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Integração de modificações geoquímicas e biológicas em áreas mineiras

Integration of geochemical and biological modifications in mining areas

Universidade de Aveiro Departamento de Geociências
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Integração de modificações geoquímicas e biológicas em áreas mineiras: águas, sedimentos e diatomáceas

Integration of geochemical and biological modifications in mining areas: waters, sediments and diatoms

Dissertação apresentada à Universidade de Aveiro e Universidade do Porto para cumprimento dos requisitos necessários à obtenção do grau conjunto de Doutor em Geociências Universidade de Aveiro / Universidade do Porto, realizada sob a orientação científica do Prof. Doutor Eduardo Anselmo Ferreira da Silva, Professor Catedrático no Departamento de Geociências da Universidade de Aveiro e da Doutora Salomé Fernandes Pinheiro de Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais pelo incansável apoio. À minha avó Isaura. Em memória do meu avô Lopes.

I dedicate this work to my parents and to my grandmother Isaura. "In memorium" of my grandfather Lopes.

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agradecimentos (continuação)

palavras-chave

Diatomáceas; *Pinnularia aljustrelica* sp. nov.; biofilmes; rios artificiais; minas de pirite; metais; pH ácido; sedimentos de corrente; água superficial; drenagem ácida de mina; fluorometria PAM; minas de New Brunswick e de Aljustrel.

resumo

O desenvolvimento de actividades mineiras ao longo de milhares de anos na região de Aljustrel, reflecte-se actualmente em vastas áreas ocupadas por escombreiras com minério, escórias e rochas encaixantes das mineralizações de sulfuretos. A geração de águas ácidas por oxidação dos sulfuretos - Drenagem Ácida de Mina (DAM) - provoca um impacte significativo na rede hidrográfica, quer na zona sul da vila de Aljustrel (Ribª. de Água Forte) quer na região norte da mesma (Ribª. da Água Azeda e Barranco do Farrobo), que se traduz em valores extremamente baixos de pH (<3) e elevadas concentrações de As, Cd, Cu, Fe, Mn, Pb, Zn e SO_4. Este estudo teve como objectivo avaliar a extensão destes impactos ambientais, integrando parâmetros geoquímicos (águas superficiais e sedimentos de corrente) e biológicos (diatomáceas). Três grupos de locais foram definidos com base na análise dos sedimentos de corrente e águas superficiais, cuja integração com as diatomáceas mostrou a mesma associação de grupos: Grupo 1- impactados, com pH ácido (1.9-5.1), conteúdo em metais alto (0.4-1975 mg L^{-1}) e águas sulfatadas ferromagnesianas, estando os metais mais biodisponíveis nas águas sob a forma catiónica (Me^{2+}); mineralogicamente os sedimentos caracterizaram-se por existência de fases filossilicatadas e sulfatadas/óxi-hidroxisulfatadas facilmente solubilizáveis que retêm grande quantidade de metais aquando da sua precipitação; o taxon dominante foi *Pinnularia aljustrelica* (espécie nova); Grupo 2- ligeiramente impactados, pH ácido fraco a neutro (5.0-6.8), conteúdo em metais pouco elevado (0.2-25 mg L^{-1}) e com águas do tipo sulfatadas ferromagnesianas a cloretadas magnesianas; os taxa dominantes foram *Brachysira neglectissima* e *Achnanthidium minutissimum*); Grupo 3- não impactados, pH alcalino (7.0-8.4), conteúdo em metais baixo (0-7 mg L^{-1}), águas essencialmente cloretadas magnesianas. Neste grupo, os metais estavam associados às fases primárias (por exemplo, sulfuretos), não se encontrando por isso tão disponíveis; verificam-se altos conteúdos em cloro o que justifica a presença de taxa típicos de águas salobras/marinhas (por exemplo, *Entomoneis paludosa*).

As amostras de diatomáceas foram estudadas do ponto de vista taxonómico (descoberta de uma nova espécie: *Pinnularia aljustrelica* Luís, Almeida et Ector sp. nov.), morfométrico (diminuição do tamanho das valvas de diatomáceas, assim como o aparecimento de formas deformadas de *Eunotia exigua* no Grupo 1 e *A. minutissimum* no Grupo 2), fisiológico (uso da Fluorometria de Pulso Modulado foi eficaz na avaliação dos efeitos dos metais/acidez na eficiência fotossintética). Foi efectuado um estudo em sistema de rios artificiais (microcosmos) que teve como objectivo simular as condições extremas existentes em Aljustrel em condições controladas de laboratório. Avaliaram-se os efeitos crónicos do Fe, SO_4^{2-} e acidez nas comunidades inoculados nos rios artificiais e a sua contribuição para a tolerância dessas comunidades à toxicidade a dois metais (Cu e Zn) através de testes agudos.

De um modo geral, os efeitos provocados pelos valores baixos de pH e pelos elevados teores em Fe e SO_4^{2-} reflectiram-se a nível das comunidades de diatomáceas, na diminuição da diversidade, no predomínio de espécies acidofílicas, na diminuição da eficiência fotossintética e no aumento das actividades enzimáticas e não enzimáticas.

**resumo
(continuação)**

Contudo, aquando da adição do Cu e do Zn, a acidez teve um efeito protector nas comunidades. Foi também efectuado um estudo comparativo entre a zona mineira de Aljustrel e a zona mineira de New Brunswick (Canadá) que apresentavam condições geológicas e de mineração semelhantes, reflectindo-se em comunidades de diatomáceas análogas, mas com uma localização geográfica e climática muito distinta

abstract

The development of mining activities over thousands of years in the region of Aljustrel is nowadays visible as a vast area of ore tailings, slag and host rocks of sulphides mineralization. The generation of acidic waters by the alteration of pyritic minerals - Acid Mine Drainage (AMD) - causes a significant impact on the river system both in the south of the village (Rib ª. Água Forte) and in the north of it (Rib ª. Água Azeda and Barranco do Farrobo), which is reflected in extremely low pH values (< 3) and high concentrations of As, Cd, Cu, Fe, Mn, Pb, Zn and sulphates. This study aimed to assess the environmental impacts extent, integrating geochemical (surface waters and stream sediments) and biological (diatoms) parameters. Three groups of sites were defined, based on sediments and water analysis, which integration with diatom data showed the same association of groups: Group 1- impacted, with acidic pH (1.9-5.1), high metal contents (0.4-1975 mg L^{-1}) and Fe-Mg-sulphate waters, being metals more bioavailable in waters in cationic form (Me^{2+}); mineralogically the sediments were characterized by phyllosilicates and sulphates/oxy-hydroxysulphate phases, easily solubilized, retaining a high amount of metals when precipitated; dominant taxon was *Pinnularia aljustrelica* (a new species); Group 2- slightly impacted, weak acid to neutral pH (5.0-6.8), metal contents not so high (0.2-25 mg L^{-1}) and Fe-Mg-sulphate to Mg-chloride waters; dominant taxa were *Brachysira neglectissima* and *Achnanthidium minutissimum*; Group 3- unimpacted, alkaline pH (7.0-8.4), low metal contents (0-7 mg L^{-1}) with Mg-chloride waters. In this group, metals were associated to the primary phases (e.g. sulphides), not so easily available; the existence of high chloride contents explained the presence of typical taxa of brackish/marine (e.g. *Entomoneis paludosa*) waters. Taxonomical aspects of the diatoms were studied (discovery of a new species: *Pinnularia aljustrelica* Luis, Almeida et Ector sp. nov.), as well as morphometric (size decrease of diatoms valves, as well as the appearance of deformed valves of *Eunotia exigua* in Group 1 and *A. minutissimum* in Group 2) and physiological (effective to assess the effects of metals/acidity in the photosynthetic efficiency through PAM Fluorometry) aspects. A study was carried out in an artificial river system (microcosm) that aimed to mimic Aljustrel's extreme conditions in controlled laboratory conditions. The chronic effects of Fe, SO_4^{2-} and acidity in field biofilms, inoculated in the artificial rivers, were evaluated as well as their contribution to the communities' tolerance to metal toxicity, through acute tests with two metals (Cu and Zn). In general, the effects caused by low pH values and high concentrations of Fe and SO_4^{2-} were reflected at the community level by the decrease in diversity, the predominance of acidophilic species, the decrease in photosynthetic efficiency and the increase of enzymatic (e.g. catalase, superoxide dismutase) and non-enzymatic activities (e.g. total glutathione and total phytochelatins). However, it was possible to verify that acidity performed a protective effect in the communities, upon Cu and Zn addition. A comparative study between Aljustrel mining area and New Brunswick mining area was carried out, both with similar mining and geological conditions, reflected in similar diatom communities in both mines, but in very different geographic and climatic areas.

"Eles não sabem, nem sonham,
que o sonho comanda a vida.
Que sempre que um homem sonha
o mundo pula e avança
como bola colorida
entre as mãos de uma criança"

António Gedeão (1906-1997), em "Movimento Perpétuo"; químico, professor, escritor divulgador de ciência, poeta.

"They don't know or dream,
That dreams command life
Whenever a man dreams
The world jumps and progresses
As a colored ball
In the hands of a child"

António Gedeão (1906-1997), in "Movimento Perpétuo"; chemist, teacher, writer, promoter of science, poet.

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List of abbreviations

ACOF	*Amphora* cf. *coffeaeformis*
ADMI	*Achnanthidium minutissimum*
AEA	Antioxidant Enzyme Activities
AFDW	Ash Free Dry Weight
AI	Autotrophic Index
AMD	Acid Mine Drainage
APX	Ascorbate Peroxidase
BNEG	*Brachysira neglectissima*
BVIT	*Brachysira vitrea*
CAT	Catalase
Chl-*a*	Chlorophyll-*a*
EARL	*Eunotia arculus*
EBIL	*Eunotia bilunaris*
EEXI	*Eunotia exigua*
EPAL	*Eunotia paludosa*
ERHO	*Eunotia rhomboidea*
ETR	Relative Electron Transport Rate
ETR_{max}	Maximum Electron Transport Rate of the ETR *vs.* E curve
Fo	Minimum Fluorescence emitted by a dark-adapted sample
FCGR	*Fragilaria capucina* var. *gracilis*
FSAX	*Frustulia saxonica*
GPAR	*Gomphonema parvulum*
GR	Glutathione Reductase
GSH	Total Glutathione
LM	Light Microscopy
NDES	*Nitzschia desertorum*
NGRE	*Navicula gregaria*
NHAN	*Nitzschia* aff. *hantzschiana*
NPAL	*Nitzchia palea*
NVEN	*Navicula veneta*
PALJ	*Pinnularia aljustrelica*
PAM	Pulse Amplitude Modulated
PCs	Phytochelatins
PSII	Photosystem II
RLCs	Rapid Light Curves
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TFLO	*Tabellaria flocculosa*
α	Initial slope of the ETR *vs.* E curve
$\Delta F/F_m'$ or Φ'_{PSII}	Effective Quantum Yield of PSII
Φ_{PSII}	Maximal Quantum Yield

CHAPTER 1

General Introduction

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1.1 INTRODUCTION

1.1.1 General overview

Aljustrel mines, as well as other mining sites from the Iberian Pyrite Belt (IPB) were considered of high level of environmental risk (Oliveira et al. 2002), due to the large volume of the tailings (~5Mt) with high amounts of metals (especially Cu, Pb and Zn) and metalloids (As, Sb) (Matos and Martins 2006). Important mining exploitation in Aljustrel was made since pre-Roman periods until today, but their greatest development was experienced in the XXth century until the mid-80s-90s. After one decade of inactivation, zinc exploitation started in 2008 however, after one year, exploitation changed to Cu until today.

Pyritic mining exploitation exposes pyrite to atmospheric conditions, in reduced form, suitable for its oxidation, producing mineralized acidic waters, with high sulphate and metal (especially, As, Cu, Fe, Mn, Pb, Zn) concentrations, with the aid of the chemolithotrophic bacteria, particularly *Acidithiobacillus ferrooxidans* (Kelly and Wood 2000) and *Leptospirillum ferrooxidans* (Romero et al. 2003) which catalyze the oxidation of reduced compounds of sulphur and Fe (II), allowing the mineral dissolution. The Acid Mine Drainage (AMD) waters are thus formed. Therefore, it is necessary to re-analyze the dispersion and distribution of the toxic chemical elements, especially when their concentrations seem anomalous, regarding the geochemical background (Oliveira et al. 2002). These elements' dispersion affects soils, sediments, waters and biota. For this reason, the environmental remediation of the Aljustrel mining area was considered as crucial. After a preliminary study period (2000-2003 and 2005) by EDM – Empresa de Desenvolvimento Mineiro, the recovery of the most affected area was initiated. Due to the complexity of the area, the recovery project was divided in 4 phases, beginning in the year of 2006 and scheduled for completion in 2014. This project will cost more than 10.000.000€ (source: EDM). However, the ongoing remediation measures are based on tailings' confinement, acid remediation using lime additives and in the construction of water-diversion systems to minimize waste water/rock contact (Maia et al. 2012). These measures are thought to be ineffective, especially for Água Forte stream, the most impacted stream (Luís et al. 2009) because they only increase the water pH but don't decrease conductivity or sulphates (Zalack et al. 2010) and create toxic sludge difficult to remove from the riverbed. In this study, it was intended to analyze how these changes - mining exploitation *vs* rehabilitation - affect biotic and abiotic components of this complex environment. Sediments are of extreme importance in this study because they accumulate

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metals over time and support epipsammic organisms (e.g. diatoms): the ones living on the top layer of sediments, the most abundant on the streams bed composed of sand. The control exerted by major sediment components (e.g. oxides of Fe and organics) to which metals are preferentially bound, the competition between sediment metals (e.g. Cu, Zn and Cd) for uptake sites in organisms, and the influence of acidic pH (Bryan and Langston 1992) make them geochemically important.

It is mandatory to understand how biological entities interact with sediment/water systems and to which extent they are affected by mining activity and recovery processes. Most water systems in the mining area are shallow streams which allow the development of biofilms, composed by bacteria, protozoa and other eukaryotes (fungi and algae), impregnated in a mucilage matrix (Lock 1993). In rivers and streams, biofilms are biological layers inhabiting the sediment interface and in contact with the flowing water. Since both water and sediments can act as sources of pollutants for benthic communities (Muñoz et al. 2009), biofilms are the first affected, and can consequently be used to detect the early effects on the ecosystem (Sabater et al. 2007). In AMD systems, diatoms (Bacillariophyceae class) are a dominant part of the biofilms, a successful and diverse algal group in aquatic media with more than 62000 genera, species and taxa at infraspecific ranks (The Catalog of Diatom Names (CAS) 2011) Their cells are indeed enclosed in a peculiar and highly differentiated silica cell wall called frustule, consisting of two parts (valves) fitting together by a series of silica bands linked along the margins (Round et al. 1990). The specific ecology of diatoms, fast replication, ubiquity and sampling easiness, favor them as indicators of water quality (Smol 2008, Van Dam et al. 1994). The sensitivity of diatom communities drove them to be used as indicators of environmental conditions, such as water quality and habitat conditions in stream and river systems (Pan et al. 1999, Soininen et al. 2004). Diatoms are now routinely used to track water quality changes related to acidification (e.g. Cumming et al. 1992) and metal contamination (e.g. Dixit et al. 1989, Luís et al. 2009). Diatoms quickly react to habitat changes both at the community and/or individual levels: morphological alterations of the silicified cell wall (Cattaneo et al. 2004). Community alterations through changes in dominant taxa (Gustavson and Wängberg 1995, Hirst et al. 2004) as well as changes in diversity (Leland and Carter 1984, Medley and Clements 1998, Luís et al. 2009) can be consequences of toxic effects. To prevent Reactive Oxygen Species (ROS) effects, cells have antioxidative systems composed of different enzymatic and non-enzymatic mechanisms. The induction of these mechanisms is considered to play an important role in the cellular defense strategy against oxidative stress caused by toxic metal

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concentrations (Collén et al. 2003, Geoffroy et al. 2004, Tripathi et al. 2006, Valavanidis et al. 2006) proving that biochemical (e.g. enzymatic and non-enzymatic methods) and physiological (e.g. photosynthetic performance of phototrophs of the biofilms through non-destructive methods) endpoints are preponderant in the early detection of toxic effects of stressors.

Experimental investigations using artificial streams allowed for a control and constant monitoring of the experiments that would not be possible in the field due to constant changes of the environmental parameters. They were already used successfully in the past to examine the effect of acidification on periphyton communities (Hendrey 1976, Hall et al. 1980) and in natural lotic ecosystems with low pH, by Hamilton and Duthie (1984) and Mulholland et al. (1986). Artificial streams have been also used to test metals effects (e.g. Serra et al. 2009, Corcoll et al. 2012).

1.1.2 Thesis objectives

The main objective of this work was to predict the geochemical and biological alterations caused by mining activities and its rehabilitation, using sediment and water characterization and improving the knowledge on the biodiversity of benthic diatom communities and species ecological preferences, from streams surrounding Aljustrel mining area, in Portugal. To attain this objective, different aspects were addressed. This thesis consists of 8 chapters, being chapter 1 a general introduction, and chapter 2 a characterization of the study area. Chapters 3 and 4 present the characteristics of biofilms in order to understand the taxonomy, diversity and physiology of diatoms inhabiting the AMD environments under study. Chapter 5 consists in a description of a microcosm test performed in the laboratory which subject biofilms to chronic and acute exposures with Fe/acidity and metals (Cu, Zn) in order to verify also as chapters 3 and 4, alterations in taxonomy, diversity, and physiology, as well as in biochemistry of biofilms. Chapters 6 and 7 integrate the geochemical parameters with the biological communities. Chapter 8: the conclusions and future directions. More specifically:

- chapter 1: a general introduction;
- chapter 2: environmental and geologic characterization of the study area;
- chapter 3: it was found that certain acidic taxa remained misidentified based on a previous work (Luís et al. 2009). To have an accurate identification of the species inhabiting these AMD waters, some species of *Pinnularia* genera were investigated and a new species - *Pinnularia aljustrelica* sp. nov. Luís, Almeida et Ector - was found;

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- chapter 4: to test accuracy of PAM fluorometry to detect AMD effects in the photosynthetic efficiency of diatom communities from contaminated sites as well as the effects on primary productivity (Chl-*a*);
- chapter 5: a microcosm test was used to understand the selection pressure exerted by acidity and how acidity and metal toxicity interact in biofilms of AMD field waters. Taxonomic, physiological and biochemical responses of biofilms to the constrains posed by these stressors were determined;
- chapter 6: the overall chemical characterization of surface waters, stream sediments and diatom communities from all 19 sites, during 5 sampling moments, was done to assess land degradation and determine how sediments and waters affected diatom communities. Finally to explore differences in diatom communities concerning their structure - taxonomic composition, relative abundance - and morphology - deformed valves - inferring how geochemical (e.g. metals *vs* acidity) factors might contribute to these alterations in the communities;
- chapter 7: to evaluate the effects of AMD on diatom communities in rivers of continental (Canada) and Mediterranean (Portugal) climates, testing the possibility of using the same biological metal assessment method in these two geographically and climatically distinct zinc mining areas;
- chapter 8: provides a summary of the main results and conclusions as well as objectives of future research.

Three of the aforementioned chapters are published (chapter 3, 4 and 7) while one is under review (chapter 5) and another is in preparation (chapter 6). The corresponding complete reference is, therefore, provided in the beginning of these chapters. Several readings of this thesis are possible because chapters 3 – 7 are mostly self-contained and independent.

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CHAPTER 2

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Study Area

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2.1 STUDY AREA

2.1.1 Location of the Aljustrel mining site and historical framework

Aljustrel mining area is located in Aljustrel, Alentejo (south-west Portugal) belonging to the Iberian Pyrite Belt (IPB), one of the greatest concentrations of massive sulphide deposits that extends in 230 km from Grândola (Portugal) to Aznalcóllar (Spain) (**Fig. 2.1**).
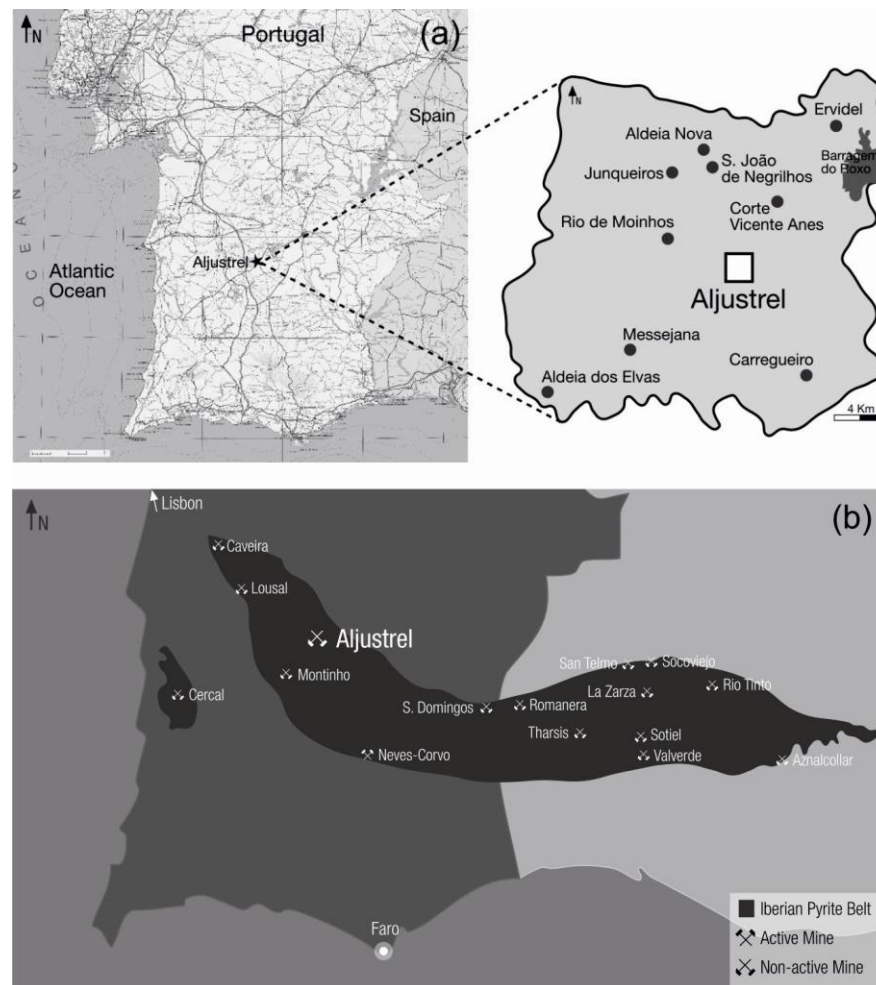
Fig. 2.1 – (a) Location of the Aljustrel study region (adapted from http://google.brand.edgar-online.com/; http://www.agenda.pt/iframe.php?subcat=ALJUSTREL), (b) location of the Iberian Pyrite Belt region (adapted from Pacheco et al. 1998, Martins et al. 2003) and of the Aljustrel study area [*in* Candeias et al. (2011)].

The Roman Lusitania had several mineral deposits, probably the most important in Western Europe that was located in the village of Vipascum or Vipasca, current Aljustrel. Since immemorial times Aljustrel is known by its mineral deposits. The existing occupations since the Copper Age, pointed out that the exploitation has begun, incipiently, 3000 years B.C. But, it was with the Roman occupation, between centuries I and IV A.D. that large-scale ore's exploitation started. Minerals were then melted on site and

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transported to Rome. Between 1876 and 1906 two impure copper tablets containing mining legislation were found, existent since the mining time of Hadrian Augustus (117-138 A.D.). There are numerous traces of this occupation, particularly in the gossan and slags of Algares mine where those two bronze tablets (Bronze Board of Vipasca) were found, assigned as "Vicus Vipascensis". During the Roman Period, the village of Valdoca was built, next to the Algares gossan and mining works were developed up to 100 m depth.

2.1.2 Aljustrel geology and mining activity

The Aljustrel region is characterized by a Palaeozoic basement of the South Portuguese Zone and by a Cenozoic sedimentary sequence of the Alto Sado Tertiary Basin. The NE–SW Messejana fault defines the SE border of this basin, which is confined to the northwestern block of the fault (**Fig. 2.2**). The Messejana fault presents an Iberian dimension and a senestral strike-slip movement of 2.5 km. A dolerite of Jurassic age is also observed along this fault (Schermerhorn et al. 1987).
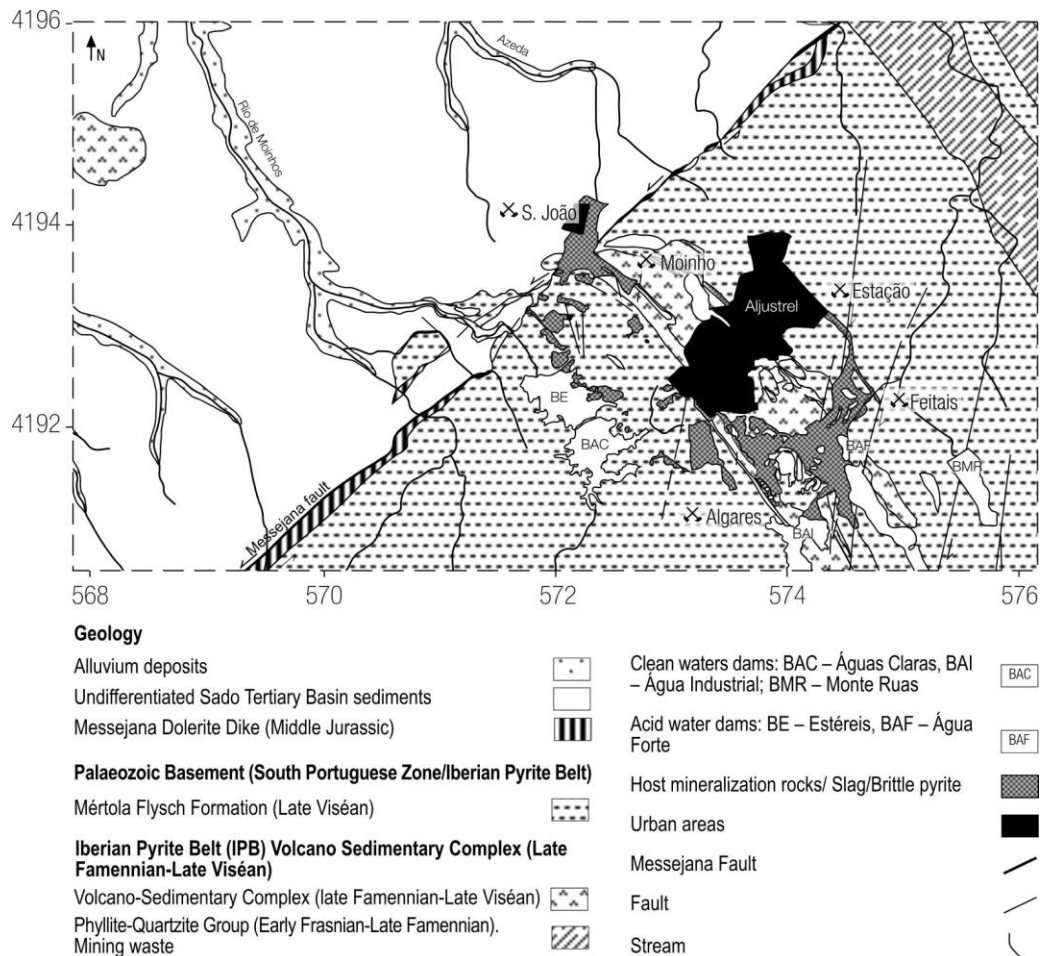
Fig. 2.2 – Geological setting (massive sulphide orebodies), UTM coordinates in km (adapted from the Portuguese Geological Map 1/50000, Schermerhorn et al. 1987) [in Candeias et al. (2011)].

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The Aljustrel's Palaeozoic stratigraphic sequence is formed by the following three main units (Andrade and Schermerhorn 1971, Schermerhorn et al. 1987, Silva et al. 1997, Leitão 1998, Matos 2005, Oliveira et al. 2006): 1) *Baixo Alentejo Flysch Group*, Mértola Formation (Upper Visean)–shales and greywackes (flysch turbidites); 2) *Iberian Pyrite Belt (IPB)*, *volcano–sedimentary complex (VSC)* (Upper Famennian–Upper Visean), represented by the following units: Paraiso Fm.– siliceous shales, phyllites, tuffites, purple shales, jaspers and cherts; Felsitic/Mine Metavolcanics sequences and Megacryst/Green Metavolcanics sequences–sericitic felsic volcanics, felsites, felsophyres, volcanic breccias, massive sulphides, feldspar megacrystals volcanics and lavas; 3) *IPB lower unit*, the sedimentary Phyllite–Quartzite Group (Frasnian–Upper Famennian), is not recognized in the Aljustrel area. The VSC Aljustrel Anticlinorium is represented by a NW–SE lineament (4.5 km length and 1.5 km across). Several NW–SE thrust are identified, mainly in the short limbs of the anticlines. The Aljustrel thrust is one of the main structures and materializes the SW contact between the VSC and the Flysch Group. Late variscan faults with NNE–SSW direction produced important dextral strike-slip movements, probably conjugated faults of the main Messejana fault. This system is represented from SE to NW by the Esteval, Azinhal, Feitais, Represa, Castelo and Moinho faults (Luís et al. 2009).

IPB is a world class volcanic-hosted massive sulphides metalogenetic province (Barriga et al. 1997, Carvalho et al. 1999, Matos and Martins 2006, Oliveira et al. 2006, Relvas et al. 2006, Tornos 2006). In the Aljustrel mining site, six massive sulphide orebodies are recognised: Moinho, Feitais, Estação, Gavião, Algares and São João. The last two were mined since Roman times (Leitão 1998, Silva et al. 1997, Matos and Martins 2003, 2006). The gossans and the supergene zones of the Algares and São João orebodies were exploited during roman era to 100 m deep (Domergue 1983). Moinho deposit was exploited by the Pirites Alentejanas Company (PA) for copper until 1993. After one decade of inactivation, zinc exploitation started in 2008 however, after one year, exploitation changed to Cu until today.

2.1.3 Environmental impact in the Aljustrel Area

The impacts observed in the Aljustrel area are related to the massive sulphide mineralization. As a result of thousands of years of pyrite ores exploitation in Aljustrel, large areas are occupied by waste tailings composed by Roman slag, pyrite ore (blocks and brittle massive pyrite ore) and volcano–sedimentary complex host rocks. Algares industrial area and São João sector present the highest volumes of mine waste, causing

impact in the environment (presence of tailings formed by reactive materials, contaminated soils and acidic waters).

The small Mn-Fe exploitations are represented by small open pits and associated ore tailings, usually with less than 1 m in thickness. Part of these small mines is still used for illegal waste disposals. The petrographic study of ore waste samples allowed the identification of interstitial chalcopyrite, sphalerite, galena, arsenopyrite and minor sulfossalts in the massive pyrite ore. The pyrite ore present high concentrations of Fe, Cu, Pb, Zn, Ag, Sb, Hg, Se, Co, Au and Cd while the roasted pyrite ore shows high concentrations of Au, Pb, Ag, Fe, Sb, Bi, Se, Cu, Zn and Mo. Roman slag presents interstitial pyrite and rare chalcopyrite with sparse distribution in the iron silicate matrix. The Roman slag had high concentrations of Pb, Cu, Zn, Fe, As and Sb elements. Roman wastes are represented by *in situ* and reworked slags, located in the upper stream sector of Água Forte stream. Some of the mining infrastructures are unsafe and potentially dangerous, like open pits, quarries, galleries and mining shafts very exposed to local urban areas (Matos and Martins 2006). The walls of São João open pit and Malpique and Moinho quarries present significant geotechnical instability.

Other wastes are present in Aljustrel, with less contaminant potential like host rocks, represented by felsic well cleaved and coherent volcanics, siliceous, purple and black shales, jaspers and cherts (volcano– sedimentary complex host rocks). Roasted pyrite ore has a very low expression confined to spotty occurrences at Algares. In the area there are several tailings containing accumulated materials with a high proportion of sulphides. The alteration of sulphides generates acidic runoff - AMD, with characteristic ferric-sulphated waters. Seepage waters resulting from the infiltration of rainfall, as well as waters resulting from acid drainage that directly contact with reactive minerals present in tailing materials. This enrichment results from chemical dissolution processes of mineralogical phases related to the oxidation of sulphides minerals (Oliveira et al. 2002). The generation of acid mine drainage (AMD) causes a significant impact on the river system namely in Água Forte Stream and Água Azeda Stream. The metabolism of the chemolithotrophic prokaryotes is responsible for the maintenance of this extreme environment throughout the continuous production of Fe^{3+} by aerobic oxidation of the ferrous iron in solution (Fernández-Remolar et al. 2004).

Presently, the EDM public company (the owner of the Portuguese mines rehabilitation program) is developing a rehabilitation program at mine-activity affected areas, before 1990 (Martins 2005, Nero 2005, Matos and Martins 2006) (**Fig.2.3**).

