15 | 49 | 4 | 33 |
| <i>Nitzschia fonticola</i> (Grunow) Grunow                            | 33 | 42 | 2 | 24 |
| <i>Nitzschia frustulum</i> (Kützing) Grunow                           | 18 | 60 | 0 | 22 |
| <i>Nitzschia inconspicua</i> Grunow                                   | 58 | 35 | 0 | 7  |
| <i>Nitzschia linearis</i> (Agardh) W. M. Smith var. <i>linearis</i>   | 27 | 40 | 0 | 33 |
| <i>Nitzschia linearis</i> var. <i>subtilis</i> (Grunow) Hustedt       | 11 | 42 | 2 | 45 |
| <i>Nitzschia microcephala</i> Grunow                                  | 0  | 33 | 4 | 64 |
| <i>Nitzschia palea</i> (Kützing) W. Smith                             | 69 | 27 | 2 | 2  |
| <i>Nitzschia palea</i> var. <i>debilis</i> (Kützing) Grunow           | 15 | 29 | 5 | 51 |
| <i>Nitzschia paleacea</i> (Grunow) Grunow                             | 22 | 58 | 4 | 16 |
| <i>Nitzschia perminuta</i> (Grunow) M. Peragallo                      | 15 | 42 | 2 | 42 |
| <i>Nitzschia recta</i> Hantzsch in Rabenhorst                         | 13 | 56 | 0 | 31 |
| <i>Nitzschia supralitorea</i> Lange-Bertalot                          | 25 | 24 | 0 | 51 |
| <i>Pinnularia microstauron</i> (Ehrenberg) Cleve                      | 11 | 36 | 0 | 53 |
| <i>Pinnularia subcapitata</i> W. Gregory                              | 4  | 13 | 2 | 82 |
| <i>Placoneis clementis</i> (Grunow) E. J. Cox                         | 13 | 29 | 2 | 56 |
| <i>Planothidium dauyi</i> (Foged) Lange-Bertalot                      | 24 | 29 | 0 | 47 |
| <i>Planothidium delicatulum</i> (Kützing) Round & Bukhtiyarova        | 7  | 33 | 2 | 58 |
| <i>Planothidium frequentissimum</i> (Lange-Bertalot) Lange-Bertalot   | 73 | 20 | 0 | 7  |
| <i>Planothidium lanceolatum</i> (Brébisson ex Kützing) Lange-Bertalot | 78 | 15 | 2 | 5  |
| <i>Planothidium rostratum</i> (Østrup) Lange-Bertalot                 | 7  | 45 | 2 | 45 |
| <i>Platessa conspicua</i> (A. Mayer) Lange-Bertalot                   | 0  | 0  | 9 | 91 |
| <i>Psammothidium helveticum</i> (Hustedt) Bukhtiyarova & Round        | 2  | 18 | 0 | 80 |
| <i>Reimeria sinuata</i> (W. Gregory) Kociolek & Stoermer              | 44 | 35 | 0 | 22 |
| <i>Rhoicosphenia abbreviata</i> (C. Agardh) Lange-Bertalot            | 18 | 51 | 4 | 27 |
| <i>Sellaphora pupula</i> (Kützing) Mereschkowsky                      | 29 | 44 | 0 | 27 |
| <i>Sellaphora seminulum</i> (Grunow) D. G. Mann                       | 53 | 40 | 0 | 7  |
| <i>Staurosira brevistriata</i> (Grunow) Grunow                        | 4  | 40 | 4 | 53 |
| <i>Staurosira venter</i> (Ehrenberg) Cleve & J. D. Möeller            | 13 | 60 | 0 | 27 |
| <i>Surirella angusta</i> Kützing                                      | 25 | 49 | 0 | 25 |
| <i>Surirella brebissonii</i> Krammer & Lange-Bertalot                 | 22 | 16 | 7 | 55 |
| <i>Tabellaria flocculosa</i> (Roth) Kützing                           | 7  | 11 | 2 | 80 |
| <i>Tryblionella apiculata</i> W. Gregory                              | 16 | 25 | 2 | 56 |
| <i>Tryblionella hungarica</i> (Grunow) Frenguelli                     | 11 | 15 | 2 | 73 |
| <i>Ulnaria biceps</i> (Kützing) Compère                               | 20 | 49 | 7 | 24 |
| <i>Ulnaria ulna</i> (Nitzsch) Compère                                 | 51 | 40 | 2 | 7  |
| <i>Ulnaria ulna</i> var. <i>acus</i> (Kützing) Lange-Bertalot         | 4  | 49 | 5 | 42 |

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**Appendix III – Results of the filters approach,  
macroinvertebrates – Chapter 3**





Frequencies (%) with which the filters approach predicted the occurrence of macroinvertebrate families at 62 sites in spring, compared with frequencies of collection. PC-taxon was predicted and collected, Pc-taxon predicted but not collected, pC-taxon not predicted but collected, pc-taxon neither predicted nor collected.