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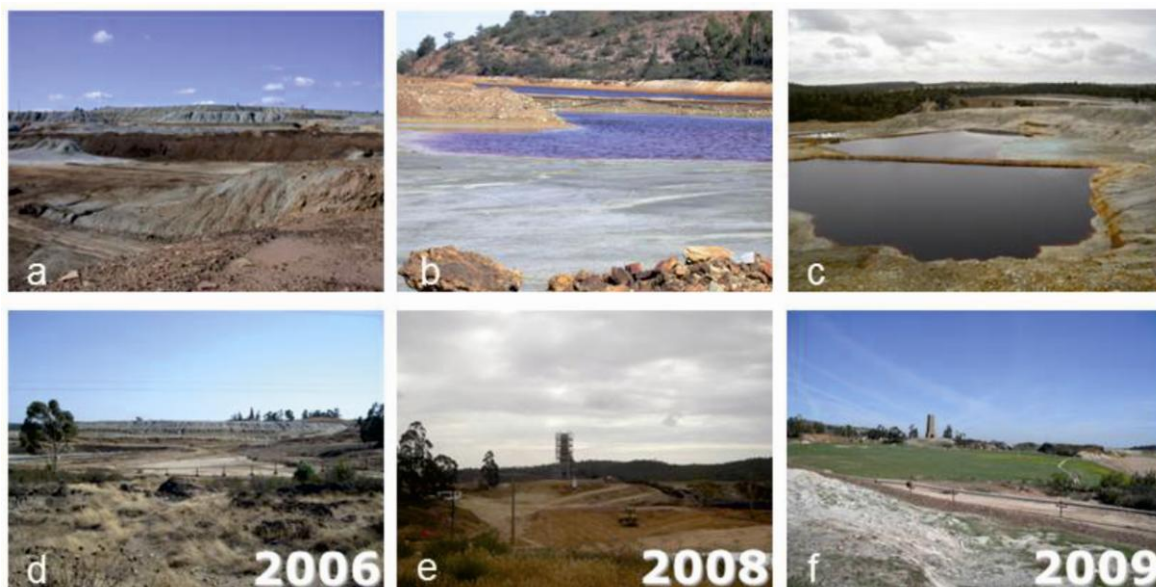
Fig. 2.3 – All images showing Algares mine in Aljustrel. Tailing (a) and acid water dams (b, c) in a non-rehabilitated period. Also a sequence of images (d, e, f) showing Algares area in non-rehabilitated (d- 2006), beginning of the rehabilitation process (e- 2008) and after one year of the rehabilitation process (f - 2009).

2.1.4 Hydrology

Sampling

The sampling sites were selected in areas under the mine influence (Água Forte and Água Azeda streams) and in areas outside the mine influence (Roxo stream and Farrobo Creek). These sites are plotted in **Fig. 2.4.**
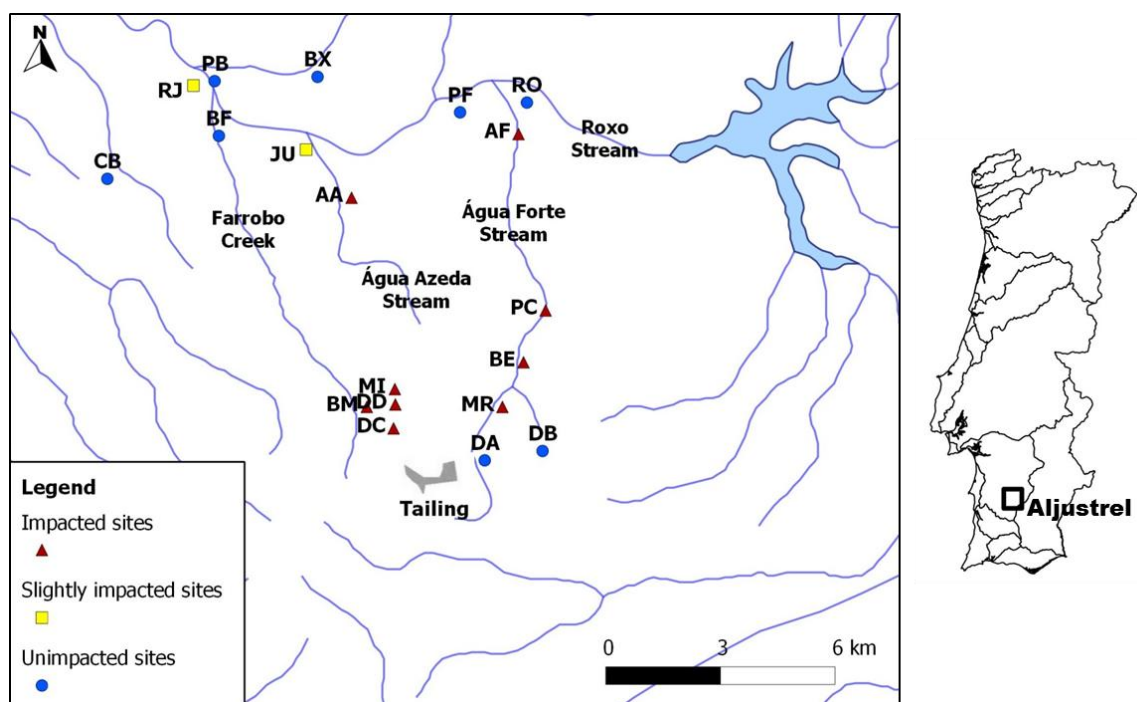
Fig. 2.4 – Sampling sites location.

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The selected sites were: 4 dams, two with clean water (DA- Águas Industriais dam, DB- Monte Ruas dam) and two impacted (DC- Mine dam DD- Estéreis dam) and 15 sites: 7 in impacted (MI- Mine, BM- Barranco Morgado, MR- Monte Ruas, BE- Porto Beja, PC- Pte Curval, AF- Água Forte, AA- Água Azeda) and 8 in unimpacted streams(RO- Roxo, PF- Porto Ferreira, JU- Jungeiros, RJ- Roxo Jusante, BX- Barranco Xacafre, BF- Barranco Farrobo, PB- Pero Bonito, CB- Canal Barrada)**.**

Roxo Stream

Roxo stream is a permanent water course, but with very irregular flow and torrential character. It is the main water stream, establishing itself as one of the sub-basins of the Sado River. Its upstream headwater area is governed by the Roxo dam, located 7 km upstream from the confluence with Água Forte stream. However, the remaining portion of Roxo stream receives not only contamination from the mining area of Aljustrel as well as contamination from the various factories installed there, especially from livestock and agri-food factories and wastes of the various villages. Outside the periods of the highest rainfall (late autumn and winter), the hydrologic contribution of Roxo stream's tributaries is practically null.

Água Forte Stream

The total area of this sub-basin is 37.8 km^2 (ICAT 1998), being most of it developed on Carboniferous land, except the final section, near the outfall of Roxo stream which runs in Tertiary's land. This stream rises upstream of the Industrial Water dam, receiving infiltration waters of this dam. It receives surface runoff from the old industrial area, possibly from the explosives factory and from Aljustrel's Waste Water Treatment Plant (WWTP) and finally, the runoff of Monte Ruas dam of agricultural use. It converges in Roxo stream near Chamenica, being of the three studied streams, the one which converges furthest upstream in the Roxo stream. Finally, in the most downstream part of its route, since the railway line, it should be highlighted the lack of direct contributions of mine pollutants. It should be mentioned the untreated discharges of Aljustrel WWTP, held in a place called Porto Malhão Popa. After this site, the mixture of acidic waters (**Fig. 2.5**) with urban waste waters occurs and the increase of pH and the decrease of metal concentrations take place until its confluence with Roxo stream. Along this impacted stream, Fe oxi-hydroxides precipitate on the stream bed (**Fig. 2.6**).

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Fig. 2.5 – Acidic water from Monte Ruas (MR) site.

Fig. 2.6 – Iron precipitates of Água Forte (AF) site.

This sub-basin is the one that shows the greatest environmental and hydrological problems. Extreme acidic water dam (Água Forte) and a clean water dam (Água Industrial) are present.

Água Azeda Stream

It is a sub-basin with a total area of 12.1 km^2 which rises near Poço do Moinho headwater zone. It drains on Carboniferous and on Tertiary lands after the S. João neighborhood until the Roxo stream.

Reasonable volumes of contaminated soils and acid waters persist, however lesser than those of Água Forte stream. Acid waters coming from industrial area are diluted with urban waste waters coming from the S. João neighborhood, being this set diluted

sometimes with big volumes of waters, treated in the industrial plant and discharged in this stream, thus waters' quality parameters are very inconstant, influenced by these discharges.

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CHAPTER 3

Pinnularia aljustrelica sp. nov. (Bacillariophyceae), a new diatom species found in acidic waters in the Aljustrel mining area (Portugal), and further observations on the taxonomy and ecology of *P. acidophila* HOFMANN et KRAMMER and *P. acoricola* HUSTEDT

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Abstract A new benthic freshwater diatom species belonging to the genus *Pinnularia* Ehren. has been recorded in the Água Forte stream surrounding the Aljustrel mining area in southern Portugal. *Pinnularia aljustrelica* Luís, Almeida et Ector sp. nov. is described as a new species based on light and scanning electron microscopy observations and on its particular habitat in an acidic environment due to acid mine drainage, high metal concentrations (As, Cd, Cu, Fe, Mn, Pb and Zn) and high sulphate and conductivity. The taxa most similar to *P. aljustrelica* are *P. acidophila* Hofmann et Krammer, *P. acoricola* Hustedt and *P. acoricola* var. *lanceolata* Hustedt, so type materials of these taxa were studied for comparative purposes. Although, the ecology of the three similar taxa is also quite similar, the new species has a combination of particular morphological characteristics studied under LM and SEM that separates it from the rest. *Pinnularia aljustrelica* has a general shape relatively similar to *P. acidophila* and *P. acoricola*, but the valve outline is not as linear as in *P. acidophila* and not as oval as in *P. acoricola*.

Keywords *Pinnularia aljustrelica*, new species, morphology, extreme acidic environment, Portugal

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3.1 INTRODUCTION

The pyrite tailings of Aljustrel (Southern Portugal) are characterized by a total lack of vegetation and the nearby streams are strongly affected by acid mine drainage (AMD) produced when pyritic minerals are exposed to weathering, resulting in very low pH (formation of sulphuric acid), high conductivity, elevated sulphate content and high concentrations of dissolved metals as arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), lead (Pb) and zinc (Zn) (Skousen et al. 1994). A new diatom species was found in this area during a survey in the 2008–2009 period. The new taxon resembled other species currently ascribed to *Pinnularia*, a genus commonly reported from similar highly acidic conditions. The acidic conditions increase bioavailability of metals (Luís et al. 2009), thus making the monitoring of the ecosystem through biological studies highly necessary. Diatoms are one of the principal groups of organisms in these affected streams; making them excellent bioindicators based on their high sensitivity to changes in environmental conditions (Bahls 1993, Stevenson and Pan 1999, Blinn and Herbst 2003). Based on literature data (Carter 1972, Whitton and Diaz 1981, Negoro 1985, Cassie and Cooper 1989, Watanabe and Asai 1995, Sabater et al. 2003), one of the most widespread taxa in acidic waters is apparently *Pinnularia acoricola* Hustedt, described in 1935 in Java from sulphate–rich waters with a pH of 2.4. *Pinnularia acoricola* var. *lanceolata* Hustedt was also discovered in Indonesia (Lake Toba, Sumatra), as a fossil freshwater diatom (Hustedt 1935). Lessmann et al. (1999) recorded *P. acoricola* in an extreme acidic lake with high concentrations of calcium, iron, aluminium, manganese and sulphate. This taxon has a variable morphology resembling *P. obscura* Krasske (Carter 1972). Both species, *P. acoricola* and *P. obscura*, were observed in waters with low pH in England (Hargreaves et al. 1975), North America (Whitton and Diaz 1981) and South Africa (Denicola 2000). Apparently, these diatoms seem to be well adapted to low pH conditions allowing them to develop large communities in otherwise inhospitable environmental conditions. The morphology of *P. acoricola* clearly influenced by the ecological conditions resulting in a high variety of sizes and shapes, e.g. *P. acoricola* var. *osoresanensis* Negoro described in Japan by Negoro (1944), then transferred at the species level by Fukushima et al. (2002) as *P. osoresanensis* (Negoro) Fukushima, Yoshitake et Ko-Bayashi. Recently, *P. acidophila* Hofmann et Krammer in Krammer (Krammer 2000) was described from Eastern Germany in a lake resulting from an opencast mining of low pH.

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The main purpose of the present work is to study the *Pinnularia* populations of the Aljustrel mining area in southern Portugal. For this purpose, the type materials of *P. acidophila*, *P. acoricola* and *P. acoricola* var. *lanceolata* were investigated in order to clarify the differences and similarities of these species with the Southern Portuguese *Pinnularia*. This detailed examination by means of light (LM) and scanning electron microscopy (SEM), as well as the particular environmental characteristics of the streams surrounding this mining area lead us to propose a new *Pinnularia* species, here described in detail.

3.2 MATERIAL AND METHODS

One litre of water was collected from the surface, as close to the centre of the stream as possible, in Ponte Monte Ruas and Porto de Beja sites (Aljustrel mining area – Southern Portugal, **Fig. 3.1**), simultaneously with the diatom sampling.
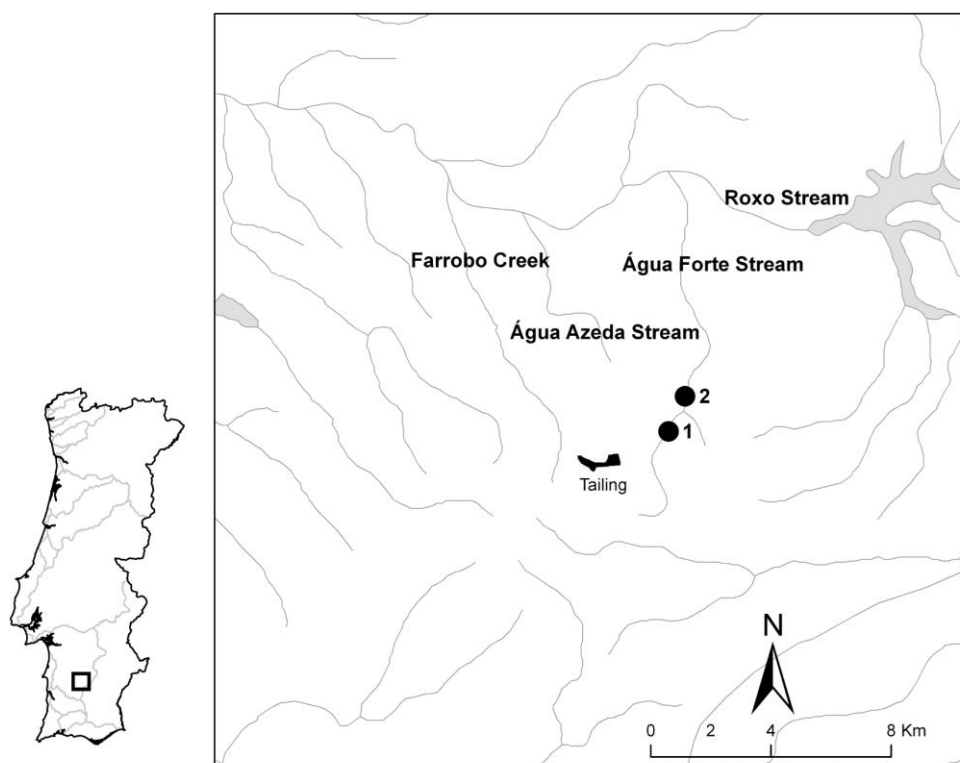
Fig. 3.1 – Sampling sites where *Pinnularia aljustrelica* was found (Água Forte stream near the Aljustrel tailing): (1) Ponte Monte Ruas; (2) Porto de Beja.

Then, the surface water samples were returned in a cool box to the laboratory and stored at 4 °C before the analyses according to the protocols proposed by German Chemists Association (1981), EPA – Environmental Protection Agency (1982) and ASTM (1984).

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Temperature (T, °C), pH and electrical conductivity (EC, µS cm^{-1}, at 25 °C) were measured directly in the field with a WTW$^{®}$ Multiline P4 set. The determination of As, Ca, Cd, Cl, Cu, Fe, K, Mg, Mn, Na, Pb, and Zn was carried out using ICP–MS. The determination of COD, NH_4^+ and PO_4^{3-} was done by spectrophotometry. For NO_3^- and SO_4^{2-} determination, ion chromatography was used (all the concentrations of the chemical elements were expressed in mg L^{-1}).

Epipsammic diatoms were collected from the sediment with a syringe, four times at Ponte Monte Ruas (site 1 – 37º52'22.87"N; 8º8'59.42"W), twice in 2008 (spring and summer) and twice in 2009 (spring and winter). Porto de Beja (site 2 – 37º53'0.69"N; 8º8'36.83"W) was sampled only once, in spring 2008. Both sites are located in the Água Forte stream that surrounds the Aljustrel mining area.

Four samples from Hofmann's and Hustedt's type material collections were also examined for this study:

– Material received from G. Hofmann (holotype material of *Pinnularia acidophila* Hofmann et Krammer, corresponding to the slide number 61B IOK in Krammer collection); Locality: Braunkohlerestsee, Lausitz, Restloch 107, 0–2 cm (lake resulting from an opencast mining, Lausitz, Eastern Germany); Collection date: 21/05/1996; Collector name: G. Hofmann.

– Material number AS403 (lectotype material of *Pinnularia acoricola* Hustedt); Locality: Java, Dieng–Plateau, D3b, Telaga Pengilan, Schwingrasen; Substratum: *Sphagnum*; Collection date: 06/03/1929; Collector name: F. Ruttner; Friedrich–Hustedt–Zentrum für Diatomeenforschung, Alfred–Wegener Institut für Polar und Meeresforschung, Bremerhaven (BRM).

– Material number AS895 (material with abundant *Pinnularia acoricola* Hustedt); Locality: Sumatra, Lake Toba, TW1d, W–Ufer Samosir, N–Sigaol; Substratum: mud; Collection date: 04/11/1929; Collector name: F. Ruttner; Friedrich–Hustedt–Zentrum für Diatomeenforschung, Alfred–Wegener Institut für Polar und Meeresforschung, Bremerhaven (BRM).

– Material number AS956 (holotype material of *Pinnularia acoricola* var. *lanceolata* Hustedt); Locality: Sumatra, Lake Toba, TD4, fossil, West Coast of Samosir Island; Substratum: diatomite; Collection date: 01/01/1929; Collector name: F. Ruttner; Friedrich–Hustedt–Zentrum für Diatomeenforschung, Alfred–Wegener Institut für Polar und Meeresforschung, Bremerhaven (BRM).

The samples from Aljustrel mining area were treated in the laboratory with nitric acid (HNO_3 65 %) and potassium dichromate ($K_2Cr_2O_7$) at room temperature for 24 h, followed

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by three centrifugations (1500 rpm) to wash the excess of acid, then treated with hydrochloric acid (HCl 37 %) during more than 24 h to disaggregate the valves, and then cleaned again, through three centrifugation cycles (1500 rpm) to wash the excess of HCl. The samples from Hofmann's and Hustedt's collections were rinsed out of fixatives, treated by oxidation with hot hydrogen peroxide (H_2O_2 120 vols.) and hydrochloric acid, and then rinsed three times with deionized water in order to obtain a suspension of clean frustules. Permanent slides were mounted using the high refractive index (1.74) medium Naphrax® (Brunel Microscopes Ltd, UK). Light microscopy observations were taken using a Leica® DMRX light microscope (DIC contrast) with a 100 x oil immersion objective and LM photographs were taken with a Leica® DC500 camera. Samples chosen for scanning electron microscopy were filtered through polycarbonate membrane filters with a 3 μm pore diameter, mounted on stubs with double sided carbon tape, sputtered with platinum (30 nm) with a Modular High Vacuum Coating System (Bal–Tec MED 020) and studied with a Hitachi SU–70, operated at 5.0 kV. Morphometric parameters were obtained from LM and SEM photographs: a total of 162 valves (110 of *Pinnularia aljustrelica* sp. nov., 23 of *P. acidophila*, 28 of *P. acoricola* and 1 of *P. acoricola* var. *lanceolata* type materials) were measured.

Morphological terminology follows Round et al. (1990) and Krammer (2000). Apart from the original descriptions of *P. acidophila*, *P. acoricola* and *P. acoricola* var. *lanceolata*, the following publications were consulted for taxonomical and ecological comparison (**Table 3.1**): Hustedt in Schmidt et al. (1934), Hustedt (1935), Negoro (1944), Carter (1972), Simonsen (1987), Krammer (2000), Jordan (2001), Wydrzycka and Lange–Bertalot (2001), Fukushima et al. (2002), Van de Vijver et al. (2002) and Metzeltin et al. (2005).

Bibliographical references of similar taxa with illustrations, which made the comparison possible with our populations, were also used e.g., Negoro (1985), Watabane and Asai (1995), Idei and Mayama (2001), Jordan (2001) and Hobbs et al. (2009). All images were digitally manipulated and plates made using Microsoft Photo Editor 3.01, Microsoft PowerPoint 2000 SP–3 and Adobe Photoshop Elements v. 2.0.

The map of the sites where the new *Pinnularia* species (**Fig. 3.1**) was found was generated using GIS software ArcGis 9.3 (ESRI 2008).

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Table 3.1 – Comparison between *Pinnularia aljustrelica* and the morphologically and ecologically most similar taxa (all data from the literature with the exception of *P. aljustrelica* Luís, Almeida et Ector).

Pinnularia (References)	*aljustrelica* LUÍS, ALMEIDA et ECTOR (This study)	*acidicola* VAN DE VIJVER et LE COHU (VAN DE VIJVER et. al. 2002)	*acidophila* HOFMANN et KRAMMER (KRAMMER 2000)	*acoricola* HUSTEDT (HUSTEDT 1935)	*acoricola* var. *elongatirhombica* WYDRZYCKA, LANGE-BERTALOT et METZELTIN (WYDRZYCKA and LANGE-BERTALOT 2001)	*acoricola* var. *lanceolata* HUSTEDT (HUSTEDT 1935, KRAMMER 2000)	*osoresanensis* (NEGORO) FUKUSHIMA, YOSHITAKE et KO-BAYASHI (NEGORO 1944, FUKUSHIMA et al. 2002)	*subacoricola* METZELTIN, LANGE-BERTALOT et GARCÍA-RODRÍGUEZ (METZELTIN et al. 2005)
Valve length (µm)	15.9–24.0	24–40	12–22	15–30	16–47	15–30	7–29	10–30
Valve width (µm)	3–4.1	4.5–7	3–3.3	4–6	4.5–7	7–7.8	3–5.5	3.5–4.5
Valve outline	Narrowly rhombic	Linear with convex sides to linear-elliptical	Linear-lanceolate; straight to weakly convex sides	Elliptico-lanceolate	Rhomboid-lanceolate and larger, gradually attenuated since the central area until the ends	More lanceolate	Linear-elliptic transapical shells	Linear-elliptic to rhombic-lanceolate
Valve apices	Rounded	Protracted	Cuneiform	Rounded	Rounded	Pointed	More or less distended middle blunt rounded ends	Less cuneately or obtusely rounded
Central area	Rhomboid	Rhomboid	Broad fascia	Medium to large circular space or a complete fascia	Medium to large circular space or a complete fascia	Large circular space or more often, a break in the striae reaching to the margin	A slight swelling in the central area	Very large because of striae limitation in the distal parts of the valve

Number of striae in 10 μm	15–18	11–12	13–16	13–15	13–16	12–13	15–19	15
Striation pattern	Striae abruptly changed from strongly convergent at the poles to strongly radial at the center	Transapical striae parallel to slightly radiate in the middle, convergent near the poles	Radiate in the middle and strongly convergent at the ends	Strongly convergent at the poles and strongly radial at the center	Strongly convergent at the poles and strongly radial at the center	Strongly convergent at the poles and strongly radial at the center	Striae transapical radiated in the medium and convergent in the poles	3-7 subpolars pairs of convergent striae and 1-3 pairs of radiate striae
Raphe	Straight; at central nodule bent to one side	Filiform, with distinct, weakly deflected central pores	Filiform, weakly deflected in the middle	Straight, slightly diverting in the central pores	Straight in the larger specimens and curved in the smaller, with central terminations curved to one side	Nearly straight; at central nodule bent to one side	Filiform	Filiform
Ecology	Present in acidic streams with pH variation of 1.9–2.2, high sulphate and metal concentrations (As, Cd, Cu, Fe, Mn, Pb, Zn) and high conductivity due to dissolved ions and not to organic contamination	Quite common in all habitats: relatively wet (>60 %) acid soils to wet mosses and acid pools and lakes with low specific conductance (<90 μS cm^{-1})	Abundant in type locality, an opencast mining lake; low pH water and 99.5 % acidobiontic taxa in association	First time found in Java (pH~3) in sulphated waters; acidophilic taxa that can change form due to high temperatures and acidic waters and in some cases, present in waters with high metal concentrations (e.g. Al, Fe)	Preference of really acidic waters and rich in minerals (sulphuric and chlorhydric acidity); huge variability of valve forms	Optimum pH is 5 but lives in waters of pH 2.6	High-saline Isobe-mineral sources (temperature 15.3 °C, pH 6.5) in the Gumma Prefecture	Different pH values in cave (reduced light regime), far from the sea

3.3. RESULTS AND DISCUSSION

***Pinnularia aljustrelica* Luís, Almeida et Ector**

Fig. 3.2 (2–21): LM, Fig. 3.4 (157–161): SEM

Diagnosis: Valvae anguste lanceolatae ad rhombicae–lanceolatae apicibus non protractis, late rotundatis. Longitudo 15.9-24.0 μm, latitudo 3.0-4.1 μm. Area axialis angusta, linearis. Area centralis rhombica formans fasciam leviter ad moderate latam, rotundam. Raphe recta poris proximalibus unilateraliter deflexis. Fissurae terminales raphis curvatae ad idem latus valvae. Striae transapicales fortiter radiatae in media parte valvae, abrupte valde convergentes ad apices, 15-18 in 10 μm.

Holotype (here designated): slide BR-4230 (National Botanic Garden, Meise, Belgium). Isotype (here designated): slide BRM-ZU8/11 (Friedrich Hustedt Diatom Collection, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany). Type locality: Ponte Monte Ruas, Água Forte stream (Ribeira da Água Forte), municipality of Aljustrel, subregion of Baixo Alentejo (District: Beja), Portugal; collector: Ana T. Luís; collection date: 07/04/2008; geographical coordinates: 4192136.37 N, (29)574774.42 W; Etymology: The specific epithet aljustrelica refers to the name of the type locality.

Light microscopy (Fig. 3.2 (2–21))

Valves narrowly lanceolate to rhombic–lanceolate, gradually tapering towards the broadly rounded, non protracted apices. Valve dimensions of the type population (n = 19): length 15.9–24.0 μm, width 3.0–4.1 μm (**Table 3.2**).

Table 3.2 – Morphometric data of the populations of *Pinnularia aljustrelica* (Água Forte stream). Minimum, maximum (and medium) values for the most important parameters: length, width and number of striae (total n = 110).

Samplings	Spring 2008	Summer 2008	Spring 2009	Winter 2009	Spring 2008	Total 2008 & 2009
Sites	Ponte Monte Ruas (**type**) (n = 19)	Ponte Monte Ruas (n = 24)	Ponte Monte Ruas (n = 18)	Ponte Monte Ruas (n = 30)	Porto de Beja (n = 19)	Ponte Monte Ruas – Porto de Beja (n=110)
Length (μm)	MIN: 15.9 MAX: 24.0 AVG: 20.7	MIN: 11.3 MAX: 25.7 AVG: 19.7	MIN: 14.3 MAX: 24.3 AVG: 18.6	MIN: 11.5 MAX: 20.0 AVG: 17.4	MIN: 14.5 MAX: 21.7 AVG 16.9	MIN: 11.3 MAX: 25.7 AVG: 18.7
Width (μm)	MIN: 3.0 MAX: 4.1 AVG: 3.6	MIN: 3.0 MAX: 3.9 AVG: 3.6	MIN: 3.0 MAX: 3.8 AVG: 3.3	MIN: 3.1 MAX: 3.7 AVG: 3.4	MIN: 3.0 MAX: 3.8 AVG: 3.3	MIN: 3.0 MAX: 4.1 AVG: 3.4
Number of striae in 10 μm	MIN: 15 MAX: 18 AVG: 16	MIN: 14 MAX: 17 AVG: 15	MIN: 16 MAX: 18 AVG: 16	MIN: 15 MAX: 18 AVG: 16	MIN: 16 MAX: 18 AVG: 17	MIN: 14 MAX: 18 AVG: 16

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Axial area narrow, linear. Central area rhomboid forming a small to moderately large, rounded fascia. Raphe straight with unilaterally deflected proximal raphe endings. Transapical striae strongly radiate near the valve centre, changing abruptly to convergent towards the apices, 15–18 in 10 μm. Other populations represented in **Fig. 3.3.**
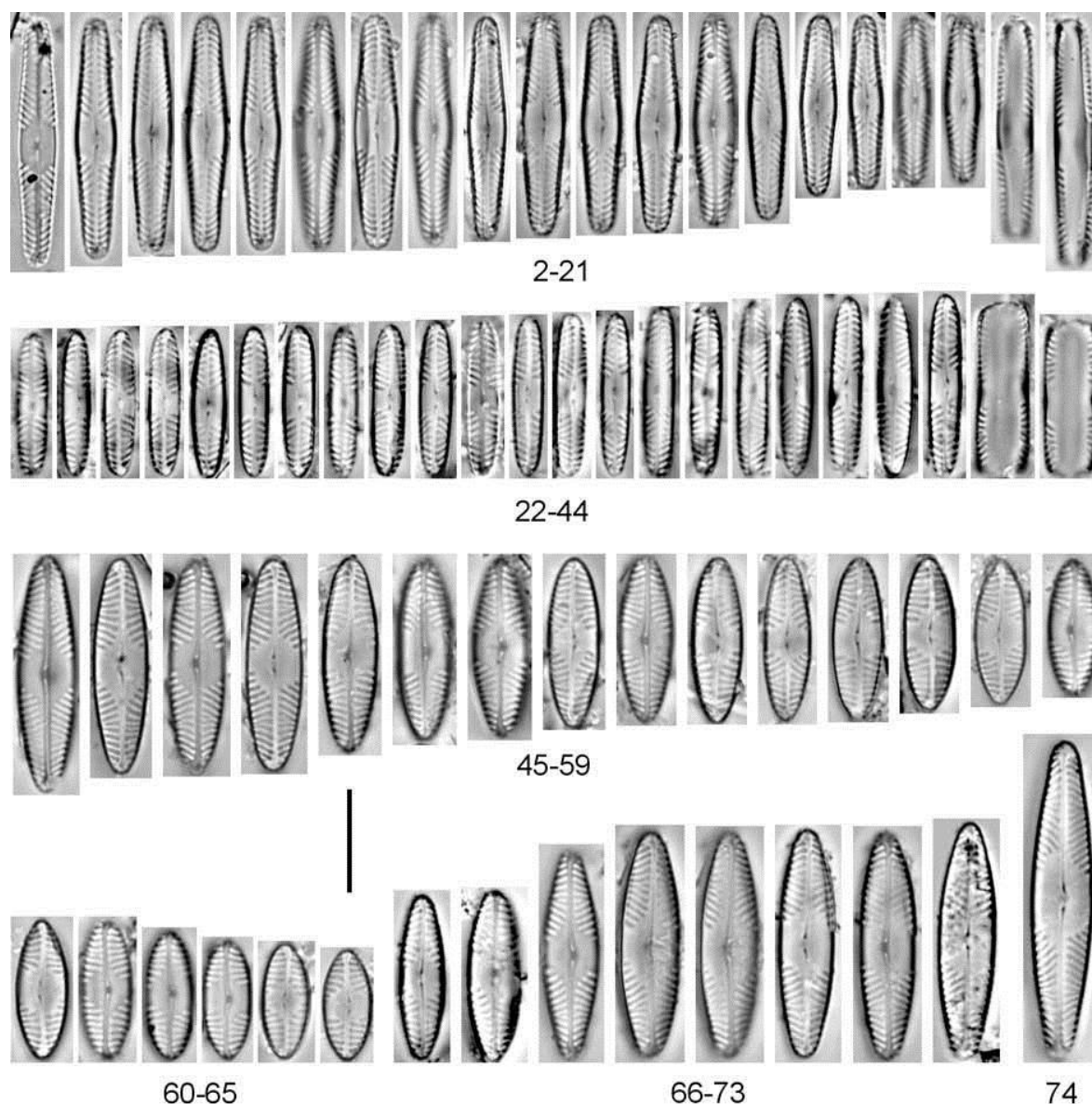
Fig. 3.2 – *Pinnularia aljustrelica* sp. nov. Luís, Almeida et Ector. Light micrographs of the type population sampled in Ponte Monte Ruas in spring 2008, Água Forte stream (Aljustrel, Portugal): (2–19) valvar view; (20–21) girdle view. 22–44: *Pinnularia acidophila* Hofmann et Krammer. Light micrographs of the type population: (22–42) valvar view; (43–44) girdle view. 45–65: *Pinnularia acoricola* Hustedt. Light micrographs of the type population in valvar view. 66–73: *Pinnularia acoricola* Hustedt. Light micrographs, in valvar view (found in the type material of *Pinnularia acoricola* var. *lanceolata* Hustedt). 74: *Pinnularia acoricola* var. *lanceolata* Hustedt. Light micrograph, in valvar view (holotype material). Scale bar 10 μm.

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75-94

95-114

115-136

137-156
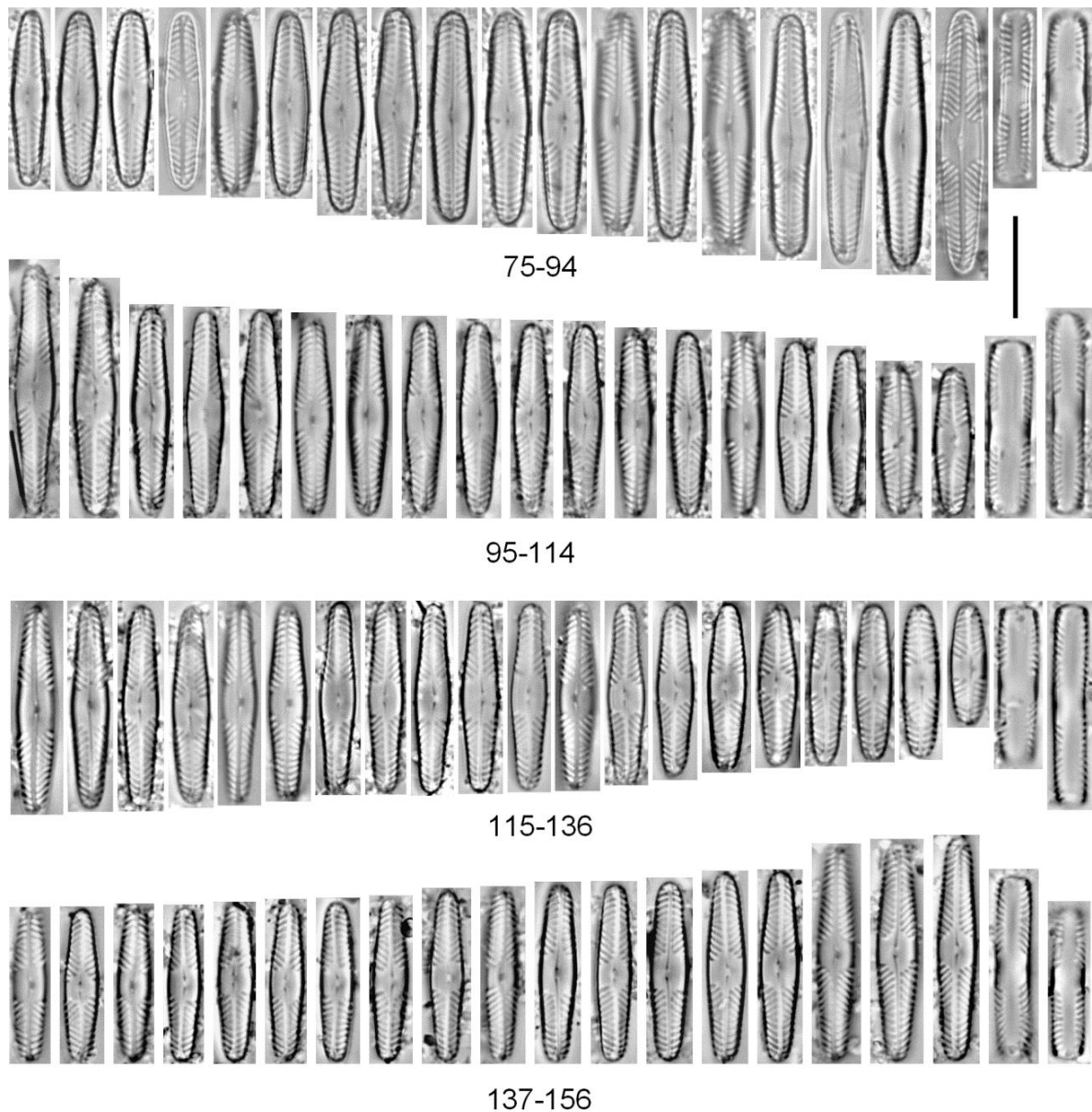
Fig. 3.3 – *Pinnularia aljustrelica* sp. nov. Luís, Almeida et Ector. 75–94: Light micrographs of the population sampled in Ponte Monte Ruas in summer 2008, Água Forte stream (Aljustrel, Portugal): (75–92) valvar view; (93–94) girdle view. 95–114: Light micrographs of the population sampled in Ponte Monte Ruas in spring 2009, Água Forte stream (Aljustrel, Portugal): (95–112) valvar view; (113–114) girdle view. 115–136. Light micrographs of the population sampled in Ponte Monte Ruas in winter 2009, Água Forte stream (Aljustrel, Portugal): (115–133) valvar view; (135–136) girdle view.137–156: Light micrographs of the population sampled in Porto de Beja in spring 2008, Água Forte stream (Aljustrel, Portugal): (137–154) valvar view; (155–156) girdle view. Scale bar 10 μm.

Scanning electron microscopy (Fig. 3.4 (157–161))

Proximal raphe endings weakly expanded, never drop–like (**Fig. 3.4** (159)). The terminal raphe endings are curved towards the secondary side of the valves while the central pores are deflected to the primary side of the valve (**Fig. 3.4** (157)). Each alveolus composed of 3–5 rows of very small, rounded pores, usually externally covered by a membrane (**Fig. 3.4** (160, 161)). Internally, the raphe fissure shows no intermission near

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the central nodule (**Fig. 3.4** (160)) and the distal raphe endings terminate on small helictoglossae (**Fig. 3.4** (161)).
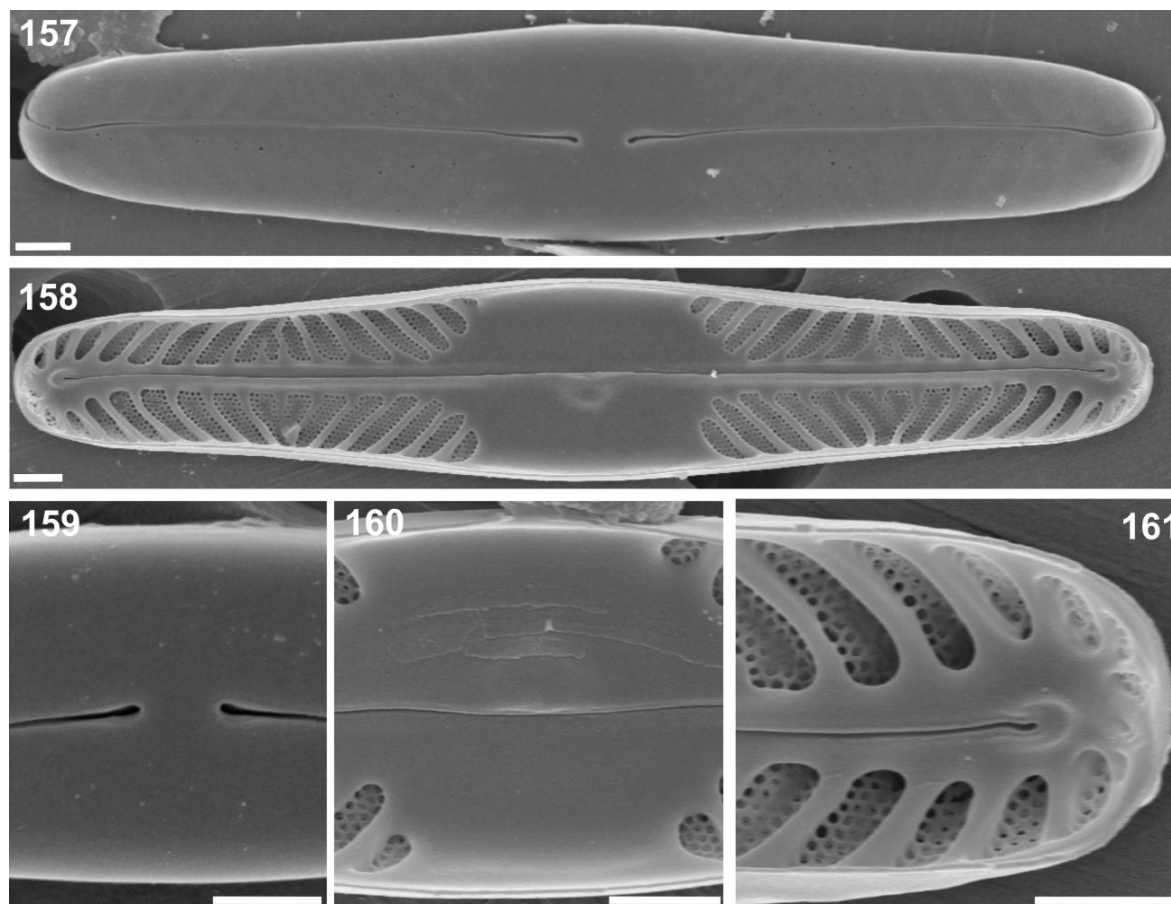
Fig. 3.4 – *Pinnularia aljustrelica* sp. nov. Luís, Almeida et Ector. Scanning electron micrographs of the type population sampled in Ponte Monte Ruas in spring 2008, Água Forte stream (Aljustrel, Portugal): (157) external valve view: terminal fissure curved in the same direction and the terminal raphe endings deflected to the primary side; (158) internal valve view; (159) external view of the raphe with the proximal endings weakly expanded; (160) internally, the raphe fissure shows no intermission near the central nodule; (161) internal view of the apex, showing the distal raphe endings terminating on small helictoglossae, the areolae features of the striae and the structure of the alveoli. Scale bars 1 μm.

Morphometric variability

The type population of *Pinnularia aljustrelica* shows a length range between 15.9 and 24.0 μm, a width of 3.0 to 4.1 μm and a stria density of 15-18 in 10 μm. The other three populations observed at Ponte Monte Ruas presented a slightly different range in valve dimensions (**Table 3.2**).

During the summer of 2008 (**Fig. 3.3** (75-94)), valves showed a length ranging from 11.3 to 25.7 μm, a width of 3.0 to 3.9 μm and a stria density of 14-17 in 10 μm. In spring 2009 (**Fig. 3.3** (95-114)) the valve length varied between 14.3–24.3 μm with a width of 3.0-3.8 μm and 16-18 striae in 10 μm. Finally, in the sample taken in winter 2009 (**Fig 3.3** (115-136), **Fig. 3.7** (173-177)), the observed length varied between 11.5-20.0 μm, while

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the width ranged between 3.1-3.7 µm and the striae were 15-18 in 10 µm. The Porto de Beja population (**Fig 3.3** (137-156)) had a range in length of 14.5 to 21.7 µm, width of 3.0 to 3.8 µm and 16-18 striae in 10 µm. A total of 110 valves were measured for all populations (length of 11.3 to 25.7 µm, width of 3.0 to 4.1 µm, stria density of 14-18 in 10 µm).

Ecology, distribution and associated diatom flora

The ecological preferences of the new *Pinnularia* species were inferred based on environmental variables from two sites of the Água Forte stream in the Aljustrel mining area (Portugal). *Pinnularia aljustrelica* dominates the investigated stream Água Forte. **Table 3.3** shows all environmental parameters. Both sites have highly acidic waters with a rather constant low pH (1.9-2.3), high sulphate levels (16995-36900 mg L^{-1} SO_4^{2-}), high metal concentrations (As: 1.90-52.17 mg L^{-1}; Cd: 1.62-2.48 mg L^{-1}; Cu: 130-348 mg L^{-1}; Fe: 3688-6173 mg L^{-1}; Mn: 66-204 mg L^{-1}; Pb: <0.01-1.20 mg L^{-1}; Zn: 766-1202 mg L^{-1}) and high conductivity (12200-17140 µS cm^{-1}) caused by dissolved ions in the water. The water temperature varied between 9.1-29.2 ⁰C. In total, *P. aljustrelica* was recorded in two sites of the Água Forte stream at five sampling moments with abundances above 91 %.

Table 3.3 – Values of 13 physical and chemical variables and 7 trace metals, for the two sampling sites, in spring and summer of 2008 and spring and winter of 2009 (COD: Chemical Oxygen Demand).

Samplings	Spring 2008 Ponte Monte	Summer 2008 Ponte Monte	Spring 2009 Ponte Monte	Winter 2009 Ponte Monte	Spring 2008 Porto de Beja
pH	1.9	2.3	2.1	2.0	2.2
Conductivity (µS cm^{-1})	17140	14200	12200	14100	12670
Temperature (⁰C)	18.5	29.2	18.6	9.1	21.0
NH_4^+ (mg L^{-1})	0	0	0	0	0
NO_3^- (mg L^{-1})	0	295	0	546	0
PO_4^{3-} (mg l^{-1})	<0.02	0.24	<0.02	2.00	<0.02
COD (mg L^{-1})	722	657	14	30	166
Cl^- (mg L^{-1})	157	184	122	74	144
SO_4^{2-} (mg L^{-1})	28239	16995	22601	36900	20169
Na^+ (mg L^{-1})	63	88	47	40	65
K^+ (mg L^{-1})	<5000	<5	<5000	8	<5000
Mg^{2+} (mg L^{-1})	953	438	668	366	652
Ca^{2+} (mg L^{-1})	539	250	424	434	375
Si^{3+} (mg L^{-1})	89	45	59	34	83
As (mg L^{-1})	48.50	30.00	1.90	52.17	21.70
Cd (mg L^{-1})	2.48	1.70	1.80	2.10	1.62
Cu (mg L^{-1})	348	130	265	216	248
Fe (mg L^{-1})	6173	3688	6157	4842	4543
Mn (mg L^{-1})	204	89	117	66	142
Pb (mg L^{-1})	<0.01	0.01	0.30	1.20	<0.01
Zn (mg L^{-1})	1202	970	766	1112	776

Comparison with related taxa

The most similar taxa to *Pinnularia aljustrelica* are *P. acidophila* and *P. acoricola* (**Table 3.4**).

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Table 3.4 – Morphometric data of the type populations of *Pinnularia aljustrelica*, *P. acidophila* and *P. acoricola.* Minimum, maximum (and medium) values for the most important parameters: length, width and number of striae.

Type material	*Pinnularia aljustrelica* LUÍS, ALMEIDA et ECTOR	*Pinnularia acidophila* HOFMANN et KRAMMER	*Pinnularia acoricola* HUSTEDT
n	19	23	28
Length (µm)	MIN: 15.9 MAX: 24.0 AVG: 20.7	MIN: 13.6 MAX: 17.3 AVG 15.5	MIN: 10.6 MAX: 22.9 AVG: 16.2
Width (µm)	MIN: 3.0 MAX: 4.1 AVG: 3.6	MIN: 2.5 MAX: 3.1 AVG: 2.8	MIN: 4.3 MAX: 5.6 AVG 4.8
Number of striae in 10 µm	MIN: 15 MAX: 18 AVG: 16	MIN: 15 MAX: 18 AVG: 17	MIN: 14 MAX: 18 AVG: 16

The first taxon in this comparison is *P. acidophila* (**Fig 3.2** (22–44), **Fig. 3.5** (162–166)).
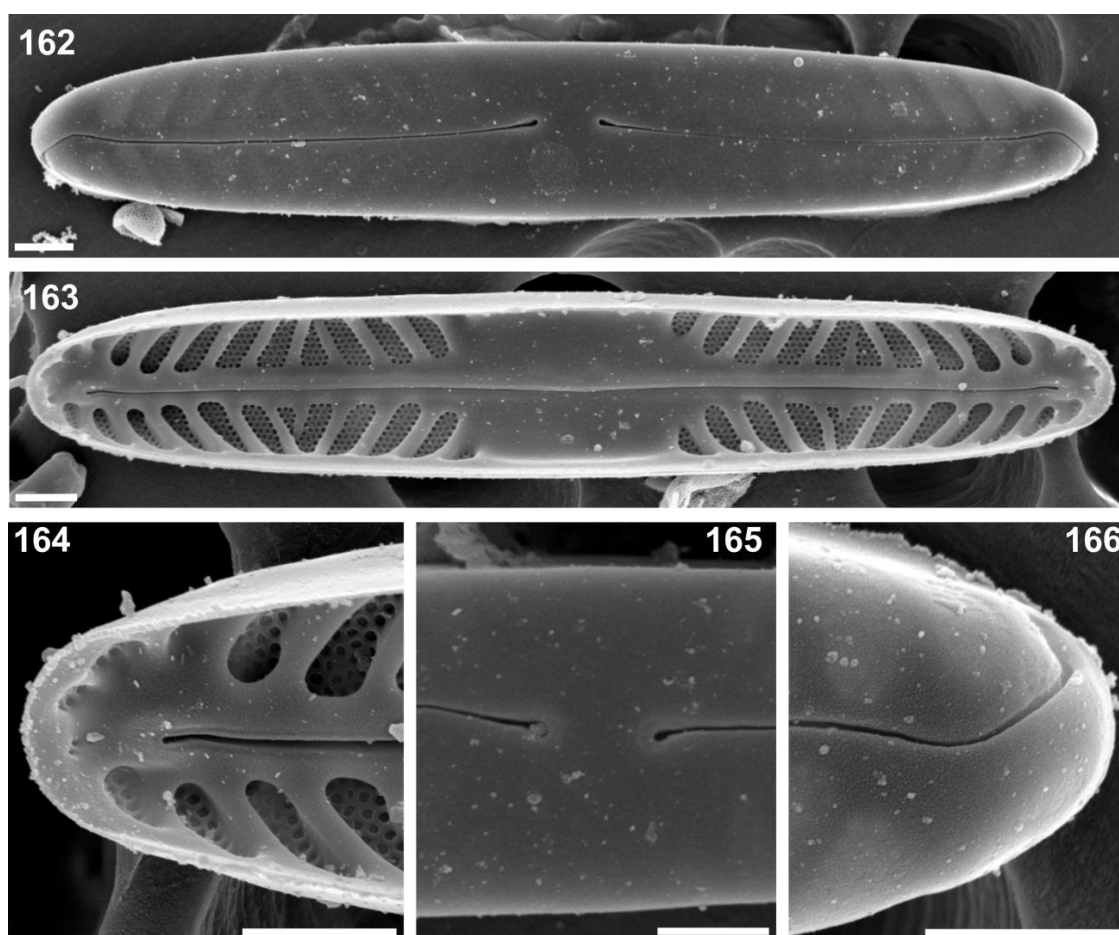
Fig. 3.5 – *Pinnularia acidophila* Hofmann et Krammer. Scanning electron micrographs of the type population: (162) external view of the entire valve; (163) internal view of the entire valve; (164) internal view of the apex, showing the raphe termination, the areolae features of the striae and the structure of the alveoli; (165) external view of the raphe in the central area; (166) external view of the apex, showing the distal raphe ending. Scale bars 1 µm.

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Our analysis of the type material of *P. acidophila* shows some differences in valve length (13.6–17.3 µm), width (2.5–3.1 µm) and number of striae in 10 µm (15–18) compared with the original description of Krammer (2000): 12–22 µm in length, 3.0–3.3 in width, 13–16 striae in 10 µm. The main differences between *P. acidophila* and *P. aljustrelica* include a narrower valve width and a more linear to lanceolate valve outline lacking the larger width in the central area (**Fig 3.2** (22–42)) instead of lanceolate to rhombic shape in *P. aljustrelica* (**Fig 3.2** (2–19)). The valve apices in *P. acidophila* are cuneiform and never rounded, and the raphe is filiform and weakly deflected in the middle. *Pinnularia acoricola* was described in 1935 by Hustedt. Carter emended the original description in 1972 based on the extremely high variability in shape and size of *P. acoricola*: the valves range from lanceolate to oval with a length varying between 8 to 34 µm and a width of 3 to 6 µm. The apices are rounded and the stria density varied between 13 and 17 in 10 µm. Our analyses of the *P. acoricola* type material (**Fig 3.2** (45–65), **Fig. 3.6** (167–172)) revealed a range of 10.6–22.9 µm in length, 4.3–5.6 µm in width and 14–18 striae in 10 µm (**Table 3.4**).
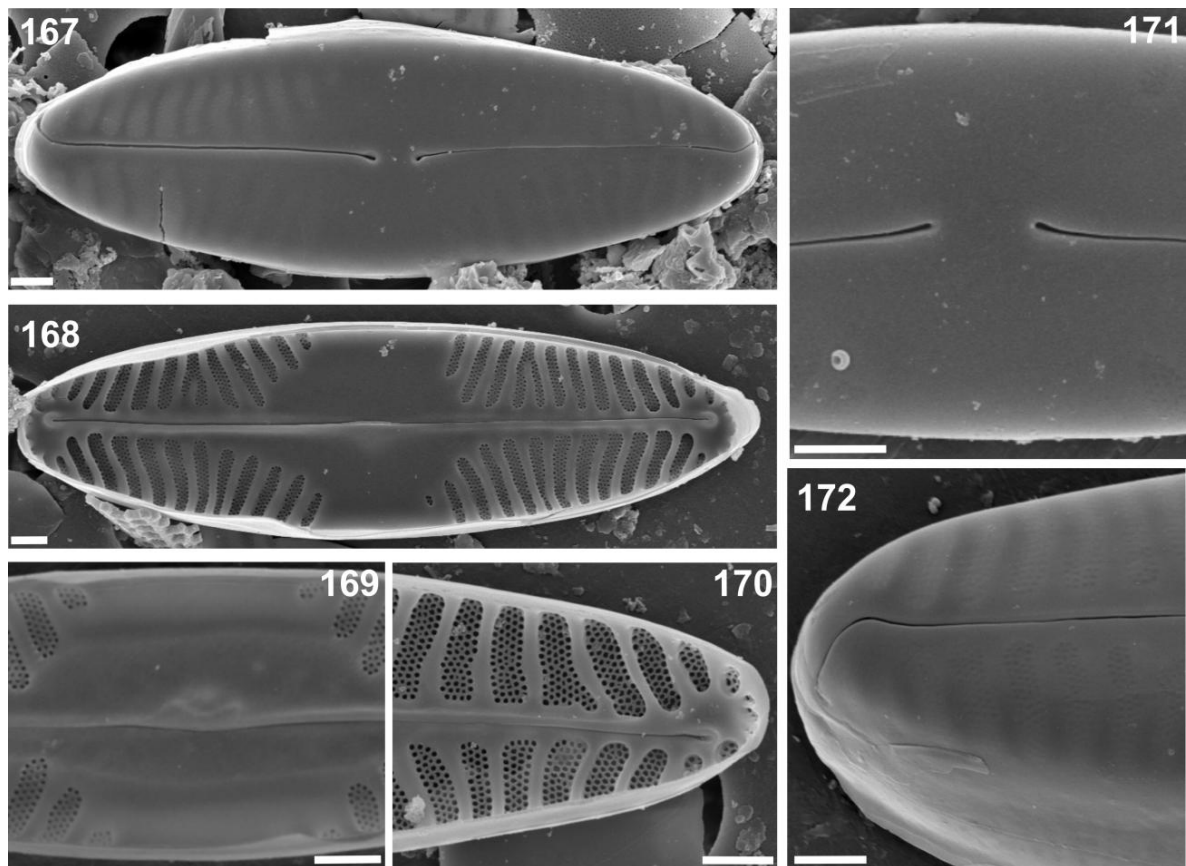
Fig. 3.6 – *Pinnularia acoricola* Hustedt. Scanning electron micrographs of the type population: (167) external view of the entire valve; (168) internal view of the entire valve; (169) internal view of the raphe in the central area; (170) internal view of the apex, showing the raphe termination, the areolae features of the striae and the structure of the alveoli; (171) external view of the raphe in the central area; (172) external view of the apex, showing the distal raphe ending. Scale bars 1 µm.

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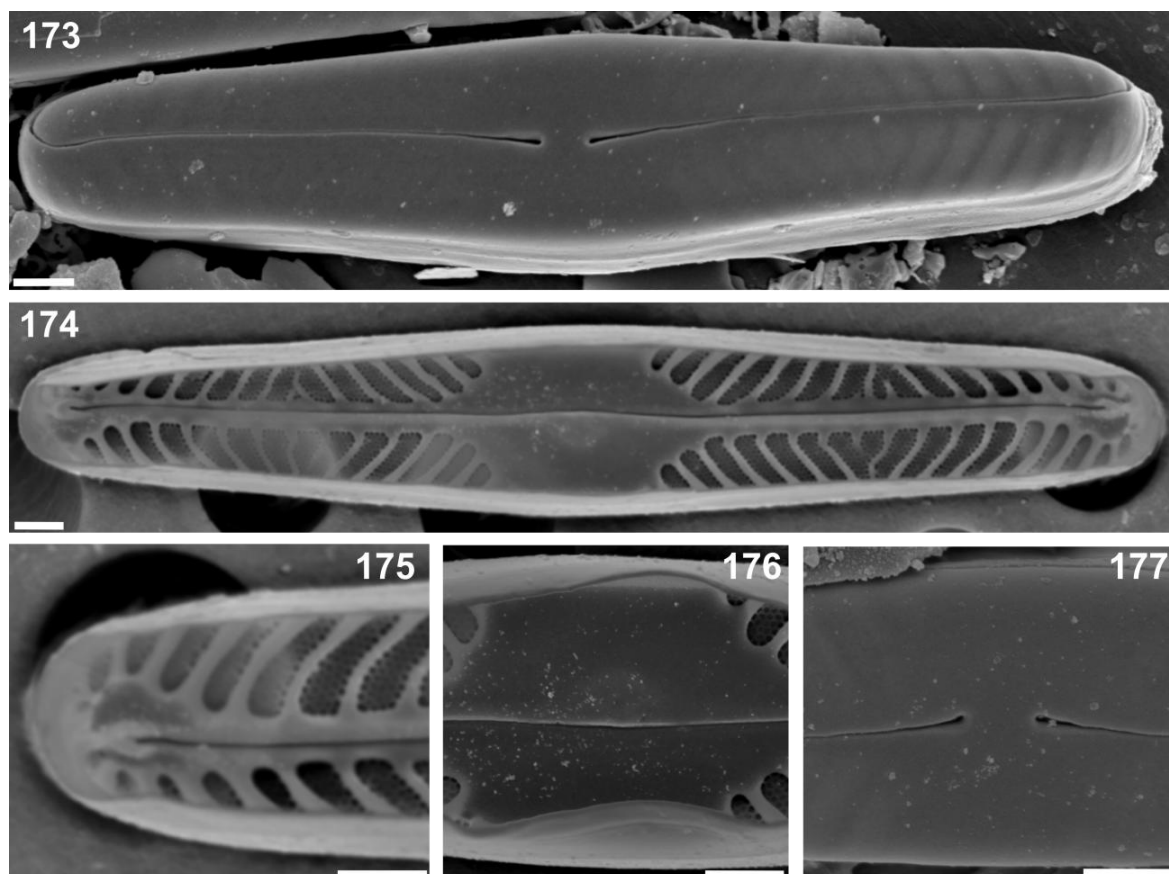
Fig. 3.7 – *Pinnularia aljustrelica* Luís, Almeida et Ector. Scanning electron micrographs of the population sampled in Ponte Monte Ruas site in winter 2009, Água Forte stream (Aljustrel, Portugal): (173) external view of the entire valve; (174) internal view of the entire valve; (175) internal view of the apex, showing the raphe termination and the areolae features of the striae; (176) internal view of the raphe in the central area; (177) external view of the raphe in the central area. Scale bars 1 μm.

Nevertheless, *P. acoricola* differs sufficiently from *P. aljustrelica* by the valve outline. *Pinnularia acoricola* has an elliptic-lanceolate valve outline instead of the more lanceolate to narrowly rhombic–lanceolate valves of *P. aljustrelica* furthermore, the valves are wider than those of *P. aljustrelica*.

Pinnularia acoricola var. *lanceolata* (**Fig. 3.2** (74)), a third taxon used for comparison, recently recombined under the name of *P. acoricolatoba* Kulikovskiy, Lange-Bertalot et Metzeltin (Kulikovskiy et al. 2010), has acutely rounded apices combined with a typical lanceolate valve outline contrary to *P. aljustrelica* that has broadly rounded apices and a more rhombic–lanceolate outline. Moreover, the central area in *P. acoricola* var. *lanceolata* forms a rather elliptically–shaped fascia, a feature never observed in *P. aljustrelica*. *Pinnularia acoricola* var. *lanceolata* valves are larger, wider and have a lower number of striae in 10 μm than *P. aljustrelica* sp. nov. In the type slide of *P. acoricola* var. *lanceolata* we only found one valve (**Fig. 3.2** (74)) that maybe can be identified as *P. acoricola* var. *lanceolata*, which means that the variety is very rare. **Fig. 3.2** (66–73)

represents *P. acoricola* var. *acoricola* valves found on the type slide of *P. acoricola* var. *lanceolata*. Fortunately Simonsen (1987, pl. 254, figs 18–21) could illustrate four valves of *P. acoricola* var. *lanceolata* (Holotype: P1/4, Tobasee, Sumatra, TD4, marked specimens named on the label); this variety differs from the nominate variety of *P. acoricola* by much larger size dimensions, rhombic–lanceolate area and more widely spaced central raphe endings (Kulikovskiy et al. 2010). Several authors (Whitton and Satake 1996, Sabater et al. 2003, Gerhardt et al. 2008, Hobbs et al. 2009, Urrea-Clos and Sabater 2009) reported *P. acoricola* as an abundant taxon in AMD affected sites, the species being often reported as a common taxon in acidic environments all over the world: in Java sulphuric streams with pH of 2.8 to 3.0 (Carter 1972), in Europe with pH of 1.5 to 3 (Whitton and Diaz 1981) and in New Zealand waters with pH below 1 (Cassie and Cooper 1989). Negoro (1985) regularly found this species in acidic habitats in Japan, with pH 2–4 and temperatures of 20.5-46.8 ºC. According to Whitton et al. (2000), *P. acoricola* has been recorded at by far the lowest pH values for any diatom, with records as low as pH 0.2, though critical studies to confirm viability have not yet been made for the lowest pH values. Watanabe and Asai (1995) reported this taxon also from Japan, in sites having a pH below 1.1 whereas Sabater et al. (2003) found this taxon in an acid stream in south–western Spain, the Río Tinto, located in the same geological unit Iberian Pyrite Belt (IPB) as the Aljustrel mining area. Jordan (2001) also reported that *P. acoricola* was found in acidic waters, with a pH optimum around 5, in thermal waters, frequently associated with high iron concentrations. The waters where Negoro (1944) found the species had high concentrations of aluminium and iron. All Great Britain localities, where this species was found, are typically acidic pounds, that, due to mining activity, have high concentrations of copper, iron and zinc (J.R. Carter, pers. comm.). It is highly likely that this acidobiontic species may not always correspond to *P. acoricola* and might have been confused in the past with other *Pinnularia* species.

3.4 ACKNOWLEDGMENTS

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CHAPTER 4

Photosynthetic activity and ecology of benthic diatom communities from streams affected by Acid Mine Drainage (AMD) in pyritic mines

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Abstract The surrounding streams of the Aljustrel mining area (Alentejo Region, Southern Portugal) are strongly affected by acid mine drainage (AMD), as a result of the weathering of the tailing's sulphide minerals. Benthic diatoms (Bacillariophyceae) and water samples were collected seasonally, during one year (2008/09), at 13 sampling sites (5 impacted and 8 unimpacted). Benthic diatom assemblages were studied using Pulse Amplitude Modulated (PAM) fluorometry to assess the effects of the metals/acidity on the photosynthetic efficiency. The information provided by Chl-*a* fluorescence parameters (e.g. α, ETR_{max}) is an early warning signal of biological effects after acute exposure to chemicals, damaging either directly or indirectly Photosystem II (PSII). Statistical analysis (Two-way and Factorial ANOVAs) found significant differences on Chl-*a* fluorescence parameters of impacted and unimpacted sites. Photosynthetic efficiency and biomass (Chl-*a*) were lower for benthic diatom assemblages in impacted streams (Água Forte and Água Azeda), especially during summer, due to very low pH (2.3) and the concentration effect of Cu (348 mg L^{-1}), Fe (6173 mg L^{-1}) and Zn (1728 mg L^{-1}) in waters and sediments. Diatom diversity was low at these sites, mainly dominated by *Pinnularia aljustrelica*, the main contributor to the high similarity between impacted sites (81 % of similarity by SIMPER). This is a typical species from acidic waters with high metal concentrations. Maximum values of Chl-*a* fluorescence parameters (α, ETR_{max}) were found for winter at the unimpacted sites in the Canal da Barrada and Roxo streams due to: 1) higher distance from mine input, 2) pH differences, 3) the existence of brackish/marine species (e.g. *Entomoneis paludosa* and *Haslea spicula*), supported by high ions content in the waters. When the analysis was based on the structure of diatom assemblages dominated by *Achnanthidium minutissimum*, two groups were highlighted: JU-PB and RJ-BF (uncontaminated), indicating seasonal differences. This study confirmed PAM fluorescence as an effective method for the rapid and non-destructive estimation of photosynthetic performance in diatom communities of AMD impacted streams from pyritic mining areas.

Keywords AMD contamination, pyritic mines, diatoms, PAM fluorometry, RLCs, Chl-*a*.

4.1 INTRODUCTION

The streams surrounding the Aljustrel mining area (Alentejo Region, Southern Portugal) are highly affected by acid mine drainage (AMD), as a consequence of the erosion of tailing materials during the long history of mining activity in the region. The presence of chemolithotrophic microorganisms (mainly bacteria) in the waters causes the acceleration of metal sulphides oxidation and dissolution reactions, and also the decrease of water pH and the increase of sulphate contents and dissolved metal concentrations (Nordstrom et al. 1999), especially copper (Cu), iron (Fe) and zinc (Zn) (Luís et al. 2009). Thus, primary producers are exposed to lethal levels of pH and metals that lead to a decrease in species richness and diversity (e.g. Mulholland et al. 1986, Verb and Vis 2000 a). Therefore, communities tend to be restricted to tolerant organisms, which are able to survive in these extreme and variable conditions.