| Family           | PC | Pc | pC | pc |
|------------------|----|----|----|----|
| Aeshnidae        | 31 | 31 | 3  | 35 |
| Ancylidae        | 63 | 29 | 5  | 3  |
| Aphelocheiridae  | 2  | 15 | 2  | 82 |
| Asellidae        | 21 | 47 | 8  | 24 |
| Athericidae      | 45 | 45 | 3  | 6  |
| Atyidae          | 42 | 50 | 2  | 6  |
| Baetidae         | 79 | 15 | 6  | 0  |
| Brachycentridae  | 5  | 60 | 0  | 35 |
| Caenidae         | 69 | 23 | 5  | 3  |
| Calamoceratidae  | 19 | 53 | 3  | 24 |
| Calopterygidae   | 40 | 32 | 5  | 23 |
| Capniidae        | 10 | 19 | 2  | 69 |
| Ceratopogonidae  | 48 | 35 | 3  | 13 |
| Chironomidae     | 94 | 0  | 6  | 0  |
| Chloroperlidae   | 0  | 15 | 0  | 85 |
| Coenagrionidae   | 19 | 56 | 3  | 21 |
| Cordulegastridae | 26 | 39 | 3  | 32 |
| Corixidae        | 11 | 77 | 3  | 8  |
| Dixidae          | 13 | 45 | 2  | 40 |
| Dolichopodidae   | 8  | 44 | 2  | 47 |
| Dryopidae        | 44 | 47 | 3  | 6  |
| Dugesiidae       | 52 | 40 | 2  | 6  |
| Dytiscidae       | 44 | 34 | 3  | 19 |
| Elmidae          | 68 | 24 | 3  | 5  |
| Empididae        | 45 | 37 | 2  | 16 |
| Ephemerellidae   | 42 | 39 | 3  | 16 |
| Gerridae         | 21 | 66 | 2  | 11 |
| Glossosomatidae  | 15 | 48 | 2  | 35 |
| Goeridae         | 5  | 8  | 0  | 87 |
| Gomphidae        | 39 | 32 | 10 | 19 |
| Gyrinidae        | 10 | 34 | 0  | 56 |
| Haliplidae       | 32 | 48 | 3  | 16 |
| Helophoridae     | 34 | 45 | 2  | 19 |
| Heptageniidae    | 19 | 45 | 2  | 34 |
| Hirudinea        | 48 | 44 | 5  | 3  |
| Hydraenidae      | 27 | 47 | 2  | 24 |
| Hydrobiidae      | 61 | 34 | 3  | 2  |
| Hydrometridae    | 2  | 45 | 2  | 52 |

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|                   |    |    |   |    |
|-------------------|----|----|---|----|
| Hydrophilidae     | 34 | 52 | 2 | 13 |
| Hydropsichidae    | 61 | 31 | 5 | 3  |
| Hydroptilidae     | 44 | 48 | 3 | 5  |
| Lepidostomatidae  | 8  | 16 | 2 | 74 |
| Leptoceridae      | 13 | 60 | 0 | 27 |
| Leptophlebiidae   | 37 | 47 | 5 | 11 |
| Leuctridae        | 29 | 44 | 3 | 24 |
| Libellulidae      | 6  | 39 | 0 | 55 |
| Limnephilidae     | 10 | 32 | 2 | 56 |
| Limoniidae        | 45 | 48 | 3 | 3  |
| Lymnaeidae        | 31 | 58 | 0 | 11 |
| Naucoridae        | 2  | 37 | 0 | 61 |
| Nemouridae        | 19 | 27 | 3 | 50 |
| Notonectidae      | 10 | 40 | 2 | 48 |
| Oligochaeta       | 89 | 5  | 6 | 0  |
| Pediciidae        | 34 | 23 | 2 | 39 |
| Perlidae          | 0  | 27 | 0 | 73 |
| Perlodidae        | 21 | 48 | 2 | 29 |
| Philopotamidae    | 15 | 47 | 0 | 39 |
| Physidae          | 61 | 34 | 2 | 3  |
| Planariidae       | 3  | 10 | 0 | 87 |
| Planorbidae       | 47 | 47 | 2 | 5  |
| Platycnemididae   | 11 | 58 | 0 | 31 |
| Polycentropodidae | 16 | 74 | 3 | 6  |
| Psychodidae       | 24 | 37 | 3 | 35 |
| Psychomyiidae     | 32 | 52 | 3 | 13 |
| Rhagionidae       | 10 | 31 | 0 | 60 |
| Rhyacophilidae    | 34 | 48 | 3 | 15 |
| Scirtidae         | 5  | 29 | 0 | 66 |
| Sericostomatidae  | 6  | 50 | 2 | 42 |
| Simuliidae        | 79 | 13 | 3 | 5  |
| Sphaeriidae       | 32 | 47 | 2 | 1  |
| Tabanidae         | 10 | 71 | 3 | 16 |
| Taeniopterygidae  | 10 | 19 | 0 | 71 |
| Tipulidae         | 45 | 45 | 3 | 6  |

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## **Appendix IV – Experimental set– Chapter 6**



Photographs of the mesocosm channels. Detailed view of the substrates used for the diatom assemblages colization.