One of the most widespread and tolerant algal group is the diatoms (Bacillariophyceae). They are also well known as excellent bioindicators due to their high sensitivity to changes in environmental conditions (Stevenson and Pan 1999, Blinn and Herbst 2003). In these environments, most species are tolerant to metals. This fact has been reported under field and laboratory conditions several times (e.g. Leland and Carter 1984, Morin et al. 2007). In waters with low pH, metal contamination is often associated with acidification (Dixit et al. 1991), making it difficult to separate the effects on the biota. Diatoms respond to metals and acidity stresses at community level, changing dominant taxa or diversity patterns, as well as at the individual level, decreasing cell size or increasing frustule deformities (Corcoll et al. 2012). In variable climates, such as the Mediterranean (hot summers: 30 °C, cold winters: 12 °C, and short springs and autumns – 1 to 2 months), changes in diatom assemblages may occur both at the structural (i.e. taxonomic composition, diversity patterns) as well as at the physiological level (i.e. photosynthetic activity).

Photosynthetic activity of diatoms can also be affected by contamination. These variations can be monitored using Pulse Amplitude Modulated (PAM) fluorometry, which is a rapid, very sensitive, nondestructive and non-intrusive method, based on the measurement of *in vivo* chlorophyll-*a* (Chl-*a*) fluorescence. Originally introduced by Schreiber et al. (1986) for the study of the photosynthetic processes in terrestrial plants, it was later applied to concentrated microalgae suspensions (Ting and Owens 1992, Hofstraad et al. 1994) and lastly applied to microphytobenthos (Serôdio et al. 1997). PAM fluorometry can be applied to assess the effect of contamination on the photosynthesis

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process, as toxic concentrations of metals and other ions may inhibit electron transport rate (Ralph et al. 2007, Corcoll 2011). PAM has been used in ecotoxicological experiments using biofilms (e.g. García-Meza et al. 2005, Serra et al. 2009 a, Corcoll et al. 2011) and is most appropriate for algal-metal bioassays, especially when the metals affect the photosynthetic efficiency (i.e. Cu) prior to any other physiological processes (e.g. Pérèz et al. 2006). It was recently found that rapid Zn bioaccumulation (after few hours of exposure) by biofilms was related to a decrease of photosynthetic efficiency and an increase of protection mechanisms through the xanthophyll cycle (Corcoll et al. 2012). PAM fluorometry allows the construction of steady-state light-response curves (LC) relating the steady state photosynthetic electron transport rates (ETR) with incident photon irradiance (E) (Genty et al. 1989). Rapid light curves (RLCs) have been frequently adopted (Schreiber et al. 1997, White and Critchley 1999), allowing for the rapid (< 2 min) and non-invasive assessment of the light response of PSII activity (Serôdio et al. 2006). RLCs have been used to study the photosynthetic performance and photoacclimation of other aquatic photosynthetic organisms, such as ice microalgae, seagrasses or corals (Serôdio et al. 2006).

The objectives of this study were to: (1) test the use of PAM fluorometry to detect the effects of AMD on the photosynthetic efficiency of the diatom community from contaminated and uncontaminated sites along a seasonal cycle; and (2) check for effects of AMD on primary productivity (Chl-*a*) and on structural changes of diatom assemblages. For that, diatoms were collected from three different substrata (boulders, plants, sediments) at 13 sampling sites during four different seasons over a 1-year period (2008/09) in the Aljustrel mining area, Portugal.

4.2 STUDY AREA

Located in the Alentejo region in the south of Portugal, the Aljustrel mine (37°52′ 37.90″ N, 8°10′54.64″ W) is one of the main important mining sites in the Portuguese part of the Iberian Pyrite Belt (IPB). Nowadays the exploited pyritic material is used to extract zinc. The hydrographic network is characterized by four local drainage basins: (1) Roxo, main stream (with Canal da Barrada and Xacafre creek as secondary streams); (2) Farrôbo creek, which is affected by the Sto. Antão industrial area, where the ore processing plant, the Estéreis (acid water dam) and the Águas Claras (clean water dam) are located; (3) Água Azeda stream is affected by the São João mining sector (tailings and open pit) and by the São João urban area (domestic effluents); (4) Água Forte stream

is affected by the Algares large brittle pyrite and slag tailings, with an extremely acidic water dam (Água Forte dam) and another dam with clean water (Água Industrial dam). Impacted sites are located in basins 3 and 4 that are the most affected by the mine discharges. However, all these water basins are located downstream of the mining area and are clearly affected by mining activity. **Figure 4.1** shows the distribution of sampling sites in the study area.
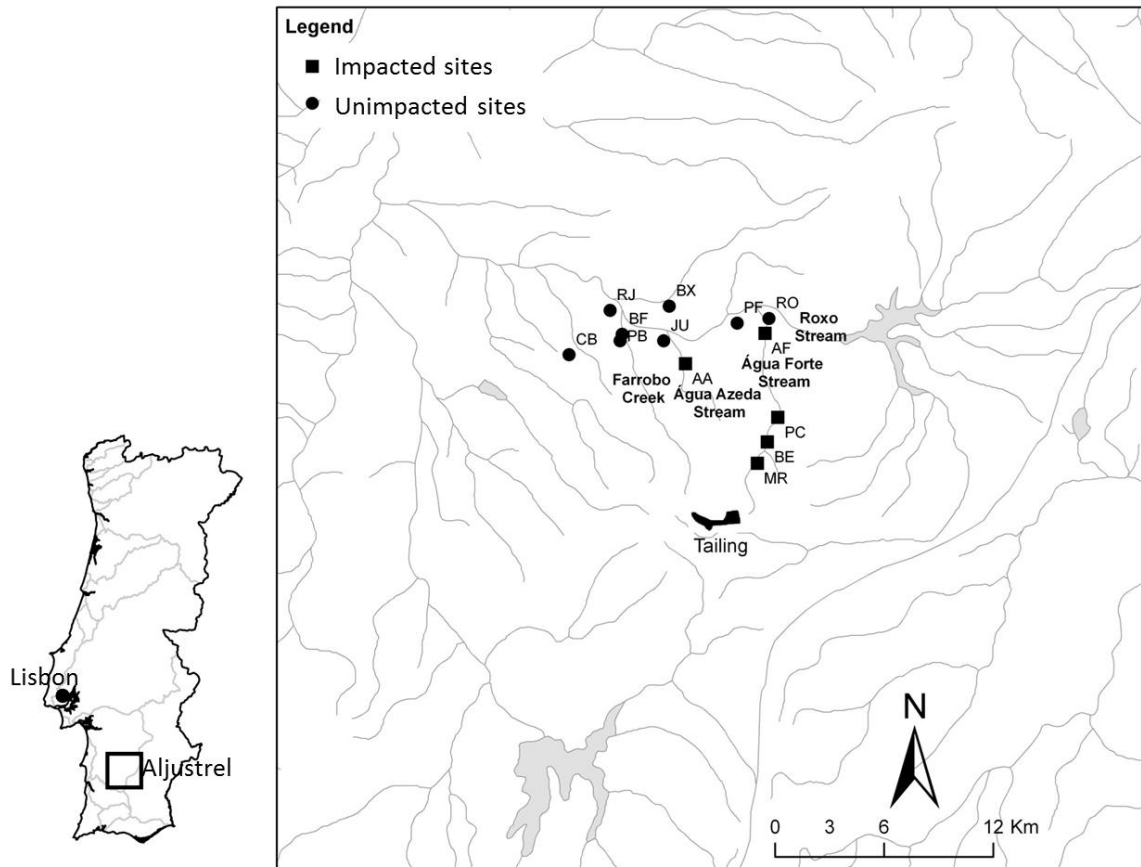
Fig. 4.1 – The location of sampling sites in the study area: Monte Ruas (MR), Porto Beja (BE), Ponte Curval (PC), Água Forte (AF), Água Azeda (AA), Jungeiros (JU), Pero Bonito (PB), Roxo Jusante (RJ), Barranco Farrobo (BF), Porto Ferreira (PF), Roxo (RO), Barranco Xacafre (BX) and Canal da Barrada (CB).

4.3 MATERIAL AND METHODS

Sampling

Diatom communities were sampled in 13 sampling sites: impacted – MR (Monte Ruas), PB (Porto Beja), PC (Ponte Curval), AF (Água Forte), AA (Água Azeda); unimpacted – JU (Jungeiros), PB (Pero Bonito), RJ (Roxo Jusante), BF (Barranco Farrobo), PF (Porto Ferreira), RO (Roxo), BX (Barranco Xacafre) and CB (Canal da Barrada). Sampling was carried out seasonally, during one year (2008/09) and according to national (INAG 2008) and international (European Committee for Standardization 2003)

norms. Epilithic diatom samples were obtained by scraping the upper surface of the boulders with a toothbrush, epiphytic samples were collected by squeezing the plants from the margins of the streams and epipsammic samples were obtained by removing the upper layer of the sediment with a syringe. Pools of stagnant water and shaded sites were avoided. The samples were collected in duplicate, one kept alive and the other preserved with formalin solution (5 %).

Taxonomical analyses

The preserved samples were treated with HNO_3 (65 %) and potassium dichromate ($K_2Cr_2O_7$), at room temperature for 24 hours, in order to eliminate the organic matter and to obtain clean diatom suspensions, followed by three centrifugations (1500 rpm) to rinse the samples. Permanent slides were mounted using the high refractive index (1.74) medium Naphrax® (Brunel Microscopes Ltd, UK). Diatom identifications were taken with a Leitz Biomed 20 EB light microscope using a 100x objective (A.N. 1.32); about 400 valves were counted for each sample. Nomenclature followed Krammer and Lange-Bertalot (1986, 1988, 1991 a, b) and Prygiel and Coste (2000).

Chlorophyll fluorescence – Rapid light curves (RLCs)

In order to characterize the photosynthetic performance of diatoms, Chl-*a* fluorescence was measured in dark-adapted microalgal suspensions using a PAM fluorometer comprising a computer-operated PAM-control unit (Walz, Effeltrich, Germany) and a WATER-EDF-universal emitter-detector unit (Gademann Instruments, Würzburg, Germany). Fluorescence measurements were carried out using a 6-mm diameter Fluid Light Guide fiberoptics bundle connected to a fluorescence cuvette (KS-101, Walz, Effeltrich, Germany) (Serôdio 2004). Rapid Light Curves (RLCs) were constructed by exposing the samples to 8 increasing actinic light levels from 72 to 920 µmol quanta m^{-2} s^{-1} and were carried out applying those light steps during 10 – 20 s and completed within 1.5 – 2 min. The relative electron transport rate (ETR) was calculated for each level of actinic light, using the delivered actinic irradiance (E) and the effective quantum yield of PSII ($\Delta F/Fm'$) by ETR = E * $\Delta F/F_m$' (Serôdio et al. 2005). The light response of microalgae was characterized by fitting the model of Platt et al. (1980) to ETR *versus* E curves and by estimating α, the initial slope of the light curve, and the maximum relative electron transport rate (ETR_{max}) of the ETR *vs* E curve. The model was fitted iteratively using MS Excel Solver and the curve fitted well (r > 0.90) in all cases.

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Biomass

Chl-*a* was extracted from sediment samples in 90 % aqueous acetone and quantified spectrophotometrically following the method of Lorenzen (1967), adapted to sediment samples by Plante-Cuny (1974).

Statistical analysis

A factorial ANOVA was carried out to test the effect of season (spring, summer, autumn, winter) and site (impacted or unimpacted) on Chl-*a* fluorescence parameters (α and ETR_{max}). A two-way ANOVA was performed to test the effect of season (summer, spring, autumn and winter) and site (MR, PC, AF, AA) on α and ETR_{max} within the impacted samples. A two-way ANOVA was performed to test the effect of season (summer, spring, autumn and winter) and site (JU, PB, RJ, BF, PF, RO, BX, CB) on α and ETR_{max} within all the unimpacted samples. A two-way ANOVA was performed to test the effect of season (summer, spring, autumn and winter) and site (JU, PB, RJ, BF) on α and ETR_{max} within the unimpacted samples with a common species. Prior to analysis, assumptions were verified and data transformed when necessary. Tukey's test was used for post hoc comparison of the main effects when significant differences were found. ANOVAs were performed on STATISTICA (v8, StatSoft Inc, USA) and following Sokal and Rohlf (1981).

Statistical differences between species of diatom communities of impacted and unimpacted sites were tested with a distance-based permutational multivariate analysis of variance, PERMANOVA test (Primer 6; Primer-E Ltd, Plymouth, UK). Hierarchical agglomerative clustering analysis was performed on a Bray-Curtis similarity matrix in order to visualize the differences. SIMPER analysis was used to determine the species responsible for the largest contribution to the Bray-Curtis dissimilarity in diatoms abundance between impacted and unimpacted sites.

4.4 RESULTS

Table 4.1 shows the physical characteristics and physicochemical parameters of waters from the 13 sampling sites, measured during a one year period (2008/09).

Table 4.1 – Physical characteristics (water colour, stream vegetation, substrate type) and physico-chemical (As, Cd, Cu, Fe, Mn, Zn, SO_4^{2-}, pH, conductivity) parameters of waters from the 13 sampling sites, measured during one year period (2008/09). Maximum and minimum values of metals and SO_4^{2-} are expressed in mg L^{-1} and conductivity in µs cm^{-1}.

Site name	Site abbr	Water colour	Stream Veget	Substrate type	As	Cd	Cu	Fe	Mn	Zn	SO_4^{2-}	pH	Conductivity
Monte Ruas	MR	Red (dissolved Fe)	No	Sediment	1.3-52.2	1.7-3.5	130-348	1592-6173	66-203	970-1728	17049-36900	1.9-2.3	14100-17140
Porto Beja	BE	Red (dissolved Fe)	No	Sediment	4.9-41.7	1.2-2.0	155-248	1834-4542	59-142	633-1047	11135-39124	1.9-2.2	7700-13200
Ponte Curval	PC	Yellow (FeOH deposits)	Yes	Sediment	0.010-4.8	0.02-0.5	0.021-99	0.01-2104	2.4-11.4	0.32-341	405-4054	2.8-6.5	1898-5000
Água Forte	AF	Yellow (FeOH deposits)	Yes	Sediment	0.004-0.011	0.04-0.14	3.2-16.6	10.0-97.7	5.4-15.4	23.5-57.4	733-2064	2.6-3.3	1764-3670
Água Azeda	AA	Yellow (FeOH deposits)	Yes	Sediment	0.002-0.012	0.03-0.1	0.7-2.1	0.01-1.9	6.2-14.9	11.5-30.4	11526-30449	4.2-5.3	1870-3180
Jungeiros	JU	Clean	Yes	Sediment	0.002-0.008	0.002-0.027	0.012-0.102	0.01-0.23	1.0-4.1	0.18-14.9	347-546	6.1-6.8	1800-2430
Pero Bonito	PB	Grey	Yes	Boulder	0.007-0.010	d.l.	0.002-0.011	0.010-0.05	0.7-1.3	0.001-0.031	207-292	7.7-8.4	2700-3810
Roxo Jusante	RJ	Clean	Yes	Sediment/ Plant	0.002-0.011	d.l.	0.030-0.170	0.010-2.10	0.04-3.7	0.4-4.7	302-560	6.2-6.8	1720-2350
Barranco Farrobo	BF	Clean	Yes	Sediment	0.004-0.010	d.l.	0.001-0.016	0.01-0.08	0.013-1.5	0.001-0.03	10-28	8.2-8.4	2250-2650
Porto Ferreira	PF	Clean	Yes	Sediment/ Plant	0.004-0.006	d.l.	0.001-0.008	0.01-0.07	0.01-0.1	0.001-0.2	46-66	7.4-8.0	1920-2510
Roxo	RO	Yellowish	Yes	Sediment/ Plant	0.003-0.007	d.l.	0.001-0.002	0.01-0.04	0.06-0.35	0.0005-0.01	38-67	7.3-8.0	1412-1530
Barranco Xacafre	BX	Yellowish	Yes	Sediment/ Plant	0.003-0.009	d.l.	0.001-0.005	0.01-0.08	0.001-0.1	0.001-0.01	60-91	7.6-8.2	1850-3690
Canal Barrada	CB	Yellowish	Yes	Sediment	0.006-0.012	d.l.	0.001-0.012	0.01-0.47	0.06-1.1	0.005-0.02	84-154	6.8-7.7	3890-5290

Site abbr, Site abbreviation; Stream Veget, Stream vegetation. When YES, it is composed mainly by *Juncus effusus*; Impacted sites in dark grey colour; Unimpacted sites in light grey colour; the substrate used in each sampling site was one of the one or two available in column 5; d.l.- detection limit.

Variation of RLCs with season and AMD impact

A clear seasonal pattern was observed in RLCs measured on all diatom samples (**Fig. 4.2**). Variations in RLC pattern were observed between seasons for both, α and ETR_{max}. The exception was site CB, unimpacted, where almost no oscillations on the pattern were observed throughout the year (curves overlapped, **Fig. 4.2**, CB).
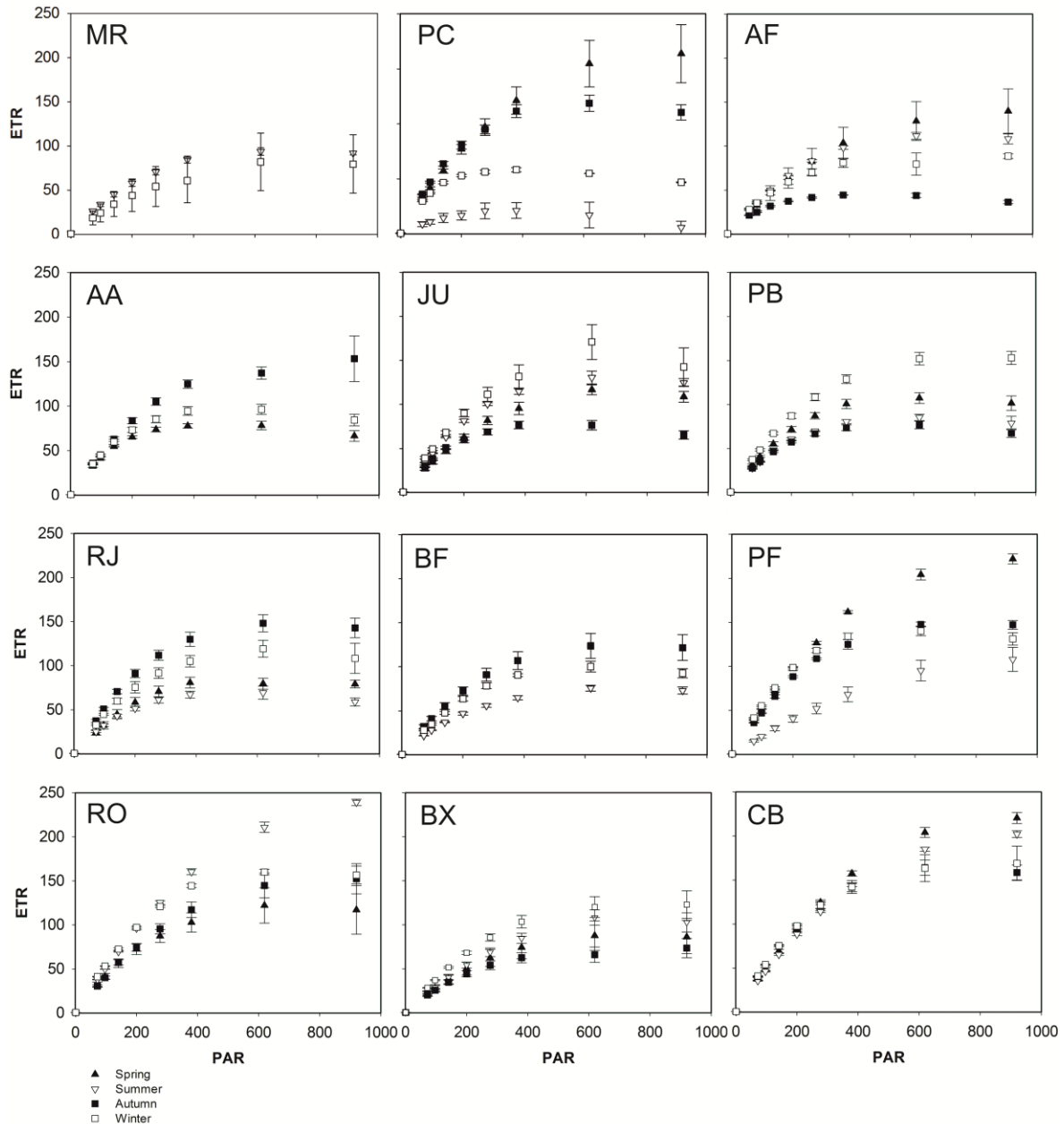
Fig. 4.2 – Variation of rapid light curves (RLCs) with site and season. Impacted sites are MR, PC, AF, AA and unimpacted are JU, PB, RJ, BF, PF, RO, BX, CB. Error bars represent one standard error. For key to site abbreviations see **Table 4.1**.

Values per season of Chl-*a* fluorescence parameters (α and ETR_{max}) are shown in **Table 4.2**, and were often higher in unimpacted sites than in the impacted ones. In

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general, the lowest values of α belonged to summer and the highest to winter. However, no specific seasonal pattern, for ETR_{max}, in the unimpacted sites was observed. It was not possible to measure RLC in the samples of site BE (impacted site).

Table 4.2 – Variation of RLC parameters, α and ETRmax, with site and season (2008/2009); impacted sites in dark grey color, unimpacted sites in light grey color.

	α				ETR_{max}			
Sites	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
MR	-	0.39	-	0.28	-	96.00	-	81.00
BE	-	-	-	-	-	-	-	-
PC	0.48	0.17	0.56	0.43	168.01	20.30	121.60	63.86
AF	0.39	0.44	0.29	0.40	140.61	113.27	47.92	89.82
AA	0.49	-	0.53	0.54	84.34	-	139.90	100.84
JU	0.42	0.54	0.45	0.61	115.36	132.03	80.84	157.97
PB	0.49	0.42	0.42	0.57	115.11	88.18	79.56	115.80
RJ	0.40	0.38	0.61	0.52	86.13	71.72	150.11	119.06
BF	-	0.31	0.48	0.43	-	76.35	126.08	100.58
PF	0.24	0.60	0.57	0.68	116.50	224.67	151.06	145.40
RO	0.49	0.58	0.47	0.65	128.51	246.74	153.61	163.97
BX	0.33	0.35	0.29	0.59	89.50	106.97	72.90	167.00
CB	0.59	0.54	0.65	0.64	225.43	204.87	165.54	172.20

Table 4.3 shows the results for the factorial ANOVA of the effects of season (Se) and site (Si) on Chl-*a* fluorescence parameters (α and ETR_{max}).

Table 4.3 – Factorial ANOVA of the effects of season (Se) and site (Si) on Chl*a* fluorescence parameters (α and ETR_{max}).

Source of variation	α				ETR_{max}			
	df	MS	*F*	p	df	MS	*F*	p
Effect								
Se	3	0.072	6.313	0.001	3	6.60	1.456	0.231
Si	1	0.125	10.887	0.001	1	99.31	21.907	<0.001
Interaction								
Se*Si	3	0.047	4.147	0.008	3	27.66	6.101	<0.001
Error	118	0.011	-	-	109	4.53	-	

There was a significant effect of both season and site on α (**Table 4.3**, *p* = 0.001). Moreover, interaction between season and site was significant on the initial slope of the light curve generated by diatom samples (**Table 4.3**, *p* < 0.01). Considering the significant effect of season or the interaction between season and site on α, posthoc comparison showed mainly differences between winter and the other seasons (Tukey's test: *p < 0.05*).

The maximum relative electron transport rate (ETR_{max}) was significantly lower in impacted sites and also varied with the interaction between season and site (**Table 4.3**, *p* < 0.001). During the year, ETR_{max} did not vary with season (Table **4.3**, *p* = 0.231), however for impacted sites, effects of season were registered between summer and spring (Tukey's test: *p < 0.05*). For all the unimpacted sites, ETR_{max} did not vary significantly between seasons (Tukeys' test: *p* > 0.05). Different statistical results were

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found for site (two-way ANOVA: $p < 0.001$), showing that electron transport rate was higher in unimpacted sites such as PF, RO and CB. No statistical differences were found in either α or ETR_{max} (two-way ANOVA: $p = 0.081$) within the impacted sites (MR, PC, AF and AA). No photosynthetic activity was observed in samples of site BE (impacted site).

ETR_{max} and α variation in the unimpacted sites with a common species

Two groups of sites, JU-PB and RJ-BF, stood out in the statistical analysis, considering the factor "common species" *Achnanthidium minutissimum*. Highly significant differences in α among season (two-way ANOVA: $p < 0.001$) and site (two-way ANOVA: $p < 0.01$) were found. However, changes in ETR_{max} were only significant among seasons (two-way ANOVA: $p < 0.01$, winter and summer).

Differences in diatom communities between impacted and unimpacted sites

A quantitative analysis of the diatom data, PERMANOVA showed highly significant differences in species composition between impacted and unimpacted sites (Pseudo-F = 12.561, $p < 0.001$, df = 1) and cluster analysis also showed this clear separation (**Fig. 4.3**). The samples of impacted sites were generally put together in the right part of the cluster, in contrast with the left side, which grouped the samples of the unimpacted sites.
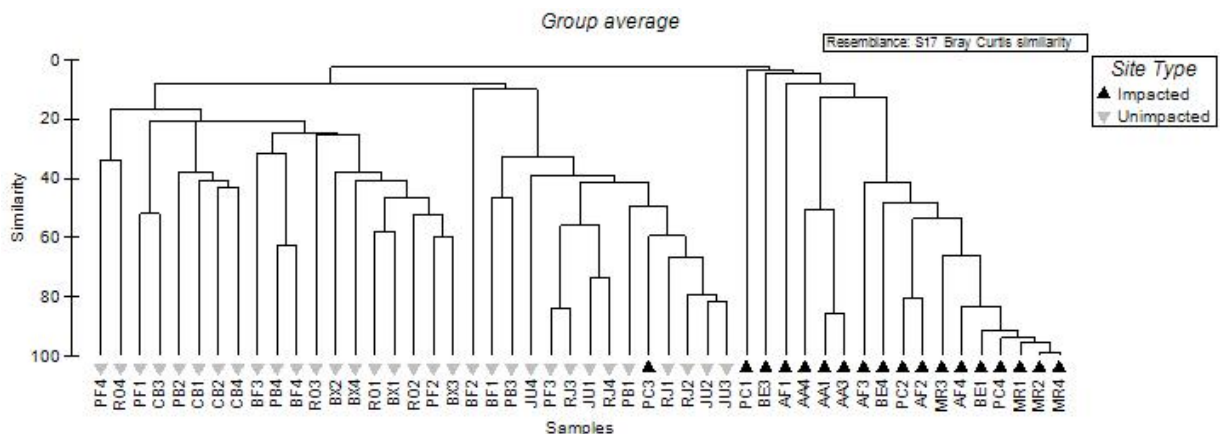
Fig. 4.3 – Dendrogram from Bray-Curtis cluster analysis of diatoms species abundances (no transformation) of impacted and unimpacted sites. For key to site abbreviations see **Table 4.1**.

Contaminated sites (MR, BE, PC, AF, AA) were separated from unimpacted samples except for the site PC, where PC3 (autumn) is located together with the samples from uncontaminated sites (RJ and PB). Moreover, contaminated sites showed low diversity of diatom taxa, compared with unimpacted ones. AMD affected sites were dominated by typical species of acidic waters (Luís et al. 2009, Luís et al. 2012) such as *Pinnularia aljustrelica* Luís, Almeida et Ector (Luís et al. 2012) (dominant in sites MR, BE, PC and AF), *Eunotia exigua* (Brebisson ex Kützing) Rabenhorst (dominant in site AF) and

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Nitzschia hantzschiana Rabenhorst (dominant in site AA). This was also confirmed with SIMPER analysis, which showed that *P. aljustrelica*, contributed ca. 81 % to the similarity between impacted sites. SIMPER analysis also indicated this species as making the largest contributions to the dissimilarity in abundance between sites, considering the estimated average dissimilarity of 97.05 %. Comparison of impacted and unimpacted sites showed that besides *P. aljustrelica* (24 %), *Achnanthidium minutissimum* Kützing (Czarnecki) and *N. hantzschiana* each contributed ca. 10 % to the dissimilarity. These three species as well as the contribution of *Navicula gregaria* Donkin (5 %) and *E. exigua* (3.5 %) made up more than 50 % of the cumulative contribution to the differences between impacted and unimpacted sites.

The most different unimpacted sites, namely CB and PF, presented similar dominant species in all seasons: *Entomoneis paludosa* (W. Smith) Reimer, *Nitzschia desertorum* Hustedt, *Haslea spicula* (Hickie) Bukhtiyarova*, Nitzschia capitellata* Hustedt in A. Schmidt et al. and *Nitzschia palea* (Kützing) W. Smith. The first three species are typical of brackish waters. *N. gregaria* (also a brackish taxon) was the dominant (and almost unique) species at site RO.

Biomass

Chl-*a* presented irregular seasonal variation throughout the year for all sites (**Fig. 4.4**).
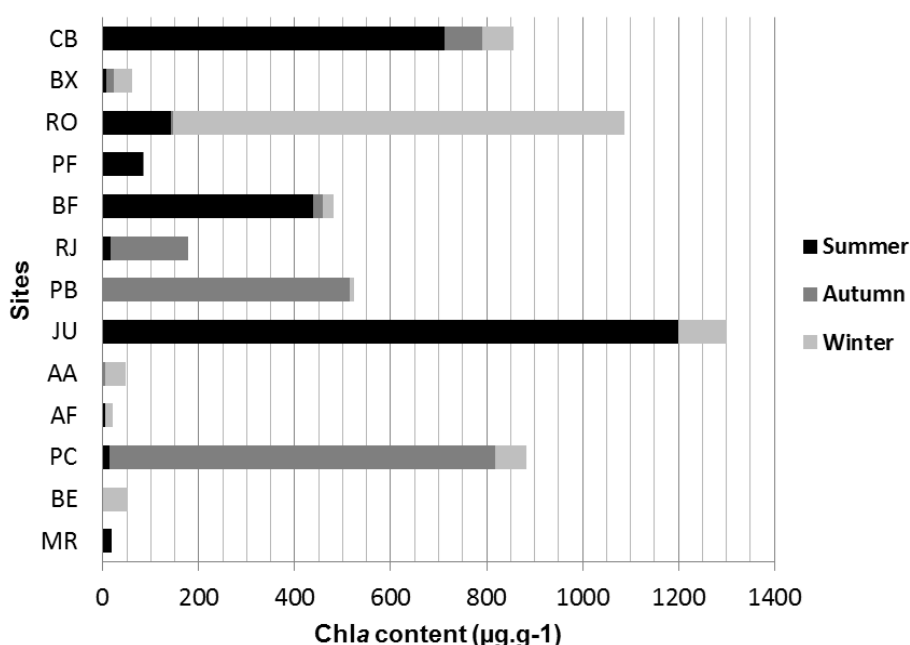
Fig. 4.4 – Seasonal chlorophyll-*a* (Chl-*a*) content (μg g^{-1} of dry sediment) of sediment samples from the 13 sampling sites. For key to site abbreviations see **Table 4.1**.

In the five impacted sites (MR, BE, PC, AF, AA), Chl-*a* ranged between 5.41 µg g^{-1} to 65.74 µg g^{-1} (site PC presented an atypical high value of 804 µg g^{-1} in autumn). Thus at the impacted sites, low algal biomass (reported as Chl-*a*), typical of AMD streams, was found and the lowest values were registered in summer. Whereas in the unimpacted ones, it ranged between 2.93 µg g^{-1} to 1200 µg g^{-1}, with the highest values registered in summer, at sites JU, BF and CB and in autumn in sites PC, PB and RJ. RO was the only site with a high value during winter.

4.5 DISCUSSION

The parameters α and ETR$_{max}$ were both analyzed in order to evaluate the global changes in the photosynthetic activity of the diatom communities in AMD impacted and unimpacted sites. α quantifies the efficiency of light capture and ETR$_{max}$ expresses the maximum photosynthetic capacity, obtained when the photosynthetic rate is limited by the activity of the electron transport chain or Calvin cycle enzymes (Ralph and Gademann 2005, Serôdio et al. 2006).

Diatom assemblages showed significant variations in photosynthetic performance, considering the effect of season or site. Both light-limited and light-saturated parts of the RLCs responded markedly to changes in ambient irradiance. The increase in E was followed by a decrease in α, evident as a decline in the light limited ETR values of the RLCs measured following the exposure to high irradiances.

Regarding α, significant differences were observed among sites and seasons; winter was the most different. The significant interaction between site-season indicates that changes in Chl-*a* fluorescence parameters are regulated mainly by the combination of these two factors. In winter, more adapted communities (high α values) related to decreases in the effects of metals and acidity caused by heavy rain (IPMA 2009; and personal observation/ field data) were found. In contrast, summer was the season with the lowest α values, especially in the impacted sites, due to high light conditions. Light is a key factor for algal growth and consequently for algal biomass. However, negative responses to high light conditions (in summer) have been frequently reported for microalgae in both planktonic and benthic environments (Sigee 2005). Other studies have also shown a decrease in photosynthetic efficiency of diatoms as a photoprotection mechanism (Coelho et al. 2011). High metal concentrations and acidity in waters and stream sediments (mainly FeOH deposits) due to concentration effect of these stressors in summer, also affected the epipsammic diatom communities, the most frequently

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analyzed in this study. In algae, almost all Chl-*a* fluorescence is associated to PSII (Falkowski and Kiefer 1985), thus the decrease of photosynthetic efficiency of diatom communities indicates the inactivation of parts of the PSII reaction centers (García-Meza et al. 2005).

In our study, this may be a result of the exposure of diatom assemblages to acidity and Cu, Fe and Zn. These are the main metals present in waters from impacted sites, such as MR, BE, PC, AF or AA (**Table 4.1**). In this context, Serra et al. (2009 b) determined ETR to evaluate the photosynthetic performance decrease of biofilms during a Cu retention experiment. Temporary inhibition of ETR was also observed by Corcoll et al. (2011) after a few hours of Zn exposure, which induced the reduction of the photosynthetic efficiency (Rijstenbil et al.1994, Corcoll et al 2012). According to this study and related studies (e.g. Corcoll et al. 2012), Chl-*a* fluorescence parameters and photosynthetic pigments of biofilms are suitable as functional endpoints of early toxicity or acute exposure. However, long-term exposure leads to community adaptation, mainly by changes in species composition (Serra et al. 2009 a), which might not be reflected in the photosynthetic efficiency. Moreover, it could also influence other functional processes as seen in streams affected by other sources of acidity, namely organic matter processing or nutrient uptake (Niyogi et al. 1999, Simon et al. 2009). No significant differences were registered for α during our study for the five mine impacted sites, which implies that the chemical differences (spatial or temporal) were not sufficient to differentiate diatom responses at this level. This may also suggest that benthic diatom assemblages from those AMD affected streams are adapted, which is indicative of chronic adaptation to acidic and metal contamination. However, site BE showed a particular behavior. Despite having a stable community mainly composed by *Pinnularia aljustrelica*, no fluorescence was registered from the collected samples. It may indicate that photosynthetic efficiency was too low to be detected. In general, the impacted sites showed the lowest Chl*a* contents, which means that algal biomass (measured as Chl-*a*) was lower in all AMD affected sites than in the unimpacted sites. This difference was already reported by other authors (Mulholland et al. 1986), who have also pointed out that the observed response was more complex than a simple biomass reduction with the increasing severity of AMD contamination (Smucker and Vis 2011). Lower Chl-*a* content may also indicate that sites were dominated by Cyanobacteria, which have already been reported as more resistant to metals exposure than diatoms (Ivorra et al. 2000, Corcoll et al. 2012).

Regarding, ETR_{max}, seasonal important differences between spring and summer for the impacted sites were verified. The highest values of ETR_{max} were displayed in spring

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and the lowest in summer (**Table 4.2**). Diatom blooms tend to be very seasonal and are most likely to occur in the spring. In this study, spring communities even if dominated by a small number of taxa (e.g. *P. aljustrelica*, *Eunotia exigua*) showed higher abundances of individuals within these species than summer samples. Electron transport rate was higher in unimpacted sites, like PF, RO and CB, which was supported by ETR curves and higher values of ETR_{max}.

These results can be related to several factors, namely: water pH, conductivity or species composition. The water pH is similar in unimpacted sites, such as PF, RO and CB (**Table 4.1**), but different from the other unimpacted sites and from impacted ones. PF, RO and CB particularly, were characterized by slightly alkaline waters (pH: 6.8 – 8.0). Moreover, sites PB, BF and BX were also characterized by more alkaline waters (7.6 – 8.4), while sites such as PC, AF and AA (impacted) were characterized by acidic waters (pH: 2.6 – 5.3) (**Table 4.1**). On the other hand, high conductivity was also observed in unimpacted sites such as PF, RO, but especially in the site CB (3890 – 5290 $\mu S\ cm^{-1}$). Moreover, high conductivity also affected species composition. The dominance of brackish to marine species such as *Entomoneis paludosa* (Patrick and Reimer 1975), *Haslea spicula* (Gell et al. 2007, Krammer and Lange-Bertalot 1986), *Nitzschia desertorum* (Hustedt 1979 in Simonsen 1987) and *Navicula gregaria* Donkin (Donkin 1861) was supported by the occurrence of high conductivities (caused by the presence of dissolved ions in waters, especially SO_4^{2-}). Thus, *Entomoneis* is a large marine epipelic genus, with just a few representative species from fresh and brackish inland waters. One of the most representative species of this genus is *E. paludosa* (W. Smith) Reimer in Patrick and Reimer (1975) considered by Hustedt (1957) as mesohalobe, often found in waters with moderate to high conductivities. This taxon was already reported in Portugal in the Tagus estuary by Ribeiro et al. (2003). *H. spicula* was reported as abundant in core sediments post-1960 of lakes, which might be due to an increase in sulphur salt concentrations (Gell et al. 2007) and also to high conductivity. In Krammer and Lange-Bertalot (1986) it is described as cosmopolitan along the seacoast and freshwater courses with high electrolytic contents. *N. desertorum* is also brackish and typical of waters with organic contamination (Gasse 1986). *N. gregaria* is also a brackish taxon (Potapova 2011) capable of colonizing freshwaters as well. Sites CB, PF and RO were located upstream of the acid mine drainage input which can also be another important factor discriminating these three sites. pH, conductivity and mine input distance are determinant in the species composition (in this case, brackish to marine species), which will then also affect the photosynthetic efficiency.

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Cluster analysis separated contaminated sites (MR, BE, PC, AF, AA) from unimpacted sites, except for the site PC3 (autumn sample) which is located together with the samples from uncontaminated sites (RJ and PB) due to *Achnanthidium minutissimum* (ADMI) dominance (53 %). However, PC3 presented also *P. aljustrelica* (35 %), the typical species of acidic waters (Luís et al. 2009, 2012), dominant in the sites MR, BE, PC and AF of Água Forte stream. The ecology of this species is similar to *Pinnularia acoricola*'s but morphological differences were found that discriminates it from *P. acoricola* (Luís et al. 2012). *E. exigua* is also an abundant taxon in this stream, especially in site AF. It is acidobiontic according to Van Dam et al. (1994) and is one of the most common species in rivers and lakes influenced by AMD, with pH < 5.0 (Kwandrans 1993). In the present study, samples with low pH and high metal concentrations also showed high *E. exigua* relative abundances (dominant in site AF), as reported in other studies (DeNicola 2000, Passy 2006). *Nitzschia hantzschiana* dominated in Água Azeda stream (site AA). This taxon has been identified as a dominant species after AMD influence input in mining sites, indicating its ability to live in acidic waters, despite being sometimes rare (Hofmann 1994). These acidic environments with high metal concentrations showed low diversity and species richness as referred in other studies (Mulholland et al. 1986, Corcoll et al. 2012). Changes in biofilm composition – from sensitive to tolerant species – have been reported as a tolerance mechanism to metal contamination (Corcoll et al. 2012). Of the three previous species found in the impacted sites, *P. aljustrelica* was the most significant species driving the similarity between impacted sites. This is clearly related to its capacity to survive the particular environment found in the streams surrounding this mining area, with pyritic minerals exposed to weathering, resulting in very low pH (1.9 – 4.2), high conductivity, elevated sulphate contents and high concentrations of metals, especially Cu, Fe and Zn. Comparison of impacted and unimpacted sites showed that besides *P. aljustrelica* contribution to the dissimilarity between impacted and unimpacted sites, dissimilarity was also supported by the contribution of species such as *N. hantzschiana* or *A. minutissimum*, which is in agreement with species ecology and occurrence.

A. minutissimum (ADMI) appears in four of the unimpacted sites, which discriminate two groups, JU-PB and RJ-BF. This grouping may be due to site proximity, which indicates a probable ecological pattern due to closeness or to seasonal differences between the two groups. Some authors have described *A. minutissimum* occurrence in disturbed waters, being the first taxon colonizing the different habitats (e.g. rocks, sediments) (Sabater 2000) and capable of invading open areas due to changes in environmental conditions (Peterson and Stevenson 1992, DeNicola 2000). Thus, this

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species is abundant in a wide variety of habitats and environmental conditions (Beaver 1981, Verb and Vis 2000 b). However, based on the present study, we can consider *A. minutissimum* as neutrophilic and avoiding high metal concentrations, because it was present at sites with no metal contamination. Although, other studies (Medley and Clements 1998, Gold et al. 2002) have shown a positive correlation between high densities of *A. minutissimum* and metal contamination (e.g. Cu, Fe, Pb, Zn). Its tolerance to heavy metals can be due to its small size (Cattaneo et al. 1998). Thus, *A. minutissimum* might be carefully used as a heavy metal bioindicator. This contradictory information can be due to other environmental parameters, for example, pH. Van Dam et al. (1981) considered *A. minutissimum* as a circumneutral taxon, but also capable of withstanding large pH oscillations in some mine-impacted Ohio streams (Verb and Vis 2000 b) and in an acidic lake with severe metal contamination (Ruggiu et al. 1998). According to Van Dam et al. (1994), *A. minutissimum* is very common in pH < 5.0. Other authors refer to it as cosmopolitan, being present in waters with pH ~ 7 (neutrophilic).

This study contributed to enlarge the knowledge on structural and physiological changes of photosynthetic microorganisms, such as diatoms, in AMD affected and non-affected streams from pyritic mining areas.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

Experimental evaluation of the contribution of acidic pH and Fe concentration to the structure, function and tolerance to metals (Cu and Zn) exposure in fluvial biofilms

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Abstract An indoor channel system was colonised with fluvial biofilms to study the chronic effects of Fe and acidic pH and their contribution to community (in)tolerance to metal toxicity by short-term experiments with Cu and Zn. Biofilms were subjected to four different treatments during 8 weeks aiming to reproduce the water chemistry in the surrounding streams of Aljustrel mining area (Alentejo, Portugal): 1 mg Fe L^{-1}+700 mg SO$_4^{2-}$ L^{-1} and acidic pH (*Acid+Fe* treatment), and comparing it to the most common water chemistry: lower Fe and SO$_4^{2-}$ concentrations and alkaline pH: (*Non-Exposed* treatment), and their respective controls: high Fe and SO$_4^{2-}$ at alkaline pH (*Fe* treatment) and lower Fe and SO$_4^{2-}$ at acidic pH: (*Acid* treatment). During chronic exposure, acidic pH affected growth negatively, based on low values of algal biomass and the autotrophic index (AI), high values of the antioxidant enzyme activities (AEAs) and low diversity diatom communities, dominated by acidophilic species (*Pinnularia aljustrelica*) in acidic treatments, being the effects more marked with high Fe and SO$_4^{2-}$ (*Acid+Fe* treatment). Co-tolerance to metals (Cu and Zn) was also shown in biofilms from the acidic treatments (*Acid* and *Acid+Fe*), contrasting with the higher sensitivity described in the *Fe* treatment (at alkaline pH). We can conclude that the Aljustrel mining area acidic environment limits algal growth and exerts a strong selection pressure on the community composition which is in turn more tolerant to metal exposure.

Keywords biofilms, diatoms, artificial streams, pyritic mines, metals, acidic pH

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5.1 INTRODUCTION

The streams surrounding the Aljustrel mining area (Alentejo Region, Southern Portugal: N 37º52'37.90'', W 8º10'54.64'') in the Iberian Pyritic Belt are affected by Acidic Mine Drainage (AMD) as a result of the sulphidic minerals (mainly pyrite) oxidation. This process, with many steps, involves iron oxidation in the minerals. The acidophilic chemolithotrophic microorganisms, mainly bacteria (González-Toril et al. 2003) present in these environments have the potential to accelerate the oxidation and dissolution reactions of the minerals, resulting in high sulphate contents, high dissolved metal concentrations, as arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), zinc (Zn) and H^+ ions released as mineral acidity (Skousen et al. 1994; Nordstrom and Alpers 1999).

Biofilm studies provide reliable information on biochemical and physiological changes, indicating an initial stress response of the organisms, that can be used as an early indicator of ecosystem damage (Sabater et al. 2007), providing also an ecotoxicological approach with higher ecological realism than just single species studies (Clements and Newman 2002, Guasch et al. 2010 b). In addition biofilms can also respond to chronic exposure to toxicants (Gold et al. 2003). Several studies have highlighted the biofilm sensitivity to both acidity and heavy metals (e.g. Guasch et al. 2002, Sabater 2002, Ivorra et al. 2002, Pinto et al. 2003, Baker et al. 2004). Diatoms (Bacillariophyceae) are part of the algae composing the biofilm and offer advantages in monitoring pH effects due to their short generation time and species-specific acid tolerance. pH is affecting metal bioavailability either by changing the metal speciation or complexing it at the biofilm's matrix and cell surfaces (Sabater 2002). Metal toxicity also changes the community structure (e.g. Ferreira da Silva et al. 2009). It is expected that the type of pollution of Aljustrel mining area induce alterations in the biofilm due to chronic exposure to a mixture of metals (especially Fe) and acidic pH. In fact, a diatom community dominated by few diatom taxa as *Pinnularia aljustrelica* Luis, Almeida et Ector (Luís et al. 2012), *Eunotia exigua* (Brebisson ex Kützing) Rabenhorst and *Nitzschia a.f.f. hantzschiana* Rabenhorst, typical species of acidic waters with high metal contents has already been described (Luís et al. 2009, Luís et al. 2012).

Assessing contaminants impact on lotic systems is complicated due to the co-occurrence of several stress factors. Experimental investigations using artificial streams to examine the effect of acidification on periphyton communities have been conducted by Hendrey (1976) and Hall et al. (1980), and in natural lotic ecosystem with low pH, by Hamilton and Duthie (1984), and Mulholland et al. (1986). Therefore, use of microcosms

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may be a pertinent alternative to a field study as this system allows a gradient of pH/metals contamination to be simulated under controlled conditions (Clements and Newman 2002).

In this study, we tried to mimic the extreme chemical environment described in the Aljustrel mining area: high contents of iron (up to 1 mg L^{-1}), sulphates (700 mg L^{-1}) and acidic pH (3.5-4.0). These stress conditions were tested in order to evaluate their specific effects on biofilms growth and therefore, testing the hypothesis of the biofilms response to the constrains posed by the chemical conditions of acidic pH and Fe compared to the most common situation of alkaline pH with or without Fe. These chemical conditions co-occur with high metal contents, thus it is not possible to test, under field conditions their specific effects, leading to un-consistent conclusions about metal toxicity. We hypothesize that the selection pressure exerted by this extreme chemical environment is mainly driven by acidity and that the selected community, will also be tolerant (co-tolerant) to metals. Low pH may effectively reduce toxicity at the cell surface via competition between metals and H^+ ions (Riseng et al. 1991).

Thus, to test our hypothesis, artificial streams were used to grow biofilms under different chemical environments and compare them in terms of diversity and diatoms species composition, algal biomass, total biomass, photosynthetic parameters, antioxidant enzyme activities (AEAs), and finally antioxidant non-enzymatic mechanisms (glutathione (GSH) and phytochelatins (PCs)). Furthermore, their sensitivity to metals was also assessed using short-term tests.

This study will contribute to understand the selection pressure exerted by acidity and how acidity and metal toxicity interact in biofilms of AMD field waters. This study will also aid to define a strategy to improve the conditions of streams in the Aljustrel mining area, as well as other similar mining areas by increasing our understanding in acidity and metals toxic effects.

5.2 MATERIAL AND METHODS

Experimental indoor channel system

The experiments were carried out in an indoor channel system consisting of 8 Perspex channels, each with 170 cm long and 9 cm wide, as described in Serra et al. (2009). Each treatment was composed by 2 channels named as replicates (R1 and R2). Dechlorinated tap water, previously filtered through an active carbon filter, was supplied from 8 L carboys located at the end of each channel and recirculated at a rate of 1 L min^{-1} through centrifuge pumps. Light was provided by LEDS (130 µE m^{-2} s^{-1}) with a 12 h:12 h

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light:dark and the temperature was kept between 17°C and 20°C. Two water replacements per week were done, to maintain the good nutrients supply to the biofilms.

Periphyton communities' colonization and maturation

During 8 weeks (1 March to 27 April 2010), indoor channels were colonised with fluvial biofilms under different chemical conditions: Four different treatments were applied: one with low addition of iron and sulphate (0.01 mg L^{-1} Fe; 300 mg L^{-1} SO_4^{2-}) and alkaline pH (8.0 in the field), referred to as *Non-Exposed* (*Non-Exp*); the second with the same iron and sulphate concentrations but with the addition of sulphuric acid to reduce pH (3.5 as in the field), *Acid* treatment; the third with high iron and sulphate (1 mg L^{-1} Fe; 700 mg L^{-1} SO_4^{2-}) and alkaline pH, referred to as *Fe* treatment; and the fourth with the same iron and sulphate concentrations as the previous (*Fe* treatment) but with the addition of sulphuric acid to reduce pH: *Acid+Fe* treatment. Fluvial biofilms were obtained from an iron spring: Font Sant Verdaguer (Guasch et al. 2012) and from Água Forte and Pero Bonito streams (Aljustrel, Portugal), the natural systems used to define the chemical conditions of *Fe* (iron spring), *Acid+Fe* (Água Forte) and *Non-Exp* (Pero Bonito) treatments. A mixture of the three inocula was added in each replicate.

Colonization was carried out during five weeks (adding inocula in the channels, once a week, in one of the two water replacements) and biofilms were allowed to colonise the glass substratum surface (2 glass types: 7.5 cm x 2 cm and 12 x 8.5 cm), placed at the bottom of each channel; three extra weeks were needed to obtain mature periphyton communities. Water from the channels was completely replaced twice a week and macronutrients: 100 mg L^{-1} of calcium as $Ca(NO_3)_2$, 60 mg L^{-1} of magnesium as $MgSO_4.7H_2O$ and 100 mg L^{-1} of sodium as NaCl and micronutrients: 0.03 mg L^{-1} of phosphate (nominal concentration) as KH_2PO_4 and 0.015 mg L^{-1} of silica as Na_2SiO_3 were added at each water renewal to avoid nutrient depletion and to simulate field conditions.

Endpoints sampling

During the 8 weeks, the physicochemical parameters (temperature, pH, dissolved oxygen and conductivity) were measured using a multi-parametric probe (WTW METERS, Weilheim, Germany) before and after each water renewal (twice a week). In weeks 3, 6 and 8, 10 mL of water was taken to determine phosphate, anions and cations; for Fe, Cu and Zn analyses, 5 mL of water was taken in weeks 7 and 8. All samples were immediately filtered using 0.2 µm nylon membrane filters (Whatman) and just the samples for metal analysis were acidified with 1% nitric acid (65% suprapure, Merck). Samples

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were stored at 4 ºC until analysis. In weeks 6 and 8, one glass of each replicate was sampled to analyse: Algal biomass (Chlorophyll-*a* (Chl-*a*)), diatom composition, photosynthetic performance through Chl-*a* fluorescence parameters (Fo, ΦPSII, Φ'PSII). The fluorescence measurements were carried out using undisturbed biofilm. Maximal quantum yield (ΦPSII) and effective quantum yield (Φ'PSII) parameters were used as photosynthesis capacity and photosynthesis efficiency indicators. Fo was used as an alternative non-destructive measure of algal biomass. Antioxidant enzymatic activities (catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) were taken. As well as non-enzymatic activities to minimize the reactive oxygen species: Phytochelatins (PCs), total gluthatione (GSH) and total biomass (Ash-Free-Dry-Weight, AFDW).

Metal (M) toxicity assay

After growth, under the four different treatments, their effects in biofilm's sensitivity to metals (Zn and Cu) were investigated. A 24h acute toxicity test (Dose/Response (D/R) in 26-27/04/2010) was performed during the last week. Biofilms of the four treatments were scraped and distributed in glass vials containing three increasing Cu and Zn concentrations: controls (no addition); concentration 1 (75 µg L^{-1} Cu and 500 µg L^{-1} Zn); 2 (600 µg L^{-1} Cu and 4000 µg L^{-1} Zn) and 3 (1200 µg L^{-1} Cu and 8000 µg L^{-1} Zn). Each glass vial contained 11.5 mL of water from the 4 treatments previously tested during 8 weeks in the artificial channels, 2 ml of a scraped biofilm mixture (biofilm from 6 scrapped glasses, each 2x7.5 cm in 20 mL of treatment water) and 0.75 ml of the corresponding Cu and Zn concentration. Samples were incubated during 24 h under the same conditions as in the colonization, using a single-speed orbital mixer (KS260 Basic, IKA®) to maintain a constant agitation of 100 rpm. After 24 h of exposure, two replicates were sampled for AEA, PCs and GSH analyses to determine acute toxicity of Cu and Zn at concentration 1 (75 µg L^{-1} Cu and 500 Zn µg L^{-1}) and Chl-*a* fluorescence measurements were made for the three metal concentration and controls. AEA, PCs and GSH were focused on the lowest concentration to expect antioxidant responses because at high concentrations enzymes may not respond anymore due to cell damage effect of the toxicant which may also affect the enzymes response (Bonnineau et al. 2011). Water samples used to analyse dissolved metals (Fe, Cu, Zn) were filtered and preserved as described above.

Chemical analysis

Metal concentrations (Fe, Cu, Zn) in water were analysed by inductively coupled plasma mass spectrometry ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE).

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The accuracy of the analytical method was checked periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway). Phosphate concentration (PO_4^{3-}) was analysed using the Murphy and Riley (1992) and dissolved major cations (Ca^{2+}, K^+, Mg^{2+}, Na^+) and anions (Cl^-, SO_4^{2-}, NO_3^-) were analysed by ion-chromatography (761 Compact IC, METROHM Ltd., Herisau, Switzerland).

Biofilm analysis

Algal biomass: Chlorophyll-*a* (Chl-*a*) concentration was used as a measure of algal biomass. Chl-a concentration was obtained after extraction with 90% acetone and measured spectrophotometrically following Jeffrey and Humphrey (1975).

Total biofilm biomass: was measured as ash free dry weight (AFDW). 15 cm^2 of biofilm was scraped and then dried for 24h at 105ºC in order to calculate dry matter (DW). Afterwards, samples were combusted for 4h, in an oven at 450ºC (Hobersal MOD MF12-124, Spain) and then weighted again to calculate the mineral matter (AW). The AFDW was calculated subtracting the mineral matter from the total dry matter and after divided by the area of the glass slide. It was expressed in mg cm^{-2}.

AFDW values were used with Chl-*a* values as a measure of the trophic status in streams, expressed as Autotrophic Index (AI). According to USEPA-RBP (Barbour et al. 1999), the formula to calculate the AI, is: AI = Chl-*a* (mg cm^{-2})/AFDW (mg cm^{-2}).

Metals (Fe, Cu, Zn) quantification in biofilms: biofilms were harvested by centrifugation at 4000×g for 10 min, washed in distilled H_2O, and centrifuged again. In order to obtain the Fe, Cu and Zn bounded to the frustule, the method described by Purchase et al. (1997), with some modifications, was used; the cell pellet obtained was suspended in 2 ml of 0.1M HNO_3, sonicated in an ultrasonic bath for 5 min, centrifuged at 14.000×g for 10 min and collected into a microtube. The pellet was re-suspended in 1ml of distilled H_2O and transferred to Teflon bombs. Cells were digested overnight, at 115 ºC, with 2 ml of an equivolume mixture of concentrated H_2SO_4 and concentrated HNO_3. The cooled digested was filled up to 5 ml, using 1M HNO_3, and the concentration of Fe, Cu and Zn (equivalent to that taken up by cells) was determined by ICP-AES (inductively coupled plasma-atomic emission spectroscopy) model Jobin Yvon Jy Plus.

Diatom treatment and identification: biofilm samples were fist fixed with 4% formaldehyde in order to preserve the sample for diatom study. Then after removing the formaldehyde, the samples were treated with HNO_3 (65%) and potassium dichromate ($K_2Cr_2O_7$), at room temperature/24 hours in order to eliminate the organic matter and to obtain clean diatom suspensions, followed by three centrifugations (1500 rpm) to rinse the

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samples. Permanent slides were mounted using the high refractive index (1.74) medium Naphrax® (Brunel Microscopes Ltd, UK). Light microscopy identifications were taken with a Leitz Biomed 20 EB microscope using a 100x objective (A.N. 1.32); 400 valves were counted for each sample. Morphological terminology followed Krammer and Lange-Bertalot (1986, 1988, 1991 a, b).

Chlorophyll-a fluorescence parameters: measurements were carried out by PhytoPAM (Pulse Amplitude Modulated) fluorometer (Heinz Walz GmbH) according to the procedure described in Corcoll et al. (2011) used to measure maximal quantum yield (Φ_{PSII}), effective quantum yield (Φ'_{PSII}) and Fo of dark-adapted cells (at 665 nm). The Fo of dark-adapted cells at 665 nm, is proportional to Chl-*a* concentration (Rysgaard et al. 2001); thus, Fo was used to follow the growth of the biofilm during the colonization. All fluorescence parameters were calculated from the fluorescence signal recorded at 665 nm and given as relative units of fluorescence (Genty et al. 1989).

The deconvolution of the overall fluorescence signal (Fo) into the contributions of the three main algal groups composing the biofilm, was based on the internal reference excitation spectra of a pure culture which had been validated, previously, for biofilm communities (Schmitt-Jansen and Altenburger 2008). Fo(Bl), was used as an indicator or cyanboacteria abundance. Fo(Gr) as an indicator of green-algae abundance and Fo(Br) as an indicator of diatom abundance.

Antioxidant enzymes activities (AEA): three glass substrata (15 cm^2 each) of biofilm were scrapped from each channel (considered as pseudo-replicates). Sampling and protein extraction were performed as described in Bonnineau et al. (2011). The protein concentration of supernatant was measured and used in triplicates for each sample following Bradford (1976). Results of protein concentration are expressed as µg of protein mL^{-1}. For all AEA assays the protein concentration was 4 µg of proteins and AEA were calculated as specific activities (i.e. per µg of proteins). For each assay the optimal concentration of substrate or cofactor was determined by testing the concentration in mM of H_2O_2 for the CAT and APX assay, WST-1 for the SOD assay and NADPH for the GR assay.

Catalase (CAT) activity ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) was measured spectrophotometrically at 240 nm according to Aebi (1984). 250 µL of reaction mixture were obtained by adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (40 mM final concentration) and the enzyme extract (4 µg protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25°C for 3 min. CAT activity was calculated as µmol H_2O_2 µg protein^{-1} min^{-1} (extinction coefficient, ε: 0.039 M cm^{-1}).

Ascorbate peroxidase (APX) activity was assessed by monitoring the decrease in absorbance at 290 nm at 25ºC for 2 min due to ascorbate oxidation according to Nakano & Asada (1981). 250 µL reaction mixture were obtained adding potassium phosphate buffer (pH 7.0) (80 mM final concentration); H_2O_2 (4 mM final concentration); Na-Ascorbate (1.5 mM final concentration) and the enzyme extract (4 µg protein). APX activity was calculated as µmol Ascorbate µg protein^{-1} min^{-1} (extinction coefficient, ε = 2.8 M cm^{-1}).

Superoxide dismutase (SOD) activity was measured spectrophotometrically at 450 nm according to McCord and Fridovich (1969) and Peskin and Winterbourn (2000). The 200 µL reaction mixture contained, the following, in a final concentration of: potassium phosphate buffer (50 mM, pH 8.0), diethylenetriamine (0.1 mM), hypoxanthine (0.1 mM), water-soluble tetrazolium salt (WST-1 at 0.075 mM final concentration), and the enzyme extract (4 µg protein). 6 mU mL^{-1} of xanthine oxidase were added to start the assay. WST-1 produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O^{2-} is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Therefore, the IC 50 (50% inhibition activity of SOD or SOD like materials) can be determined by this colorimetric method at 25ºC for 10 minutes. SOD activity was calculated as U/ug of protein after calculating the specific SOD activity (U) using this formula: (%inhibition×100/50)/ (protein concentration×enzymatic extract volume). SOD competes with WST-1 for superoxide anions (O^{2-}) generated by the xanthine/xanthine oxidase system. WST-1 reduction was measured at 450 nm and 25ºC for 10 min. One unit of SOD was defined as the amount of sample required for 50% inhibition of WST-1 reduction and SOD activity was expressed as U µg protein^{-1}.

Glutathione reductase (GR) activity was assessed by monitoring the decrease in absorbance at 340 nm, at 25º C and for 2 min (Schaedle and Bassham 1977). 200 µL reaction mixture was obtained adding Tris hydrochloride buffer (pH 7.5) (100 mM final concentration) and EDTA (1 mM); oxidised glutathione: GSSG (8 mM final concentration); NADPH (0.30 mM final concentration) and enzyme extract (4 µg protein). GR activity was calculated as µmol NADPH min^{-1}µg^{-1} of protein.

Non-enzymatic activities:

Phytochelatins (PCs), the fresh weight determination for each sample (two glass slides per replicate) was done and then samples were preserved at -80ºC prior to analysis. Peptide extraction was performed as described by Klapheck et al. (1994). Samples were sonicated for 20 sec with 400 µl of 0.1M HCl. The homogenates were centrifuged at 40000 x g for 10 min, at 4 ºC and the resulting supernatant was

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immediately used. The method for derivatisation of thiol compounds was performed as described earlier (Lima et al. 2006). Extracts (100 μl) were neutralised with 0.1 M NaOH, after the addition of 200 μl of 0.1M Tris–HCl buffer (pH 8.0), 1mM EDTA and 25 μl of 2mM DTE. After incubation for 1 h at room temperature, 50 μl of 20 mM mBBr were added. Derivatisation was performed in the dark, for 40 min at a temperature of 35 ºC. The reaction was stopped by the addition of 5% (v/v) acetic acid up to a total volume of 1.5 ml. Samples were stored at 4 ºC before HPLC–RP analysis.

The highly fluorescent bimane derivatives were separated by RP–HPLC (Gilson liquid chromatograph, model 306), as described earlier (Lima et al. 2006). Samples were centrifuged at 40000 $x\ g$, for 5 min, at 4 ºC, filtered, and 20 μl aliquots were injected on a RP C18 column (250 mm×4.6 mm i.d, 5 μl, Gilson). The column was equilibrated with previously degassed eluant A (0.01% aqueous TFA (v/v)) and developed by a linear gradient of 0–20% eluant B (90% acetonitrile, in 0.01% aqueous TFA) during the first 20 min, followed by an isocratic elution of 20% eluant B for the next 30 min and finally a linear gradient of 20–90% of eluant B during the last 10 min. The complete analysis was performed in 60 min. After each run, the column was washed by raising the concentration of eluant B to 100% and re-equilibrated with eluant A. Thiols were resolved and eluted at a flow rate of 1ml min^{-1} and detected by fluorescence (Jasco 821- FP Intelligent Spectrofluorometer) with excitation at 380 nm and emission at 480 nm (Lima et al. 2006). In order to overcome the problem of the decrease in derivatisation with chain length increase, adverted by other authors (Sneller et al. 2000), thiol identification was based on Cys and GSH standards (Sigma) and synthesised PCs standards (kindly offered by Dr. M.H. Zenk), with n ranging from 2 to 5.

Total Glutathione (GSH) was determined by HPLC as described above for PCs (Lima et al. 2006). GSH quantification was based on reduced GSH standards (Sigma).

Data treatment

In the acute exposure, data are presented in % of their relative controls to compare the response to chronic exposure. Potential differences between controls and treatments for physico-chemical variables (SO_4^{2-}, PO_4^{3-}, Fe in water, Fe in biofilm, conductivity and pH), Chl-a fluorescence parameters (F_0, Φ_{PSII} and Φ'_{PSII}), AI, Chl-a enzymatic activities (APX, CAT and GR) and non-enzymatic protection mechanisms (GSH) were tested using analysis of variance (ANOVA); effects were post hoc analysed by a Tukey's test if significant differences were found. This analysis was performed using Sigma Plot 12.0 software. For all the analyses, statistical significance was set at $p < 0.05$. Primer 6 software was used to do PERMANOVA, Cluster and dbRDA analyses of diatom data.

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5.3 RESULTS AND DISCUSSION

Chronic exposure

Experimental conditions and biofilm growth

The experimental setting allowed the reproduction of the chemical environment prevailing in acidic zones: low pH with high dissolved salts with a characteristic biofilm, and the comparison between these conditions and their respective controls: alkaline pH with and without increased dissolved salts (Fe and SO_4^{2-}) and low pH without salts (**Table 5.1**).

Table 5.1 – Dissolved oxygen (Oxy – mg L^{-1}), water temperature (Temp - ºC), pH and conductivity (Cond - µS cm^{-1}), dissolved salts and nutrients measured in water treatments, during the colonization. Concentrations of SO_4^{2-}, NO_3^-, PO_4^{3-}, in mg L^{-1}, PO_4^{3-}, Fe in water in µg L^{-1} and Fe in biofilm, in µg g^{-1} DW, respectively.

	Non-Exp		Fe		Acid		Acid+Fe		Two-way ANOVA		
	R1	R2	R1	R2	R1	R2	R1	R2	Fe	pH	Fe*pH
Oxy	8.88 ±0.18 (n=8)	8.93 ±0.27 (n=10)	8.74 ±0.19 (n=7)	8.83 ±0.27 (n=10)	8.71 ±0.22 (n=8)	8.81 ±0.32 (n=10)	8.66 ±0.25 (n=8)	8.81 ±0.34 (n=10)	ns	ns	ns
Temp	18.86 ±1.50 (n=8)	18.49 ±2.01 (n=10)	19.17 ±1.59 (n=7)	18.77 ±1.98 (n=10)	18.94 ±1.49 (n=8)	18.22 ±1.95 (n=10)	19.00 ±1.47 (n=8)	18.37 ±1.95 (n=10)	ns	ns	ns
pH	8.33 ±0.12 (n=8)	8.25 ±0.10 (n=10)	8.21 ±0.08 (n=7)	8.14 ±0.16 (n=10)	3.87 ±0.19 (n=8)	3.62 ±0.18 (n=10)	3.68 ±0.34 (n=8)	3.61 ±0.27 (n=10)	ns	<0.001	ns
Cond	1843 ±49 (n=8)	1973 ±124 (n=10)	2468 ±71 (n=7)	2558 ±153 (n=10)	1993 ±83 (n=8)	2043 ±113 (n=10)	2855 ±211 (n=8)	2669 ±138 (n=10)	<0.001	<0.001	<0.05
SO_4^{2-}	66.07 ±8.59 (n=4)	67.82 ±2.15 (n=4)	89.14 ±32.9 (n=4)	122.9 ±8.52 (n=4)	86.25 ±6.80 (n=4)	87.20 ±5.84 (n=3)	168.1 ±15.6 (n=3)	147.1 ±15.4 (n=4)	<0.01	<0.05	ns
Fe	236 (n=1)	258 (n=1)	270 (n=1)	259 (n=1)	335 (n=1)	319 (n=1)	2405 (n=1)	2390 (n=1)	<0.001	<0.001	<0.001
Fe biofilm	3.6 (n=1)	14.4 (n=1)	80.5 (n=1)	65 (n=1)	1.23 (n=1)	37 (n=1)	206 (n=1)	131 (n=1)	<0.01	ns	ns
PO_4^{3-}	18 ±5.59 (n=4)	19 ±7.64 (n=4)	16 ±2.86 (n=4)	16 ±4.09 (n=4)	26 ±12.0 (n=4)	24 ±5.47 (n=3)	17 ±2.32 (n=3)	14 ±1.40 (n=4)	<0.005	<0.05	ns
NO_3^-	4.79 ±0.71 (n=4)	3.82 ±0.23 (n=4)	4.49 ±0.93 (n=4)	5.28 ±0.27 (n=4)	5.14 ±1.34 (n=4)	4.39 ±0.40 (n=3)	5.22 ±0.34 (n=3)	5.09 ±0.46 (n=4)	ns	ns	ns

Each value corresponds to the average (AVG) of before and after water renewals ± standard deviation (SD); Replica 1 (R1); Replica 2 (R2); n.s. not significant.

Treatment's effect on pH was clearly achieved. Fe addition caused a marked increase in Fe concentration in water under acidic condition, but not under alkaline pH. This result was expected since Fe solubility is very low at neutral/alkaline pH, and is supported by Fe

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contents in biofilms, much higher in all Fe treatments with high pH due to retention and/or precipitation of Fe in biofilms (**Table 5.1**).

On the other hand, sulphate concentration was higher in acidic treatments since acidity was achieved by adding sulphuric acid. In addition, phosphate, while being equally added to all treatments, was affected by the two treatment factors, Fe and pH, leading to differences in P-availability that might influence biofilms growth. The influence of Fe on P-availability has already been described as one of the causes of algal growth reduction in Fe-rich environments due to an effective co-precipitation of Fe and P (e.g. Das et al. 2009, Guasch et al. 2012) in order to maintain the intracellular neutral conditions and diurnal shift of oxygen concentration in the biofilm layer (Das et al. 2009). Consequently, differences in algal growth and biofilm characteristics between treatments should be regarded as a result of the set of chemical alterations each treatment has created.

Biofilms characteristics

While algal biomass (Fo) was affected either by Fe and acidic pH, the effect of acidic pH was of much higher magnitude (**Fig. 5.1**), influencing also the community composition.
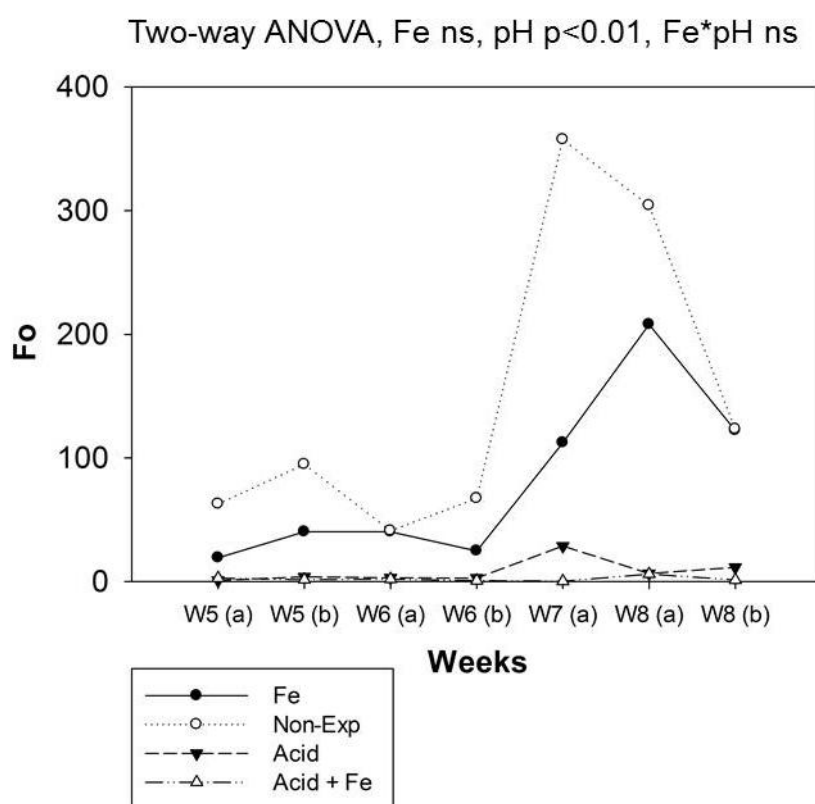
Fig. 5.1 – Fo (growth of biofilm) during colonization period. Two-way ANOVA results are also indicated.

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Fo showed the lowest values in *Acid* and *Acid+Fe* treatments, in accordance with Chl-*a* results (up to two orders of magnitude lower, **Table 5.2**).

Table 5.2 – Biofilm metrics measured at the end of the colonization. Chl-*a* ($\mu g\ cm^{-2}$), AFDW ($mg\ cm^{-2}$), Φ'_{PSII} and Φ_{PSII} (a.u., arbitrary units), PCs and GSH ($\mu mol\ g^{-1}$), CAT ($\mu mol\ H_2O_2\ \mu g\ protein^{-1}\ min^{-1}$), APX ($\mu mol$ Ascorbate $\mu g\ protein^{-1}\ min^{-1}$), GR ($\mu mol\ NADPH\ min^{-1}\ \mu g^{-1}$ of protein) and SOD (U $\mu g\ protein^{-1}$); ns: not significant.

| | Non-Exp | Fe | Acid | Acid+Fe | Two-way ANOVA | | |
					Fe	pH	Fe*pH
Chl-*a*	4.849	6.285	0.117	0.049	ns	<0.01	ns
AFDW	0.77	0.95	0.93	0.40	ns	ns	ns
Φ'_{PSII}	0.414	0.518	0.569	0.488	<0.001	<0.05	ns
Φ_{PSII}	0.174	0.243	0.141	0.324	ns	ns	ns
PCs	0	0	1.77	0	ns	ns	ns
GSH	42	32	37	43	ns	ns	ns
CAT+ 10^{-3}	57	52	130	150	ns	<0.05	ns
APX + 10^{-4}	5	4	10	20	ns	0.052	ns
GR + 10^{-6}	108	68	922	225	<0.001	<0.001	<0.001
SOD	45	45	42	47	ns	ns	ns

Also high Fe had a significant effect on Fo (demonstrated by ANOVA), always at acidic pH. The same was observed for Φ'_{PSII} (**Table 5.2**). Acidic pH and high Fe decreased Chl-*a* fluorescence emission of all open reaction centers of PSII in a non-excited status (Fo) and the toxicants were reducing electron flow in the PSII (Φ'_{PSII}). However, there were no significant alterations between treatments in Φ_{PSII}. Probably it could be linked with community adaptation. Adapted species to each treatment can maintain their photosynthetic processes for cell growing.

Differences were observed between communities especially in *Acid+Fe* treatment. In agreement with field investigations showing that acidophilic chemolithotrophic bacteria are the most adapted groups of microorganisms to these extreme conditions (González-Toril et al. 2003), heterotrophic organisms dominate in acidic treatments. While Chl-*a* was much lower at acidic pH than at alkaline pH, total biomass (AFDW) was similar among treatments, illustrating the shift from autotrophic to heterotrophic biofilms, dominated by bacteria, fungi and cyanobacteria (microscopic observations) and leading to marked differences in the Autotrophic Index (AI), **Fig. 5.2.** On the other hand, diatoms, while being also affected by acidity, were present in all treatments. From the initial three inocula mixture, each treatment selected, during colonization, the typical and dominant diatom species with ecological preferences closer to the physico-chemical characteristics of the water of the respective treatment.

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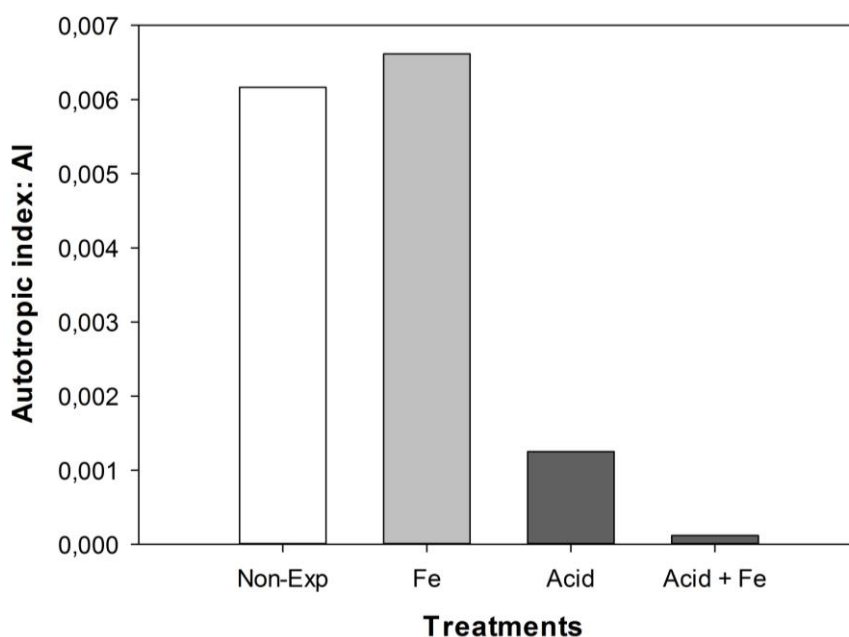
Fig. 5.2 – Autotrophic Index (AI) of each treatment at the end of colonization.

The inoculum from AMD affected streams (e.g. Ribeira Água Forte, Aljustrel, Portugal), containing a well-adapted community to chronic exposure of acidic conditions and metal contamination (mostly Fe) and dominated by few taxa, was found in the *Acid* and *Acid+Fe* treatments. In these treatments, *Pinnularia aljustrelica* dominated with abundance always above 54%. It can be concluded that acidic pH and Fe reduce diatom diversity (Mulholland et al. 1986, Planas 1996, Verb and Vis 2000) and change the assemblage structure through shifts from sensitive to tolerant taxa. Diatom communities were then analysed by Cluster and dbRDA, both showing pH as the main driving factor separating acidic from alkaline sites. PERMANOVA reinforced pH as more significant than Fe (**Fig. 5.3**).

As said above, in highly acidic environments, cells must overcome the high H^+ concentration that may lead to rapid acidification of the cytosol (Gross and Robbins 2000), as well as develop strategies to protect themselves from adverse effects commonly associated with acidity, such as decreased nutrients, increased dissolved metal concentrations (Olaueson and Stokes 1989), and limited supply of CO_2 for photosynthesis due to bicarbonate pool absence (Olaueson and Stokes 1989, Gross and Robbins 2000).

Chronic exposure to acidic pH caused also an increase in several AEAs (CAT, GR and APX) (**Table 5.2**) of these communities, which can be due to amelioration in oxidative stress to overcome the negative effects of acidic pH and Fe.

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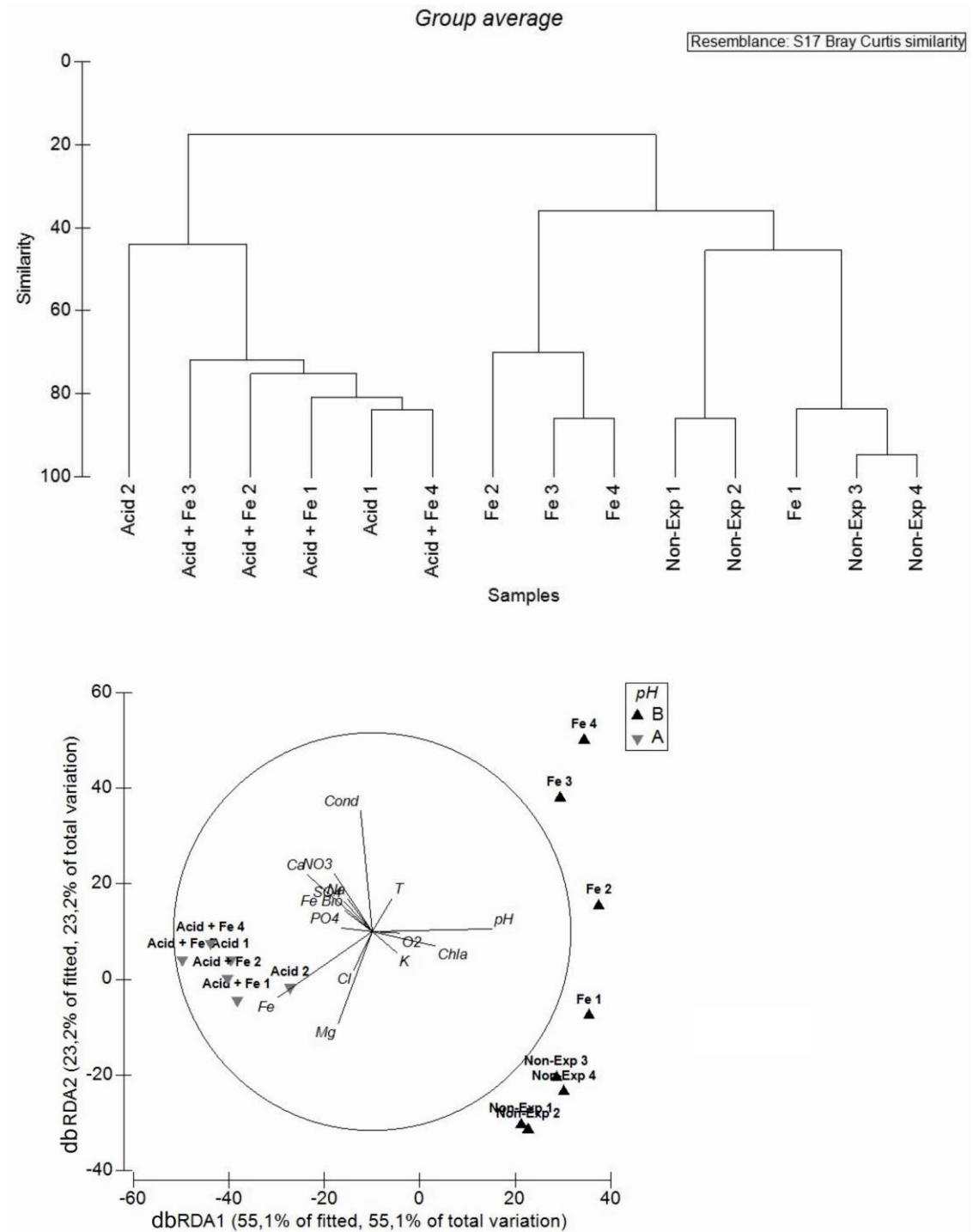
Fig. 5.3 – a**:** Dendrogram performed on a Bray-Curtis similarity matrix of diatom abundances in two sampling moments: 07/04/2012 and 21/04/2010; two replicates per sampling: 1, 2 from 07/04/2012; 3, 4 from 21/04/2010; **b** dbRDA: distance based redundancy analysis for the diatom resemblance matrix evidencing the samples disposition and the environmental variables which explain 78% of biological variation. pH separates treatments in 2 groups: **A**-Acid and **B**-Alkaline: acidic pH is more relevant in the communities discrimination than Fe.

Effect of Fe was also significant but of lower magnitude. It is also expected that Fe adaptation will be linked to photosynthesis recovery and also linked to a slight reduction in

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biomass (Guasch et al. 2010 a). It is interesting to point out that effects exerted by Fe were always higher under acidic than alkaline pH. While algal biomass was on average 1.8 times higher without Fe than with Fe at alkaline pH, it was four times higher in the same Fe conditions (without Fe than with Fe) at acidic pH, suggesting that Fe is also constraining the ability of biofilms to grow under acidic conditions. Differences in phosphate availability may partially explain this result. Additionally, GR was lower with Fe (*Fe*) than without (**Table 5.2**) and this difference (low Fe, high Fe) was also enhanced at acidic pH (*Acid+Fe*), supporting the hypothesis of additive negative effects of Fe and acidic pH reducing the capacity of biofilms to cope with oxidative stress.

Influence of chronic exposure on metal toxicity

Biofilm responses

Acute exposure to metals may lead to transitory physiological effects if the community is not adapted because metal exposure is expected to cause oxidative stress, increased activity of the enzymes that may cope with this stress, photosynthesis inhibition and finally toxicity which may damage cell defense capacities. On the other hand, low pH may reduce toxicity due competition in acute exposure between metals and H^+ ions at the cell surface (Riseng et al. 1991). Both aspects: co-tolerance and the prevailing chemical environment may explain the increase in tolerance found in our investigation. The biofilms from the two acidic treatments (*Acid+Fe* and *Acid*), after acute exposure to Cu and Zn (**Table 5.3**), had higher metal contents (**Table 5.4**) and were not inhibited, having values of Fo and Φ_{PSII} similar to non-exposed biofilms whereas those from high pH treatments (*Non-Exp* and *Fe)* were clearly inhibited (**Fig. 5.4**), thus we can conclude that adaptation to acidity led to higher tolerance to acute metal exposure.

Table 5.3 – Concentrations of Fe, Cu and Zn in water of each treatment after the Dose–Response (D/R) (tested nominal concentrations: control: no metals; 1 (75 µg L^{-1} Cu and 500 µg L^{-1} Zn); 2 (600 µg L^{-1} Cu and 4000 µg L^{-1} Zn) 3 (1200 µg L^{-1} Cu and 8000 µg L^{-1} Zn)).

			Non-Exp	Fe	Acid	Acid +Fe
	µg L^1	Nominal	R1	R1	R1	R1
control	Fe	10/1000*	236	270	335	2405
	Cu	0	3.10	3.66	3.48	4.82
	Zn	0	6.84	4.45	16.8	16.2
1	Fe	10/1000	257.60	252.11	328.35	2210.00
	Cu	75	71.74	60.45	73.95	78.90
	Zn	500	392.41	358.36	597.57	484.71
2	Fe	10/1000	253.99	85.29	1262.80	2514.02
	Cu	600	557.54	305.19	790.73	666.77
	Zn	4000	3811.49	1813.06	5624.78	4562.23
3	Fe	10/1000	120.59	278.22	334.51	2157.22
	Cu	1200	808.62	974.48	1325.87	1204.87
	Zn	8000	5101.58	7420.79	8251.73	7325.99

* Respective nominal Fe concentrations added to channel water (*Non-Exp* and *Acid*; and to *Fe* and *Acid+Fe*); R1 – Replicate 1

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Table 5.4 – Concentrations of Fe, Cu and Zn in biofilm of each treatment after the Dose–Response (D/R) for tested concentration 1 (75 µg L^{-1} Cu and 500 µg L^{-1} Zn).

µg g^{1}	Non-Exp		Fe		Acid		Acid+Fe	
	R1	R2	R1	R2	R1	R2	R1	R2
Fe (10^4)	2.79	123	509	77	2300	1180	4370	970
Cu (10^3)	2.14	10.7	7.32	5.83	27.3	61.9	47.7	15.4
Zn (10^3)	4.21	57.7	73.8	59	285	90.6	77.4	22.7

Replica 1 (R1); Replica 2 (R2)

Fig. 5.4 – Effective quantum yield (Φ'_{PSII}), Maximum quantum yield (Φ_{PSII}) and Minimum fluorescence (Fo) values (% of controls) for each treatment, corresponding to concentrations 2 (600 µg L^{-1} Cu and 4000 µg L^{-1} Zn; bars with lines) and 3 (1200 µg L^{-1} Cu and 8000 µg L^{-1} Zn; bars with no pattern) of the Dose-Response experiment. Two-way ANOVA results are also indicated.
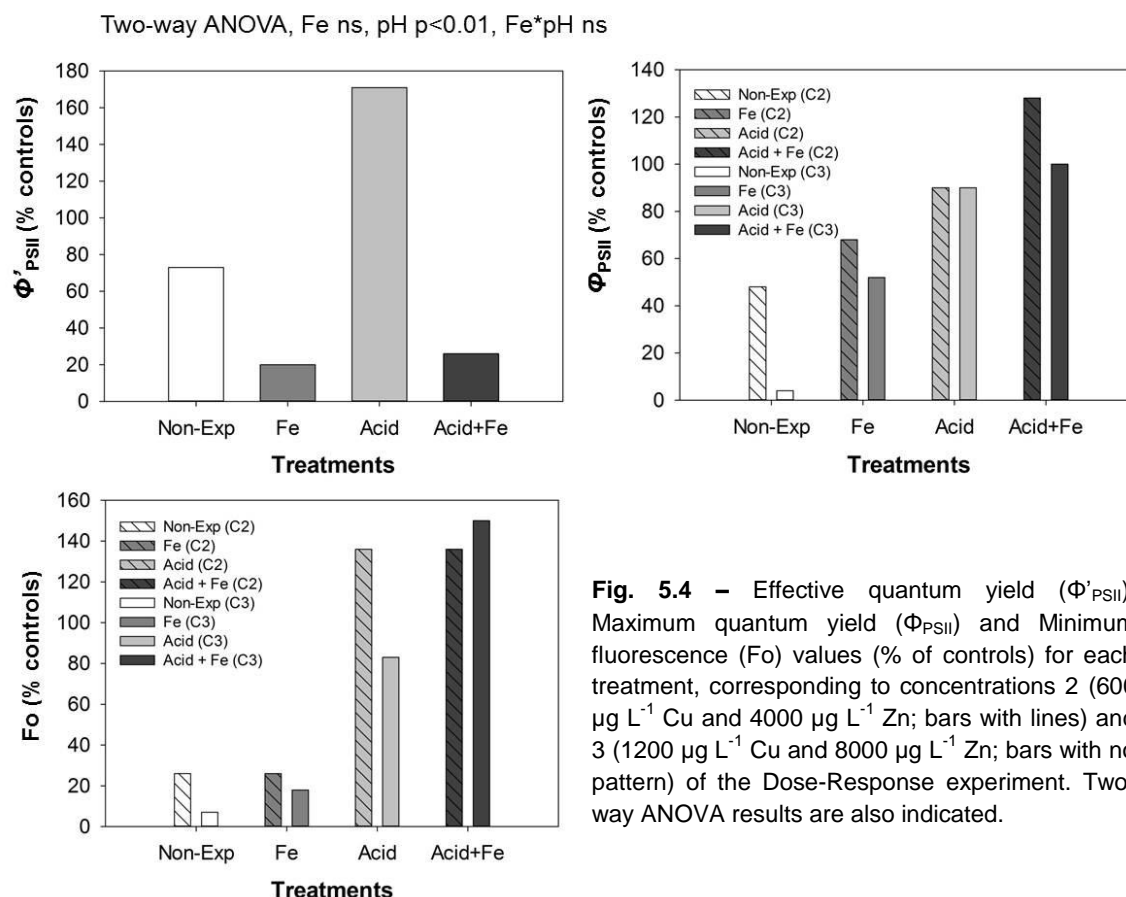
Furthermore, communities of acid treatments (*Acid*, *Acid+Fe*) didn't increase enzyme activities after metal exposure, indicating that metals were not causing oxidative stress (**Fig. 5.5**). Otherwise, at high pH treatments the opposite effect was observed, increasing CAT (ANOVA, pH p=0.022), indicating higher response to metal toxicity in cells. Due to relatively low affinity with H_2O_2, CAT is mainly involved in the removal of H_2O_2 excess, converting it into water and molecular oxygen (Noctor and Foyer 1998). In agreement with our results, Li et al. (2006), showed the increase in AEA (e.g. CAT, GPX and SOD) levels in cultures of *Pavlova viridis* (Prymnesiophyceae) after Cu-Zn treatment indicating the protective role of these enzymes against Cu-Zn induction of oxidative stress.

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Two-way ANOVA, Fe ns, pH p<0.01, Fe*pH ns

Fig. 5.5 – CAT, GR activities and GSH values of Dose-Response (D/R) for metal concentration 1 (75 µg L^{-1} Cu and 500 µg L^{-1} Zn; values in % of control) in each treatment. Two-way ANOVA results are also indicated.
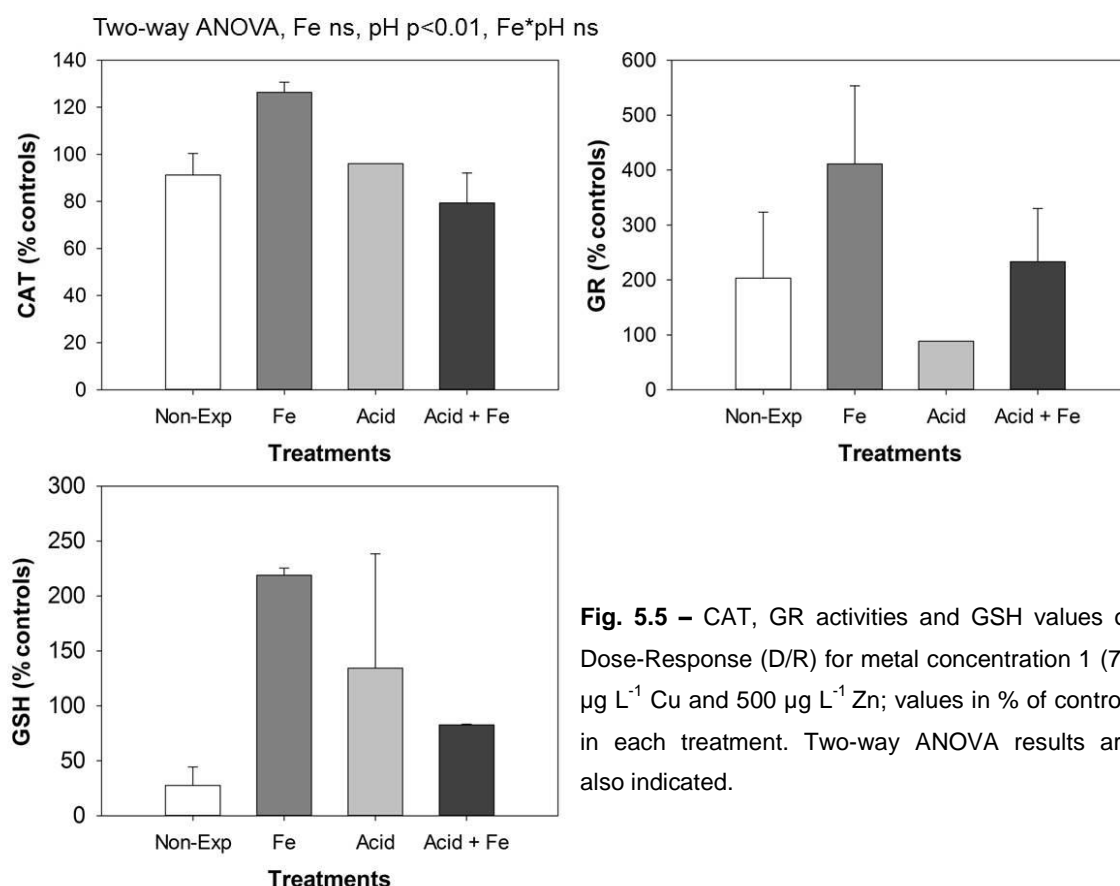
It is also interesting to point out that Fe affected Cu and Zn toxicity. Fe may be a cofactor of ROS detoxification enzymes (e.g. Andrews et al. 2003, Touati et al. 2000). For this reason, Fe regulation is strongly coupled with oxidative stress mechanisms for bacteria, cyanobacteria and microalgae (Touati et al. 2000, Estevez et al. 2001, Andrews et al. 2003, Michel et al. 2004). Higher inhibition of Φ'_{PSII} was observed in *Fe* and *Acid+Fe* treatments compared to treatments without Fe. This parameter is very sensitive to PSII-inhibiting compounds if they block the electron transport flow (e.g. Barranguet et al. 2003). In agreement with these results, metal exposure caused a marked increase of total glutathione (GSH), a non-enzymatic antioxidant protective mechanisms, in *Fe* treatment while decreasing in *Non-Exp* and acidic treatments, leading to a significant interaction between pH and Fe (ANOVA, pH*Fe p< 0.05). This increase indicates that metal exposure (Cu and Zn) induced the cells to activate non-enzymatic antioxidant protective mechanisms at high pH and high Fe concentrations. Non-enzymatic antioxidants include the major cellular redox buffer glutathione. GSH is oxidised by ROS forming oxidised glutathione (GSSG). DHA is reduced to ascorbate by the action of DHA reductase, using GSH as the reducing substrate in response to increased H_2O_2 levels (Foyer and Halliwell

1976). This reaction generates glutathione disulphide (GSSG), which is in turn re-reduced to GSH by NADPH, a reaction catalysed by glutathione reductase (GR), thus having an important role in the non-enzymatic protective system against toxic metal damage (Sabatini et al. 2009). Organisms increase the activity of GSH biosynthetic enzymes (Vanacker et al. 2000) and GSH levels in the presence of abiotic stresses (Noctor et al. 2002). What can also occur is other mechanisms, such as the production of phytochelatins (PCs) which might help cells to cope with metal toxicity, reducing the ROS produced by Zn (Gaur and Rai 2001, Le Faucheur et al. 2005). GSH is the precursor of metal-chelating peptides (e.g. PCs) (Noctor and Foyer 1998); after Cu and Zn addition, PCs were just found in *Acid* treatment (28 µmol. g^{-1}), probably supported by higher contents of Cu and Zn in biofilms in this treatment than in the other treatments.

5.4 CONCLUSIONS

During chronic exposure, acidic pH affects growth more negatively than high Fe, based on low values of Fo and AI, high values of CAT, APX, GR and low diversity of diatom communities, dominated by acidic species in the acidic treatments. This is the cost to maintain neutral the cytosolic pH, while the surrounding environment is acid. In addition, when Cu and Zn were added (acute exposure), Fo and Φ_{PSII} were not inhibited in acidic treatments (*Acid* and *Acid+Fe*) showing low metal toxicity in communities adapted to acidic pH. CAT activity decreased and GR activity didn't show any change in these treatments, showing well adapted communities to low pH. Φ'_{PSII}, which was less affected by metals than Fo and Φ_{PSII}, showed higher inhibition with Fe than without. CAT and GR showed sensitivity to high Fe contents, increasing their activities in *Fe* and *Non-Exp* treatments. Whereas Φ_{PSII} did not show changes during chronic exposure, CAT and GR increased their activities in both chronic (increasing in acid treatments) and acute exposure (increasing in alkaline treatments with high Fe contents) which might be caused by high ROS levels, occurring at metal concentrations that may not be inhibitory to the photosynthetic activity of algae (Szivák et al. 2009), but detected by AEA, highlighting their higher sensitivity (Bonet et al. 2012).

Acidic pH seems to ameliorate metals' effects if the community has been pre-exposed and hence adapted to the prevailing extreme chemical environment. This may explain why acidic areas (as pyritic mines) with several high metal concentrations have algal growth with typical acidic diatoms as *Pinnularia aljustrelica,* found in high abundances.

Through this laboratory experiment, it was possible to understand which parameters affected most the diatom community. It is thus possible to extrapolate these findings to the

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complex pyritic mining areas, mainly under stable conditions since these systems are also exposed to drastic changes in water chemistry linked to high rainfall, for instance, with effects on diatom communities difficult to predict from this laboratory experiment.

5.5 ACKNOWLEDGMENTS

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CHAPTER 6

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Integrating geochemical (surface waters, stream sediments) and biological (diatoms) approaches to assess environmental impact in a pyritic mining area: Aljustrel (Alentejo, Portugal)

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Abstract Aljustrel mines (Alentejo, Portugal) were classified of high environmental hazard due to their large tailing's volume and high contents in metals (especially Cd, Cu, Ni, Pb and Zn) and metalloids (As, Sb) found in waters and sediments of their surrounding streams. The short generation time of diatoms is an advantage in the assessment of AMD impacted systems which conditions also quickly change with the environmental factors. This combined geochemical and diatom study found important results expressed in 3 groups of sites: *Group 1*, with extreme conditions of low pH (1.9-5.1), high metal concentration (0.4-1975 mg L^{-1}) and typical Fe-Mg-SO_4 waters. The taxa surviving in this group (mainly *Pinnularia aljustrelica*) were perfectly adapted to these extreme conditions, which can be due to strong silicification of the diatom frustule, once the silica contents were the highest in this group, which can grant the non-appearance of teratological (deformed) valves of diatoms. When teratologies occurred in this group (in *Eunotia exigua*) they appeared in sites of pH around 4 and metal contents started to decrease (around 40 mg L^{-1}). In waters, metals were mostly in cationic form of Me^{2+} type which may facilitate the diatom uptake. In sediments they are mainly adsorbed to the iron oxy-hydroxide phases; *Group 2*, included sites with intermediate pH (5.0-6.8) and metals (0.2-25 mg L^{-1}) conditions and with Fe-Mg-SO_4 to Mg-Cl waters, which enable the existence of typical species of uncontaminated streams (*Brachysira neglectissima, Achnanthidium minutissimum*). However, teratological forms also occurred in abundance in *A. minutissimum*. This group was still receiving some metals input, which affected these sensitive species; *Group 3*, unimpacted sites, with low metal contents (0-7.33 mg L^{-1}), high pH (7.0-8.4) and Mg-Cl waters; high contents of Cl^- which can justify the presence of brackish to marine species (e.g. *Entomoneis paludosa*) very silicified and not predisposed to deformations. In these sites there was a tendency to the association of the metals to the primary phases (e.g. sulphides) in sediments and therefore not so easily available. It was possible to conclude that for similar acidity conditions, large differences both in abundance as well as in the emergence of teratologies were explained by metals.

Keywords Surface waters, stream sediments, diatoms, metals, acidic pH, Aljustrel mine

6.1 INTRODUCTION

Acid mine drainage (AMD) is frequently found in regions with sulphide mineralizations and low contents of minerals capable of neutralizing acidity by alteration and dissolution. Mining exploitation exposes these minerals (mainly pyrite) in reduced form to atmospheric conditions suitable for their oxidation, producing mineralized acid waters with high sulphate and metal ion concentrations, resulting from mineral alteration (Kuyucak 1999, Dragisic et al. 1999.

The traditional approach to investigate the environmental impacts of an abandoned mining site is based on geochemical survey methodologies using stream sediments which can be a useful medium for trace element analyses as they can accumulate chemicals over time and surface waters which give an immediate answer about the contamination degree of the water course. Several studies in metal-polluted rivers have shown that biological indicators are needed to evaluate stream quality conditions. Diatoms are excellent bioindicators due to their short generation time and ubiquity. They respond to these toxic effects not only at the community level through shifts in dominant taxa (Gustavson and Wängberg 1995, Hirst et al. 2004) as also with changes in diversity (Leland and Carter 1984, Medley and Clements 1998, Luís et al. 2009). Past research on acidophilic and acid-tolerant algae showed they are greatly tolerant to metals exercising a diversity of mechanisms to avoid their toxic effects (Whitton 1970, Gross 2000, Lessmann et al. 2000). Frustule deformations of diatoms have been correlated with high metal concentrations (Harding and Whitton 1976, Gold et al. 2003, Cattaneo et al. 2004, Ferreira da Silva et al. 2009) and are extensively discussed in an interesting paper review published by Falasco et al. (2009) on deformations (teratologies) in diatoms. This review highlights the main causes for frustule deformations. It is very important to determine the chemical speciation of dissolved metal species in order to understand the causes of valve teratologies. If the metal is found in a more easily absorbed species or not, might determine the appearance of deformations or not in diatoms.

The Aljustrel mining area is located in the South of Portugal and belongs to one of the most important and greatest metallogenic provinces of the world, the Iberian Pyrite Belt (IPB). With an approximated area of 13800 km^2 extending from Portugal to Spain, the IPB is characterized by the occurrence of volcanogenic massive sulphide deposits (VMS-type). Two main geological units are part of the IPB, a substrate composed by phyllite and quartzite rocks and an overlying Volcano-Sedimentary Complex that hosts the mineralization (Carvalho et al. 1976, Schermerhorn 1971 a, b). The geology of this area is

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characterized by the occurrence of the volcano-sedimentary sequences in the base, overlapped by a set of schists and then by a flysch sequence. A dolerite intrusion occurs along an important fault. Over all these stratigraphic sequences a succession of fluvial and continental sediments, mainly composed of silica and carbonate were deposited.

The Aljustrel mines are one of the greatest sulphide deposits of IPB, which enclose six mineral masses: S. João do Deserto, Moinho, Açor, Algares, Estação, Fetais and Gavião. These mines have a long exploitation history, dating back to pre-Roman periods until the present time, although it was in the XX century until the mid-80s - 90s that these mines experienced their greatest development. After one decade of inactivation, zinc started to be exploited in 2008 but after one year, they quit Zn and started Cu exploitation until nowadays.

Aljustrel mines, as well as others from IPB were considered of high degree of environmental hazard (Oliveira et al. 2002), due to the large volume of tailings (~5Mt) with high amounts of metals (Cu, Pb and Zn) and metalloids (As, Sb) (Matos and Martins 2006). The dispersion of these elements affects soils, sediments and waters and also biota. Acid mine drainage are one of the most relevant impacts in these regions where sulphide mineralizations occurs and in Aljustrel there are several water streams affected by AMD. For this reason, the environmental remediation of the Aljustrel mine area were considered priority and after a study period (2000 – 2003 and in 2005) was initiated the site recovery. It is a large and complex area, thus the recovery project was divided in 4 phases, beginning in 2006 and scheduled to finish in 2014. This project will cost more than 10.000.000€ (source: EDM – Empresa de Desenvolvimento Mineiro).

The purpose of this work is (1) to perform the overall chemical characterization of the sites (surface waters and stream sediments), with the aim of assessing land degradation; (2) estimate the background values for the surface waters and stream sediments and calculate the enrichment in contaminated sites; (3) comparison of diatom communities in unimpacted sites and in sites along a strong metal gradient; (4) explore the diatom communities concerning their structure (taxonomic composition and relative abundance) and morphology (occurrence of deformed valves), inferring which factors might contribute to their occurrence.

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6.2. METHODS

Sampling and Preservation

The selected sample media were: stream sediment, surface water and biofilms (diatom assemblages). Sampling was carried out in spring, summer and autumn of 2008 and spring and winter of 2009 in 19 sites. Totally, 91 samples of water, the same number of sediment samples and 155 samples of diatoms were collected. The sampling sites were selected considering areas under the mine influence and, therefore, with clear evidence of contamination and AMD processes, and areas outside the mine influence. Respectively 4 dams, two with clean water (DA- Águas Industriais dam, DB- Monte Ruas dam) and two impacted (DC- Mine dam DD- Estéreis dam), and 15 sites in streams (MI- Mine, BM- Barranco Morgado, MR- Monte Ruas, BE- Porto Beja, PC- Pte Curval, AF- Água Forte, AA- Água Azeda, RO- Roxo, PF- Porto Ferreira, JU- Jungeiros, RJ- Roxo Jusante, BX- Barranco Xacafre, BF- Barranco Farrobo, PB- Pero Bonito, CB- Canal Barrada) in 5 sampling moments (1- spring, 2 - summer and 3 - autumn of 2008; 4 - spring and 5 - winter of 2009). These sites are represented in **Fig. 2.4** (Chapter 2).

Surface waters: to determine physical and chemical parameters of surface water, a volume of 1 L was collected from the surface, as close to the river center as possible, in acid-rinsed polyethylene bottles. Temperature (T - ºC), pH and conductivity (μS.cm^{-1}, at 25 ºC) were recorded on site, using a multiprobe WTW Multiline P4 SET. Water samples were then returned in a cool box to the laboratory and stored at 4°C before the analyses, according to the protocols defined by German Chemists Association (1981), EPA- Environmental Protection Agency (1982) and ASTM-American Society for Testing and Materials (1984). In order to determine the concentrations of trace elements, cations and anions (dissolved phase), a volume of 250 mL was taken from each sample and filtered through 0.45 μm Millipore membrane filters using an all-plastic pressurized filtration system. A sub-sample of these filtered waters, preserved with ultra-pure nitric acid (HNO_3) to prevent metals precipitation and bacterial growth, was used for trace and major cations' analysis and another portion, non-acidified, for anions' analysis.

Stream sediments: for each sampling site, 3 kg of stream sediments were collected, according to ASTM (1984) standard. In the laboratory, bed-load stream sediment samples were oven dried at a constant temperature of 40 °C until a constant weight was attained before dry sieving. Samples were disaggregated and sieved through a minus 2 mm and 0.63 mm plastic sieve and then milled in agate mill for chemical and X-ray diffraction analyses, respectively.

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Diatoms: whenever possible, 3 types of diatom samples, based on substratum criterion were collected. The epilithic diatom samples were obtained by scraping the upper surface of five boulders using a toothbrush, the epiphytic by squeezing the submerged plants from the margins of the streams and the epipsammic by removing the top layer of the sediment surface with a syringe. Following the sampling protocol (Prygiel and Coste 2000), pools of stagnant water and shaded sites were avoided. Two samples were taken, one kept alive (without preservation) and the other preserved with formalin solution (5 % final concentration).

Analytical techniques

Surface waters: the chemical analysis of water samples was carried out using inductively coupled plasma mass spectrometry (ICP-MS) for the determination of 23 elements: Al, As, B, Ba, Ca, Cd, Cl, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Na, Ni, Pb, Sb, Si, Sr and Zn and ion chromatography for SO_4^{2-} and NO_3^- (Gjerde 1986). The HCO_3^- concentration was determined by titration with H_2SO_4 at 0.16 N. Spectrophotocolorimetry was used for NH_4^+ determination (Strickland and Parsons 1972, Grasshoff's 1976, Chaussepied 1977). The last 2 parameters were determined in the Geochemistry Laboratory of the Geosciences Department at University of Aveiro. A rigorous quality control program was implemented, during water chemical analysis which included reagent blanks, duplicate samples, and certified reference materials (STANDARD WASTWATRA6). Precision and bias of the chemical analysis was less than 10 %. Samples were analyzed at ACME (ACME Anal. ISO 9002 Accredited Lab, Canada). The determination of CQO (colorimetric method (MILI 01, internal method)) and P_2O_5 (colorimetric method, adapted from Clescerl et al. 1998) took place at IDAD Laboratory at the University of Aveiro.

Stream sediments: the chemical analysis of sediments was performed by inductively coupled plasma mass spectrometry (ICP-MS) and by inductively coupled plasma emission spectrometry (ICP-ES) in the accredited laboratories ACME Lab (ACME Anal. ISO 9002 Accredited Canadian Lab-Canada). The analysis of the major elements - Al, Fe, Ca, Mg, Na, K, Si and P – and elements - Ba, Cr, Co, Mn, Sr and Ti - was made by ICP-ES and trace elements analysis - As, Cd, Cu, Pb, Sb, Ni and Zn - was run by ICP-MS after a lithium metaborate/tetraborate fusion. The mineralogy of the <63 µm fraction of 7 selected samples of stream sediments was characterized by powder X-ray diffraction (XRD) (analysis carried out in the Geosciences Department) using a Philips X'Pert MPD, equipped with an automatic divergence slit, CuKα (λ = 1.5405 Å) radiation (20 mA and 40 kV) and a Ni filter, in the range of 4 - 70º (2θº), with a step increment of 0.5 s for each

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0.02° (2θ°). Selective Sequential Chemical Extraction (SSCE) procedure was done in order to elucidate the mode of occurrence of the metals in the stream sediment samples, and establish the geochemical patterns of trace metals, which are useful for predicting their release ability into the aquatic environment and into the ecosystem. Samples MR, BE, PC, AF, AA and CB from spring 2008 were sequentially treated with different reagents so that metals with different affinities for the mineral matrix could be released (Tessier et al. 1979, Quevauviller et al. 1998, Gómez-Ariza et al. 2000). The scheme used follows a 6-step sequential extraction procedure, outlined by Cardoso Fonseca and Martin (1986) and Cardoso Fonseca and Ferreira da Silva (1998) as follows: Step 1: ammonium acetate (1M NH_4Ac, pH 4.5) - water soluble and dissolved exchangeable ions, specifically adsorbed and carbonate-bound; Step 2: hydroxylamine hydrochloride (0.1M $NH_4OH.HCl$, pH 2) - ions bound in Mn oxyhydroxides; Step 3: ammonium oxalate (dark) (0.175M $(NH_4)_2C_2O_4$–0.1 M $H_2C_2O_4$, pH 3.3 - Tamm reagent in dark) - ions linked to amorphous Fe oxides; Step 4: H_2O_2 35 % - ions associated to organic matter; Step 5:ammonium oxalate (UV) (0.175 M $(NH_4)_2C_2O_4$–0.1 M $H_2C_2O_4$, pH 3.3 - Tamm reagent under UV radiation) - ions associated to crystalline Fe oxides; Step 6: mixed-acid ($HCl+HNO_3+HF$) decomposition - ions associated to matrix elements in lattice positions, resistant oxides and some sulphides. After each reaction timing, the solutions were centrifuged and filtered. For metal speciation (Cu, Fe, Mn, Pb, and Zn) the sampled solutions were analyzed by Atomic Absorption Spectrometry (AAS) using a GBC906 spectrophotometer.

Diatoms: the live samples were examined in order to exclude dead diatoms to avoid errors in the estimation of abundances. From the second sub-sample (preserved with formalin), an aliquot was treated with HNO_3 (65 %) and potassium dichromate ($K_2Cr_2O_7$) at room temperature for 24 h, followed by three centrifugations (1500 rpm) to wash the excess of acid. Then, permanent slides of the samples were prepared using Naphrax®. Diatoms were identified and semi-quantified under the light microscope (Leitz Biomed 20 EB) using a 100 × objective (N.A. 1.32). A total of about 400 valves were counted in each sample. Taxonomy was based on Germain (1981), Krammer and Lange-Bertalot (1986, 1988, 1991 a, b), Simonsen (1987), Round et al. (1990) and Prygiel and Coste (2000). Light microscopy photographs of diatoms were taken with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan). Microphotographs were digitally manipulated and the plates were made using CorelDraw version X6.

Data Analysis

Surface waters: Principal Component Analysis (PCA) was carried out in order to reduce the dimensionality of the data set in which there were a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This reduction is achieved by transforming to a new set of variables, the principal components, which are uncorrelated, and which are ordered so that the first few retain most of the variation present in the entire original variables. The environmental data matrix used for PCA, with no transformation, was composed by: Al, As, B, Ba, Ca, Cd, Cl, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Na, Ni, Pb, Sb, Si, Sr and Zn, SO_4^{2-}, HCO_3^-, NH_4^+, P_2O_5, CQO, NO_3^-, pH, T ºC (Temp.) and conductivity. A Spearman correlation matrix was done to evaluate the correlations between diatoms and some elements. PCA and Spearman correlation matrix were done in the software Statistica version 8.0. The water samples classification was done with Piper (Piper 1944) and Ficklin (Ficklin et al. 1992) projections. The chemical and physico-chemical data were plotted in boxplots (SigmaPlot, version 12) to compare concentrations and variation between the impacted and unimpacted samples. A geochemical modeling of the water samples was made using the PHREEQC software (version 3.0, Parkhurst and Appelo 2013) for calculating the ion activity and the chemical speciation of dissolved species. The thermodynamic database used was *minteq.v4.dat* (U.S. Environmental Protection Agency 1998). A mass-balance equilibrium model has been used to calculate the elemental aqueous speciation and the stability of solid phases with respect to the dissolved constituents.

Stream sediments: descriptive statistic was made (range values, median and mean) with sediment data. The major oxides determined by ICP-ES were converted in elemental amounts for an easier comparison with water results.

Diatoms: sampling 4 (spring 2009) was not used for diatom analysis because it was not possible to count at least 400 valves in the majority of the samples. Sites MI and DD never had diatoms due to extremely adverse conditions: low light conditions and extremely high metal concentrations and low pH. To study the diatom responses (diatom matrix) to the environmental conditions (defined by 32 surface water variables), a first distance-based permutational multivariate analysis of variance (dbRDA) was done using Primer 6 software (Primer-E Ltd, Plymouth, UK). A square root transformation to retain zero values and to have a balance between the contribution of rarer and dominant species was done on the diatom data. After, a log transformation to reduce positive skew was added. dbRDA is a three-step ordination process: which first calculates a distance matrix, second runs a Principal Coordinate Analysis (PCO), third runs a Redundancy Analysis (RDA) on the eigenvalues obtained from the PCO. The matrix was composed by 244

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taxa*108 samples*32 environmental variables. However, with this first dbRDA, metals showed a multi-collinearity behavior which should be avoided: if two or more variables in X are truly collinear, they contain the same information thus, they are redundant for the purpose of the analysis.

The metal with more collinear variables ($|r| \geq 0.95$) was used as a surrogate variable, which in this study was Cu (identified in the diagram as *metal*); metal Pb and metalloids As and Sb didn't show $|r| \geq 0.95$, thus were treated as independent variables. The same square root transformed matrix of diatom data was used but excluding taxa of dams DA, DB and DC (diatom matrix now: 244 taxa*93 samples) because they overlapped to other sites due to their different physical, chemical and biological characteristics.

Thus, the final environmental matrix used for dbRDA was composed by: metals/metalloids - Cu, Pb, As, Sb - and Si, Ca, Mg, K, Cl, Na, P_2O_5, SO_4^{2-}, HCO_3^-, NH_4^+, CQO, pH, conductivity (Cond) and T °C (Temp) ($\log_{10}$ transformed). A final biological/environmental matrix: 244 taxa*93 samples*18 environmental variables was achieved to run a second dbRDA.

ANOSIM test (analysis of similarity), an analogue to the univariate 1- and 2-way ANOVA (analysis of variance) tested for differences between groups of diatoms. SIMPER analysis was used to determine the species responsible for the largest contribution to the Bray-Curtis dissimilarity in diatoms abundance between sites. The dbRDA, SIMPER and ANOSIM were run in the software Primer 6 (Primer-E Ltd, Plymouth, UK).

6.3 RESULTS

Waters

According to the modified Piper (1944) diagram (total Fe was added, given its high abundance in these waters) it was possible to distinguish 2 general groups of samples: one of impacted samples (Group 1), with Fe-Mg sulphated waters and another of unimpacted samples (Group 3), with Mg-chloride waters. There was also a transition group (Group 2), with Fe-Mg sulphated to Mg-chloride waters (**Fig. 6.1**).

Ficklin diagram (Ficklin et al. 1992), plotting the pH values versus the sum of base metals (Zn, Cu, Cd, Pb, Co and Ni) in **Fig. 6.2**.

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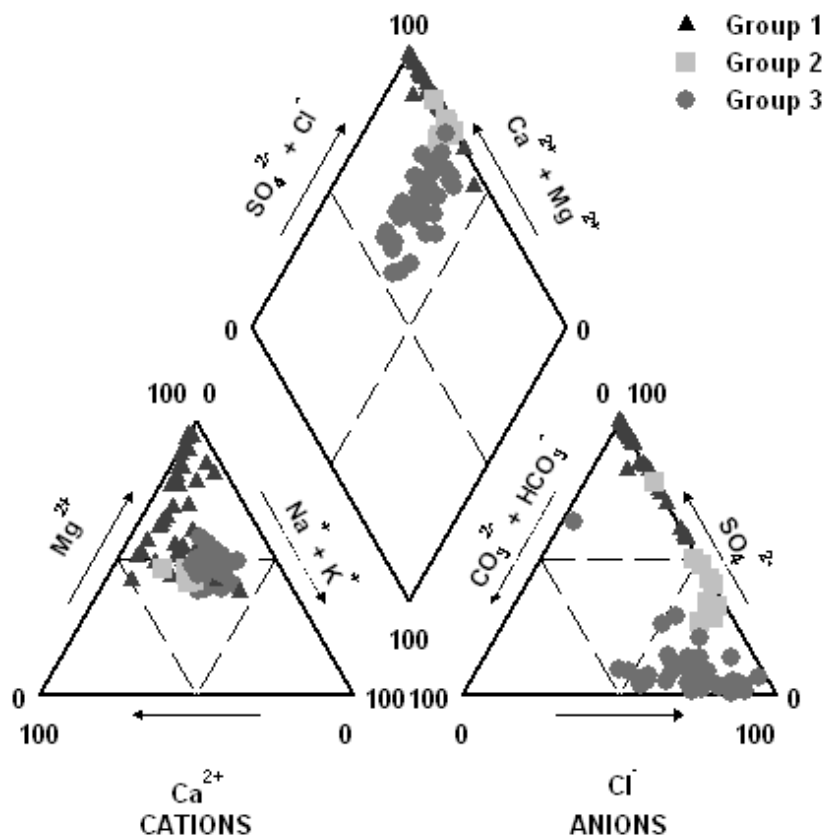
Fig. 6.1 – Piper diagram of the surface water samples.
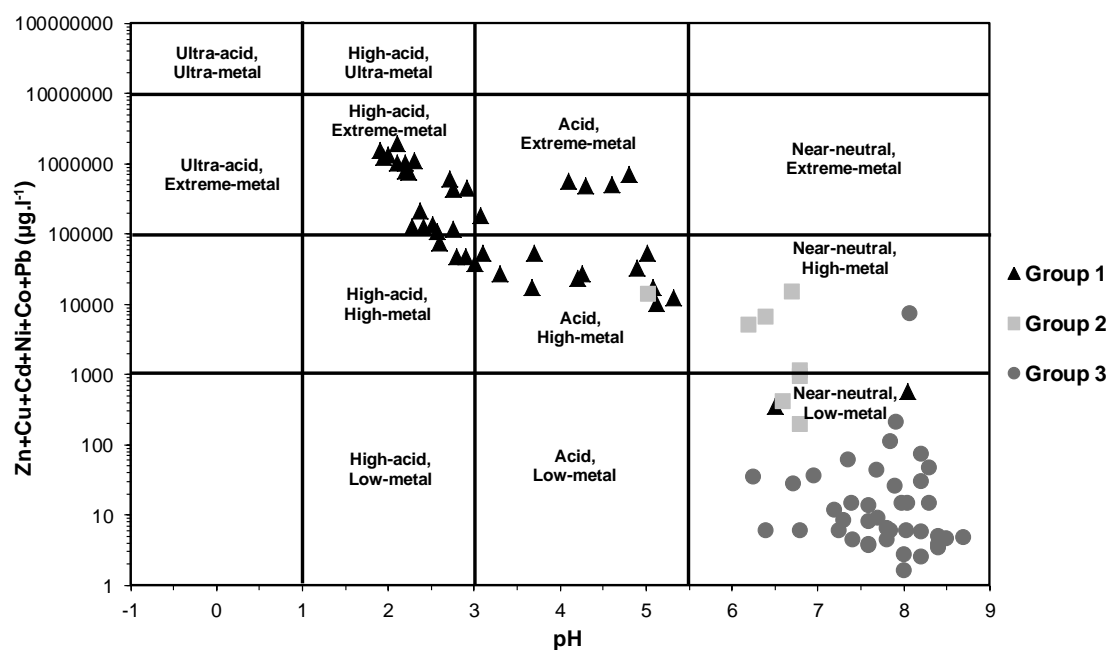
Fig. 6.2 – Ficklin diagram of the surface water samples.

According to the figure three groups of samples were defined:

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- Group 1, plotted in the High-Acid/Acid and Extreme Metal/High-Metal area represents the impacted sites from Água Forte (BM, MR, BE, PC, AF) and Água Azeda (AA) streams [the exceptions are sample BM4 (high pH and high concentration of Ca probably due to CaOH addition to control acidification) and PC2 (increase of NH_4^+ and NO_3^- concentrations, related with municipal waste water treatment station of Aljustrel town];

- Group 2, plotted in the Near-Neutral and High-Metal to Low-Metal is composed by intermediate impacted sites JU and RJ (Roxo stream, after confluence of impacted streams of Água Azeda and Água Forte) [except for sample RJ4 that is plotted in Acid and High-Metal due to AMD impact];

- Group 3, plotted in the Near-neutral, Low-metal is formed by unimpacted sites PF, RO (Roxo stream), BX, BF, PB, CB (Roxo unimpacted tributaries). The exception was sample PB1 (high pH, high metal and SO_4^{2-} concentrations). This site is not under the mine influence, thus these results can be explained by a punctual discharge with contaminated material that is afterwards diluted by the stream water.

Box plot diagrams (**Fig. 6.3**) evidenced the variation and dispersion of the elements present in the water.
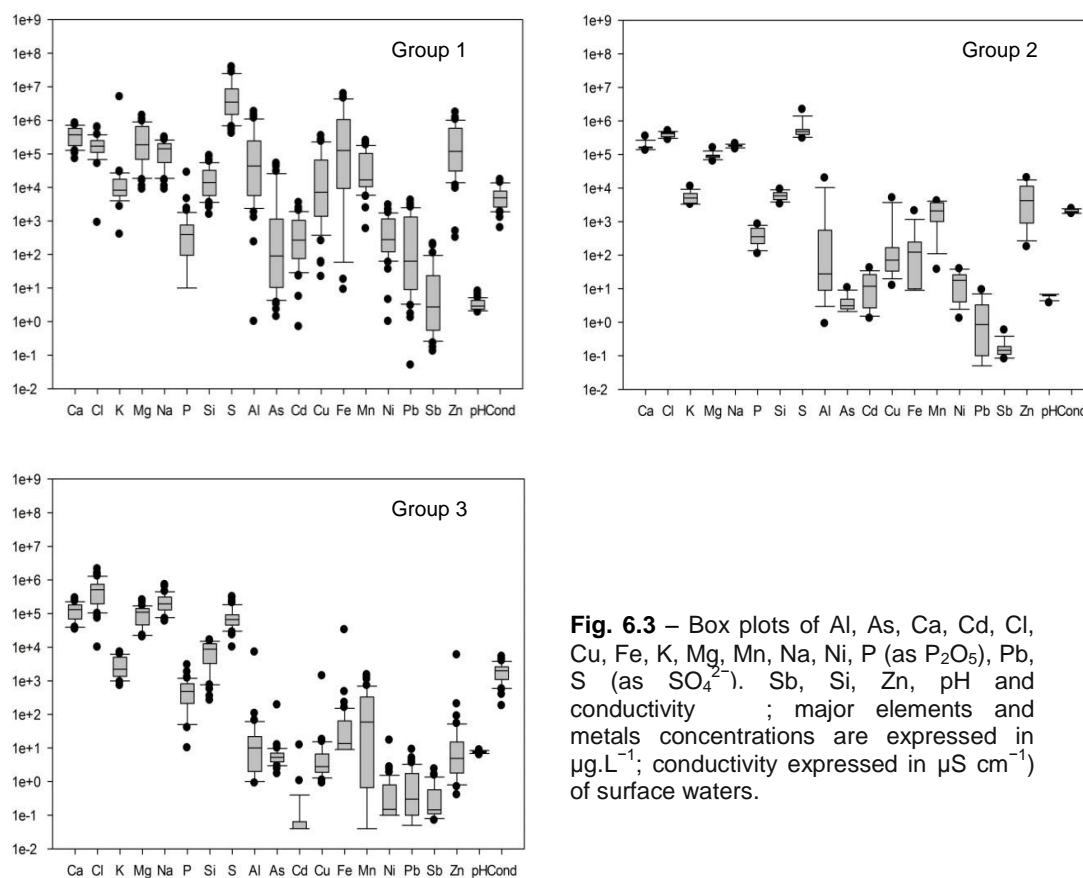
Fig. 6.3 – Box plots of Al, As, Ca, Cd, Cl, Cu, Fe, K, Mg, Mn, Na, Ni, P (as P_2O_5), Pb, S (as SO_4^{2-}). Sb, Si, Zn, pH and conductivity ; major elements and metals concentrations are expressed in $\mu g.L^{-1}$; conductivity expressed in $\mu S\ cm^{-1}$) of surface waters.

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According to the results, a higher dispersion and concentration was found, especially for metals (Al, As, Cd, Cu, Fe, Pb, Sb, Zn), SO_4^{2-} and Si (Group 1). Group 2 presented also high dispersion of the values of Al, Cd, Cu, Fe, Pb, Zn (not as high as in group 1) and in Group 3, Mn and Cl presented high concentrations for a group of unimpacted sites. Mn can be explained by punctual mine contamination and high conductivities be due to Cl dominance. PCA results are shown in **Table 6.1.**

Table 6.1 – Correlations between the variables and the PCA factors (in bold, variables with values > |0.5|), eigenvalues, percentage of explained variance and percentage of cumulative variance for surface waters (nxp=32x90).

	Factor 1	Factor 2	Factor 3
Al	**0.89**	0.24	-0.17
As	**0.70**	0.38	-0.14
B	**0.79**	-0.09	0.02
Ba	-0.41	0.03	**-0.66**
Ca	**0.53**	**-0.65**	0.13
Cd	**0.96**	0.06	-0.05
Cl	-0.40	-0.15	**-0.79**
Co	**0.98**	0.07	-0.11
Cr	**0.88**	0.07	-0.17
Cu	**0.92**	0.32	-0.14
Fe	**0.87**	0.30	-0.13
K	**0.56**	0.21	-0.25
La	**0.93**	-0.11	-0.04
Li	**0.93**	-0.16	-0.00
Mg	**0.73**	**-0.60**	-0.04
Mn	**0.81**	-0.44	0.06
Na	-0.36	-0.45	**-0.63**
Ni	**0.98**	-0.09	-0.07
P_2O_5	0.17	-0.41	0.02
Pb	0.33	**-0.70**	0.28
SO_4^{2-}	**0.92**	0.18	-0.04
Sb	**0.65**	0.17	-0.02
Si	**0.87**	0.12	-0.26
Sr	-0.03	**-0.69**	-0.41
Zn	**0.96**	0.02	-0.04
NO_3^-	**0.57**	0.28	0.02
NH_4^+	-0.11	0.07	0.41
HCO_3^-	-0.42	0.07	**-0.70**
CQO	**0.69**	-0.06	-0.11
pH	**-0.69**	0.14	-0.41
Temp	0.09	-0.44	0.07
Cond	**0.94**	-0.05	-0.18
Eigenvalues	16.54	3.23	2.86
% Explained variance	51.69	10.10	8.94
% Cumulative variance	51.69	61.79	70.73

The retention factors were based on an empirical criterion of eigenvalues >1 (Davis 1973, 1986). According to the defined criteria, three factors were retained, explaining 70.73 % of the total inertia. Percentage of explained variance and of cumulative variance of PCA factors are also shown in **Table 6.1**. Factor 1 explained the association between the variables (from the highest to the lowest correlation): Co, Ni, Cd, Zn, conductivity, La, Li, Cu, SO_4^{2-}, Al, Cr, Fe, Si, Mn, B, Mg, CQO, As, Sb, K, NO_3^- and Ca (positive scores) and also pH (negative scores), with samples from Água Forte stream contributing the most for this distribution. This factor represents the chemical signature of Aljustrel mineralization. Factor 2 explained the variables Pb and Sr (with negative scores) which were mostly related with MI and DD sites located inside the mining area; while factor 3 explained the association of variables Cl, HCO_3^-, Ba and Na, most of them related with local geology.

The associations: Ca-Mg; Cl-Na; Fe-Cu; Zn-Cd highlighted in this diagram are the same as the ones in water's PCA of Luís et al. (2009). The projection of variables on PCA's first factorial plane explained 61.79 % of the total matrix information, establishing groups of chemical elements according to their affinity (**Fig. 6.4**).
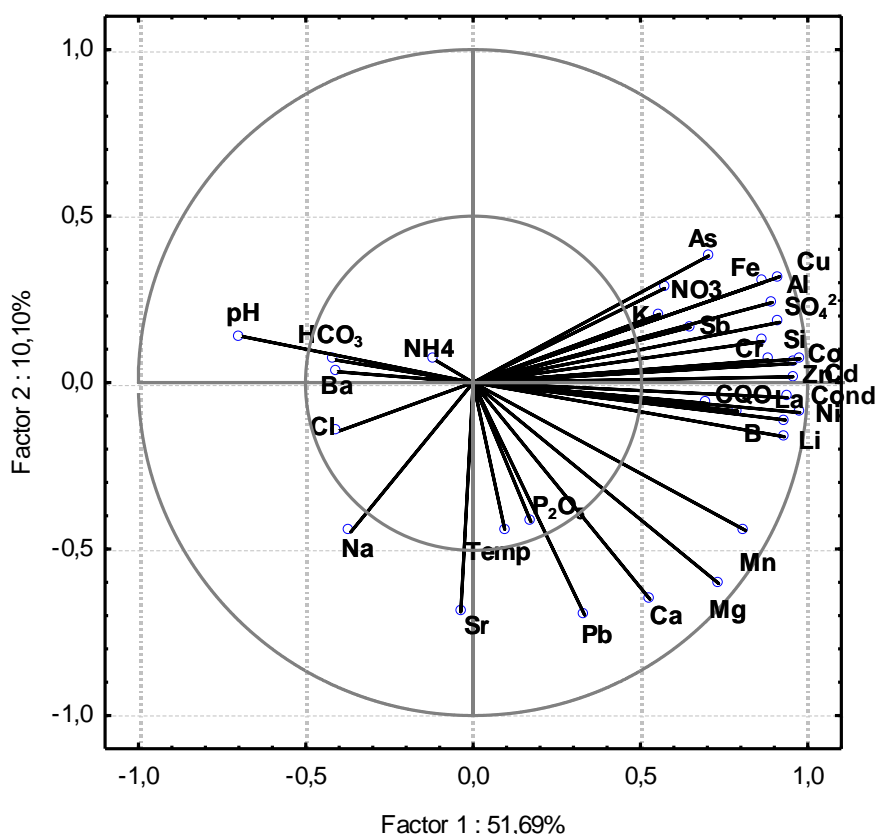
Fig. 6.4 – Principal component analysis of surface waters: projection of the variables on the first factorial plane (factor 1/factor 2).

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Sediments

Chemical data (range variation, median and average) of the stream sediments collected in impacted and in unimpacted sites in April of 2008 are shown in **Table 6.2**. Teratological forms occurred during this sampling period.

Table 6.2 – Chemical concentration (minimum, maximum, median and mean values) of elements in stream sediments from chemical elements analyzed in impacted and unimpacted sites of Aljustrel mining area (n=92).

Element	Unit	Impacted Sites			Unimpacted Sites		
		Min-Max	Median	Mean	Min-Max	Median	Mean
Si	mg kg^{-1}	14537- 361971	252781	241654	206413 - 412406	355170	343986
Al	mg kg^{-1}	3863 - 104734	43529	44203	4400 - 87693	32786	38010
Fe	mg kg^{-1}	20200 - 393217	118931	124061	11820 - 72530	35881	36240
Mg	mg kg^{-1}	905 - 13508	4161	5407	1200 - 12061	4511	5392
Ca	mg kg^{-1}	200 - 35663	2573	4324	600 - 33519	5396	8333
Na	mg kg^{-1}	74 - 15134	4859	5492	180 - 14244	4970	4872
K	mg kg^{-1}	800 - 26482	11788	11282	400 - 16105	5728	6759
P	mg kg^{-1}	22 - 2410	508	644	44 - 916	310	340
As	mg kg^{-1}	28 - 5439	981	1299	4 - 148	16	29
Ba	mg kg^{-1}	42 - 2957	394	430	36 - 470	207	218
Cd	mg kg^{-1}	0.2 - 65	1	6	0.1 - 6	0.1	0.6
Co	mg kg^{-1}	3 - 137	12	21	6 - 72	16	18
Cr	mg kg^{-1}	14 - 342	55	59	18 - 151	68	73
Cu	mg kg^{-1}	69 - 10000	393	1242	9 - 3302	22	285
Mn	mg kg^{-1}	225 - 1007	465	521	320 - 2943	1007	10323
Ni	mg kg^{-1}	6 - 62	21	24	10 - 50	19	23
Pb	mg kg^{-1}	42 - 10000	389	1343	10 - 315	30	41
Sb	mg kg^{-1}	1 - 955	22	98	0.2 - 11	0.8	1
Sr	mg kg^{-1}	7 - 181	87	84	11 - 212	61	68
Zn	mg kg^{-1}	154 - 10000	483	1683	12 - 4004	43	338

The values of metals and metalloids in impacted sites largely exceed the usual concentration in sediments (Reimann and Caritat 1998). A comparison between the chemical composition in both groups (unimpacted and impacted) showed that As, Ba, Cd, Co, Cu, Ni, Pb, Sb, Sr, Zn, Al, Fe, Mg, Na, K and P mean concentrations can range from 1 to 97 times higher in impacted than in unimpacted sites. The exceptions are Si, Ca, Mn and Cr mean concentrations which are slightly higher in the unimpacted samples. With the exception of As, Ba, P, Na, Sb and Sr, the sediments/water ratio of the metals tended to be higher in the unimpacted sites because pH increase facilitated the precipitation of these metals in sediments, while in the impacted sites the low pH values leads to the opposite, the dissolution of these metals in waters, thus the increase of concentrations in waters.

The mineralogy of the sediments obtained by XRD is presented in **Fig. 6.5**. The mineralogical content wasn't highly variable between samples from the same group.
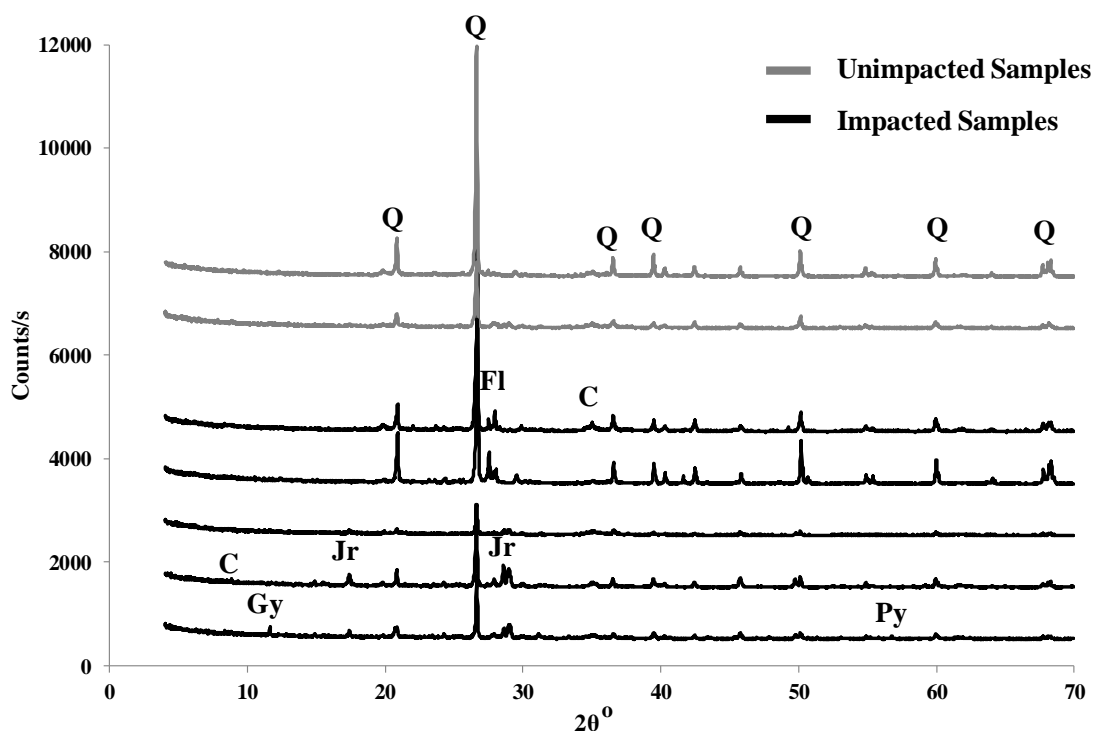
Fig. 6.5 – X-ray diffraction patterns of sediment samples from impacted and unimpacted samples. Q – quartz; Fl – feldspars; C – clay minerals; Py – pyrite; Gy – gypsum; Jr – jarosite

Comparing samples MR, BE, PC, AF and AA (impacted sites) and PB and CB (unimpacted sites) it is possible to observe that quartz and feldspars were present in all samples, which represents the residual fractions resulting from the erosion of rocks. In samples from unimpacted sites these residual fractions were dominant. Sometimes clay minerals are also identified which may result from feldspars alteration. Samples from impacted sites showed also some clay and an increase in phyllosilicates and sulphates/oxy-hydroxysulphate phases like jarosite and gypsum. As jarosite is only stable in highly acidic conditions, it is only present in samples with water pH~2, while in sample AA (pH~4-5), jarosite is not present. In some samples it was possible to identify some primary sulphides (pyrite). Probably, these sulphides didn't undergo oxidation because the oxy/hydroxides coatings precipitated on its reactive surface.

The sequential chemical extraction procedure provides information about the metal support phases. For the selected samples, the amounts of Cu, Zn, Pb, As, Fe and Mn (the most important elements in these mining areas) were extracted, with each reagent, which was determinant to infer about the main support phases responsible for the greater or lesser mobilization of the metals (**Fig. 6.6**).

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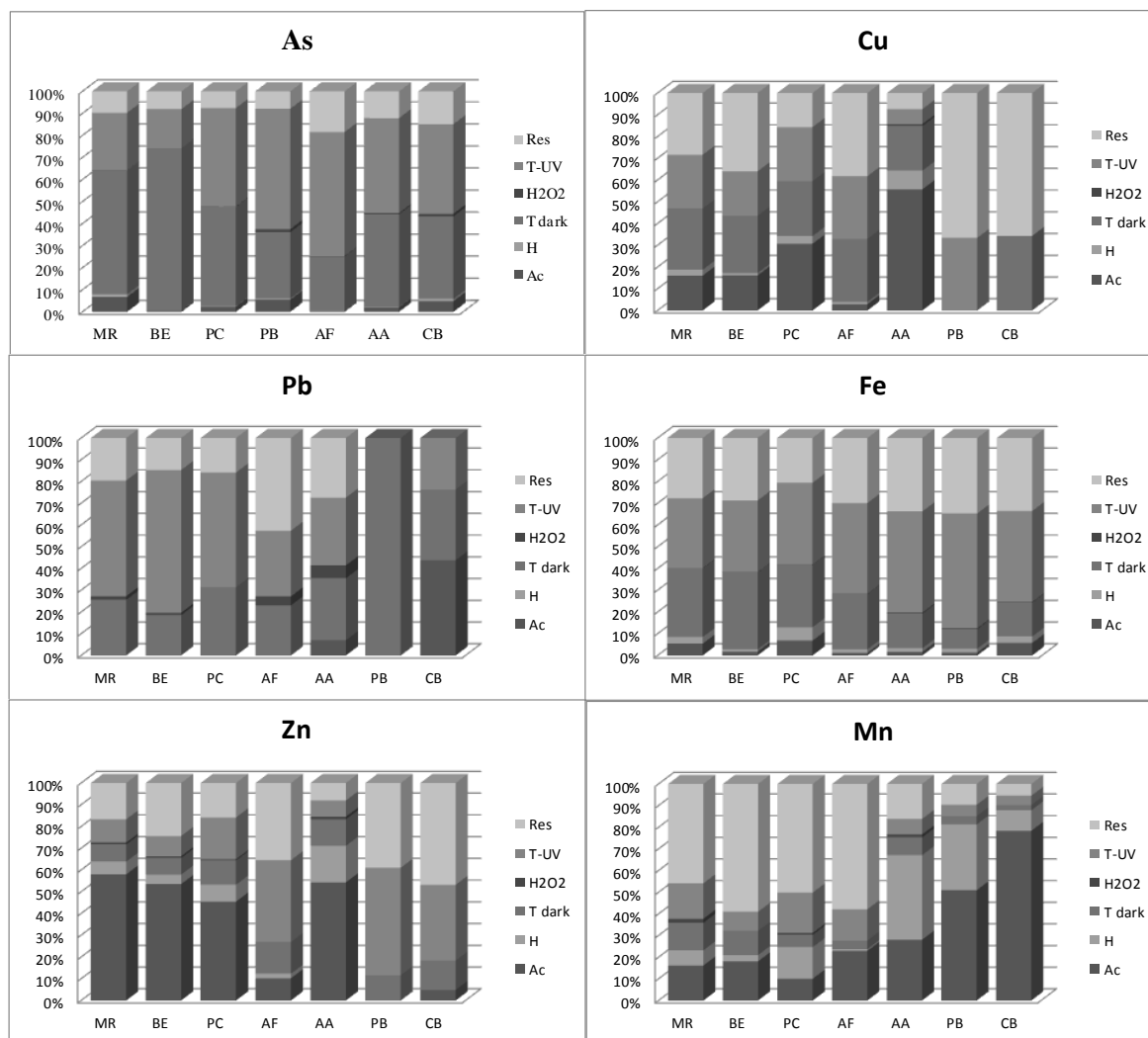
Fig. 6.6 – Percentage of extraction of As, Cu, Fe, Mn, Pb and Zn obtained by SCE procedure in sediment samples from impacted sites (MR, BE, PC, AF and AA) and unimpacted sites (PB and CB) from mining areas of Aljustrel. Ac - Ammonium acetate (1 M NH₄Ac, pH = 4.5); H - Hydroxylamine hydrochloride (0.1 M NH₂OH HCl, pH = 2); T dark - Tamm solution in darkness (0.175 M (NH₄)₂C₂O₄ - 0.1 M H₂C₂O₄, pH = 3.3); H₂O² - H₂O₂ 35 %; T-UV - Tamm solution under U.V. radiation (0.175 M (NH₄)₂C₂O₄ - 0.1 M H₂C₂O₄, pH = 3.3); Res - Mixed acid heated solution (HCl-NHO₃-HF). Abbreviations: MR- Monte Ruas, BE- Porto Beja, PC- Pte Curval, PB- Pero Bonito, AF- Água Forte, AA- Água Azeda, CB- Canal Barrada.

Copper was distributed mainly in the residual fraction (sulphides) in the unimpacted samples, but in the remaining samples it seemed to be associated with different phases as Fe-oxides/hydroxides. An amount was also extracted with ammonium acetate which is associated with the bioavailable fraction, aspect that was not verified for PB and CB samples. Zinc showed different behavior between the unimpacted and impacted samples. An amount of this metal seemed to be present in the residual fractions and in the Fe-oxyhydroxide phases, mainly in the unimpacted samples. Although, the amount of Zn extracted from the exchange fraction was very high in the impacted samples. This aspect reflects the high capacity of Zn to be mobilized. Lead was mainly associated with Fe-oxides/hydroxides phases in both types of samples, but in impacted samples it was

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possible to observe a part of Pb extracted from primary sulphides. Only in sample CB an amount of this metal was in the bioavailable fraction. In the cases of As and Fe, there was no clear distinction between the impacted and unimpacted samples. Arsenic was mainly associated with Fe-oxides/hydroxides phases, while Fe also showed an association with the primary sulphide phases, although Fe-oxides/hydroxides continued to be the main support phases. Manganese, in unimpacted samples, was highly concentrated in the bioavailable fraction, while in impacted samples its principal association was with the residual fraction (primary minerals) and with Mn-oxides.

Diatoms

Spring of 2009 (sampling 4) was very atypical not due to rainfall but probably due to some punctual mining discharge. Most sites showed even lower pH during the referred period, especially in Group 2, and no stable diatom communities were found.

The dbRDA analysis evidenced the relation between geochemical parameters and diatom communities (**Fig. 6.7**), showing the same 3 groups of samples also found in Ficklin and Piper diagrams.
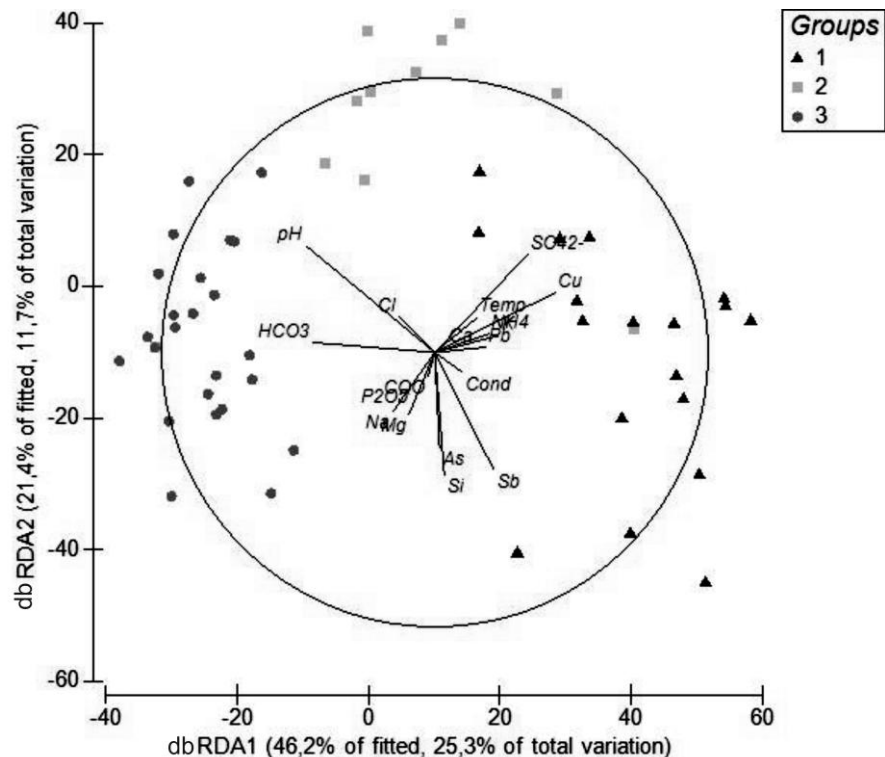
Fig. 6.7 – Distance based redundancy analysis (dbRDA) for the diatom resemblance matrix evidencing the samples disposition and the environmental variables which explain 25.3 + 11.7 % of total fitted variation. pH, SO_4^{2-}, metals (Cu as surrogate) and metalloids (As, Pb and Sb) were the main variables that divided treatments in 3 groups: 1- acid/ high metal concentrations, 2- neutral/ intermediate metal concentrations 3- neutral to alkaline/ low metal concentrations.

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According to **Fig. 6.7**, it was possible to individualize 3 groups: Group 1- impacted sites, associated with metals and SO_4^{2-}. Sites MR and BE were affected by high As (higher dissolved concentrations due to the lowest pH of these samples), Sb and Si concentrations; sites PC and AF, affected by high metal concentrations; and SO_4^{2-} was correlated with sites BM and AA. Group 2 appeared as a transitional group. In Group 3, site CB was the most distant from the other sites, due to strong correlations with Na and Mg, while sites BF, BX, PF, RO and PB were more correlated with pH and HCO_3^- due to high pH values here represented. The pH was a very important factor differentiating the 3 groups: $pH_{Group\ 1}$ = 1.9-5.1; $pH_{Group\ 2}$ = 5.0-6.8; $pH_{Group\ 3}$ = 7.0-8.4. This differentiation was possible due to the main diatom taxa: *Pinnularia aljustrelica* Luís, Almeida et Ector (PALJ), *Eunotia exigua* (Brébisson ex Kützing) Rabenhorst (EEXI) and *Nitzschia* a.f.f. *hantzschiana* Rabenhorst (NHAN) for Group 1; *Achnanthidium minutissimum* (Kützing) Czarnecki (ADMI), *Brachysira neglectissima* Lange-Bertalot (BNEG) for Group 2; *Navicula veneta* Kützing (NVEN), *Achnanthidium minutissimum* (ADMI), *Navicula gregaria* Donkin (NGRE), *Nitzschia desertorum* Hustedt (NDES) for Group 3.

SIMPER identified *Pinnularia aljustrelica* (PALJ) as contributing with 63.80 % for the similarity between impacted sites (Group 1). This species made the largest contribution to the dissimilarity in abundance between groups 1 and 3, considering the estimated average dissimilarity of 95.26 %.

ANOSIM determined the 3 groups found in dbRDA as statistically different (r = 0.88, p < 0.001).

As *Pinnularia aljustrelica* (PALJ) contributed to the similarity between impacted sites - Group 1 - in SIMPER, a Spearman correlation was calculated between this species, metals, pH and conductivity. PALJ was well correlated with As, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, SO_4^{2-}, Zn, Sb as well as with pH and conductivity (all metals and metalloids except for Pb). Samples with high PALJ relative abundances (from 65 to 100 %) showed a pH range between 2.0 and 4.0. It also contributed to the dissimilarity in diatoms between impacted (Group 1) and unimpacted sites (Group 3).

Morphological teratologies were observed in *Eunotia exigua* (EEXI), in some impacted sites (Group 1). These deformations were found at sites BM and AF, with lower metal concentrations and less acid pH than those found in sites MR and BE. EEXI deformed valves appeared in 3 samples of site AF with 4 % of counted deformed valves from a total of 60 %, 3 % in 16 % and 2.5 % in 11 %. In site BM it just appeared in one sample: 1 % deformed valves in a total of 8.5 %; pH range in sites AF and BM: 2.6-4.3. Although abundances (normal and deformed valves) of *Achnanthidium minutissimum* (ADMI) increased with the increasing of pH and decreasing of metal concentrations and

conductivity, deformations just appeared in Group 2; ADMI deformed valves appeared in site JU: 1.5 % of deformed valves in a total of 45 % and 6 % in 67 %. In site RJ: 8 % in 86 %; pH range in sites JU and RJ: 6.1-6.8. Due to the low number of samples with teratological forms, it was not possible to establish good correlations between metals/pH and teratological forms (**Fig. 6.8**) (as in Luís et al. 2011).
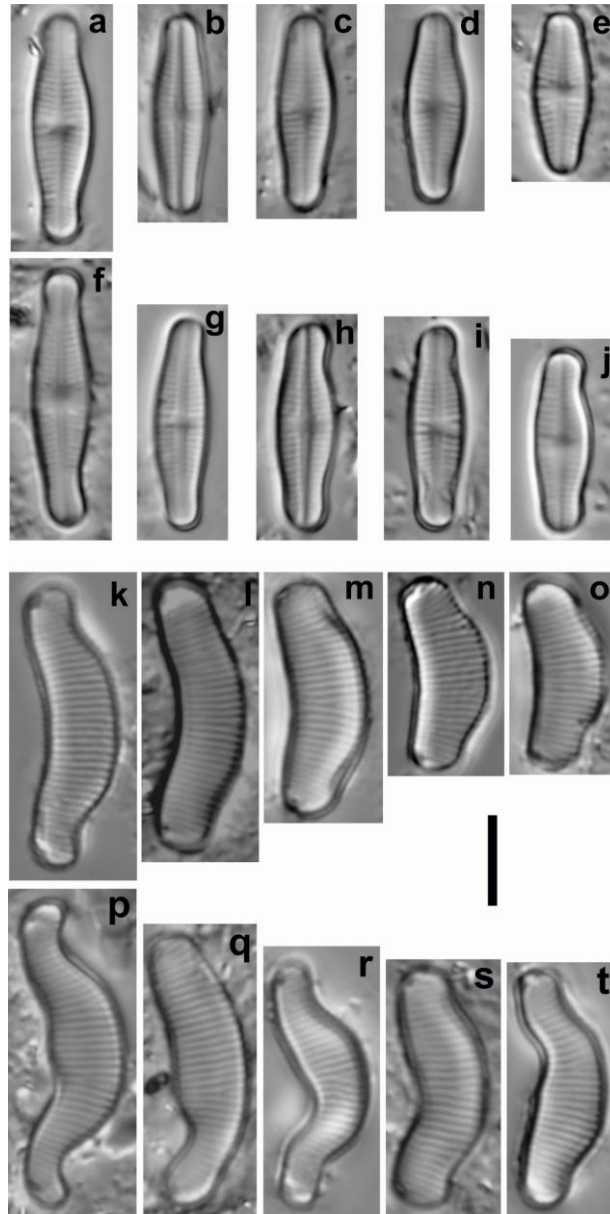
Fig. 6.8 – Light micrographs of *Achnanthidium minutissimum:* normal (a-e) and teratological (f-j) valves and of *Eunotia exigua:* normal (k-o) and teratological (p-t) valves; *Achnanthidium minutissimum* population is from RJ site, sampling 1, epiphytic sample; *Eunotia exigua* population is from site AF, sampling 1, epiphytic sample (l, p, q, s) and sampling 2, epilithic sample (k, m, n, o, r, t); magnification 3000x; scale bar 5 µm.

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The deformed frustules of ADMI were characterized by primary deformations, mainly by one more or less bent off endings.

The speciation was made using PHREEQC. Through this analysis, it was possible to see the dominance of metal species in each group of samples (**Fig. 6.9**). In Groups 1 and 2, where the teratological forms appeared, the metal is in divalent form (Me^{2+}), thus, easily absorbed, whereas in Group 3 the metals are in the form of hydroxide (Me-OH) which will hardly be absorbed.
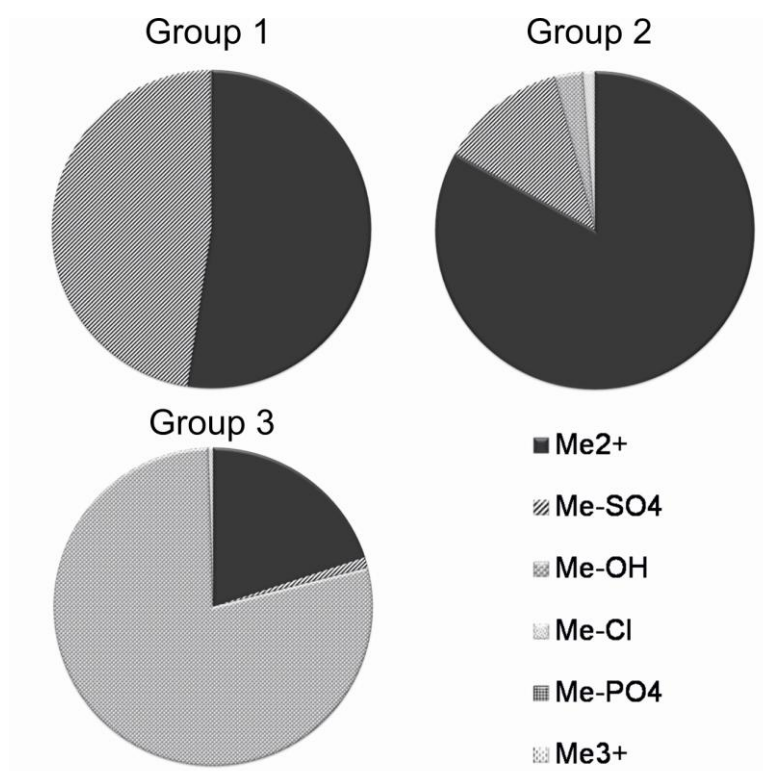
Fig. 6.9 – Circular diagrams showing the most abundant metal species in each group, obtained with PHREEQC software.

6.4 DISCUSSION

Sampling years were very atypical, with low rainfall, namely 2008 that was a very dry year, even in winter, which can explain the low variation in water chemistry between the different sampling moments. Some general associations given by water parameters were evidenced by PCA analysis: Ca-Mg representing carbonates; Cl-Na, salts; Fe-Cu, regarding primary sulphides as chalcopyrite and Fe secondary phases complexation with other metals, especially with Cu; Zn-Cd, evidencing sphalerite signatures. It was also possible to define that AMD impacted waters of Group 1, presented the highest

concentrations of metals (especially Al, Cu, Fe, Ni, Pb, Zn), metalloids (As, Sb) and SO_4^{2-} and the lowest pH. In Água Forte stream, pH, conductivity and metals were clearly influenced by rainfall. Thus, with rainfall increase, the water pH decreased, whereas in periods with less rainfall, pH tended to increase. On the contrary, conductivity and metals' concentration clearly varied inversely to pH. This fact proves that in driest periods the sulphate phases tend to precipitate, retaining metals. As these phases are extremely soluble, when the first rain occurred, the dissolution of these phases also occurred, increasing metals' concentrations and conductivity in waters (Nordstrom 2009). However, in Água Azeda stream, still belonging to Group 1 but with higher pH values and lower conductivity than those found in the previous stream, physico-chemical parameters' variation and metal levels didn't show a strong rainfall influence. Although in a first phase, pH tended to decrease with the rainfall increase, after a short period, rainfall tended to increase the pH. This fact resulted from a dilution effect as well as dissolution of other ionic species, since conductivity had an analogue behavior to pH and not conditioned by metals that tended to decrease their concentrations along the different seasons. In Roxo stream, (Groups 2 and 3), rainfall tended to cause less variation in the chemical parameters. Although in a first period, rainfall led to a little decrease in water pH, after a given moment, rainfall even increased water pH. Conductivity had an analogue behavior to pH: a pH increase didn't imply a conductivity decrease. Generally, metal concentrations which were much lower than those found in the previous streams, varied inversely to pH. High conductivity values found in these waters cannot be justified by metal contents, which were low. Thus, the major elements most probably, contributed to this increase in conductivity, mainly because of chloride concentration. This is the cause for the presence of brackish/marine species in the waters of this group. In this group, Mn presented high concentrations shown by sediments and waters results, which can be associated to the geological formation overlying the Volcano-Sedimentary Complex, composed mainly by schists, where some Mn mineralizations occur. Manganese might also have contributed to the increase of conductivity in waters of Group 1.

Seasonal variations in dams were more complicated due to the confinement of these waterbodies and constant anthropic interventions by addition of mining material or by corrective material addition in order to increase pH. However, considering chemical water analysis they were correctly placed (clean dams in Group 3 and impacted dams in Group 1). But the same was not observed for diatoms, with different communities in dams, mainly composed by planktonic species instead of benthic ones. It was interesting to notice the establishment of the same 3 groups of samples through waters and diatoms analysis. A first group, formed by impacted sites from Água Forte and Água Azeda

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streams with sulphated waters, with high/extreme metal concentrations and low pH, dominated by *Pinnularia aljustrelica* (PALJ), *Eunotia exigua* (EEXI) and *Nitzschia* aff. *hantzschiana* (NHAN). A transitional group formed by sites from Roxo stream (after the confluence of two acidic streams) with sulphated waters, neutral pH and with high/low metal contents (specially, Al, Cd, Cu, Fe, Pb, Zn) dominated by *Brachysira neglectissima* (BNEG) (misidentified as *Brachysira vitrea* in chapter 7) and *Achnanthidium minutissimum* (ADMI). Finally a third group, with sites from Roxo stream and its unimpacted tributaries, with low metal contents and high values of Cl and pH, dominated by *Navicula veneta* (NVEN), *Achnanthidium minutissimum* (ADMI), *Navicula gregaria* (NGRE), *Nitzschia desertorum* (NDES). Some brackish to marine species (e.g. NDES and *Entomoneis paludosa* (W. Smith) Reimer- EPAL) were found in this group due to high conductivities caused by Cl. Chlorine lixiviation is probably associated with its existence in undifferentiated sediments of Sado Tertiary Basin; Paleozoic Basement. The sediments/water ratio was higher in unimpacted samples (Group 3), reflecting the decrease of elements' mobility (especially for metals) with the increase of pH. In the sediments, metals were in non-soluble chemical forms, thus not so easily uptaken by diatoms.

In Group 1, especially in MR and BE samples, the high concentrations of metals and extremely low pH (1.9-2.2) limited the occurrence of a diverse diatom community. Just a particular diatom species occurred at the former sites, *Pinnularia aljustrelica*, dominating in the extreme conditions of metals and acidity of Água Forte stream (Luís et al. 2012). It is common for AMD affected streams, to be oversaturated with silica, (as evidenced by sediments mineralogy of Água Forte stream: by silicate phases) coming from silicate (e.g. feldspars and phyllosilicates) weathering, which is also an abundant compound in Fe oxy-hydroxysulphate precipitates (Sánchez-España et al. 2005). On the one hand, extra silica can contribute to the success of this species in AMD impacted streams by reinforcing the frustule. On the other hand, weak silicification of some individuals can cause teratologies. Fisher et al. (1981) in a study on diatoms cultured with high Cu concentrations have proposed that this metal might bind to sulphidryl groups on diatom cell membrane reducing silica uptake and therefore, mimicking the condition of silica limitation. Silicates dissolution can also release large quantities of their constituint elements, such Al, Mn, being those elements more available. This species seems to be very adapted to these extreme conditions. High heavy metal resistance of algae can result from the limited intracellular transport of metals at low pH (e.g.Skowroński et al. 1991). Moreover, some eukaryotic algae possess an effective mechanism of heavy metal detoxification using thiol oligopeptides such as glutathione and phytochelatins (e.g.Pawlik-Skowrońska 2003).

When the conditions arise for EEXI emergence, normal and teratological forms of this species occurred, but just in sample AF with pH: 2.8-3.3, and where less metal contents in the bioavailable fraction of the sediments were found from all the sites (by SSCE), thus the metals were already in the water column available to be assimilated by organisms. It is known that biofilms sorb metals rapidly from the water column (e.g. Newman and McIntosh 1989). Precipitation of ferric iron to ferric hydroxide in AF site was evident, which blocks sunlight and covers the stream bed with a thick red blanket "yellow boy" (Chon and Hwang 2000). It limits photosynthesis and induces stress that could cause teratologies. Minimum productivity in AMD environment is most likely due to metal stress (Niyogi et al. 2002, Hamsher et al. 2002) or soluble reactive phosphate concentration (Spijkerman et al. 2007). But low pH itself does not reduce photosynthetic activity Gyure et al. 1987 (). Regarding ecology, *Eunotia exigua* is an acidobiontic taxon (Van Dam et al. 1994) and is one of the most widespread species reported from North America, Asia and Europe in lakes and streams receiving acid mine drainage (Negoro 1985, Lessmann et al. 2000) and it has been cited in acid mine drainage waters from Rio Tinto (Urrea and Sabater 2009, Luís et al., 2009, 2011, 2013 a, b). Abnormal cells of *Eunotia exigua*, showing abnormal valve outline were found in the Irish Free State, close to a highly acid (pH 2.4) copper mine (Barber and Carter 1981). *Eunotia exigua* can thrive at pH levels down to 2.2. (DeNicola 2000).

An important taxon of Group 2 and 3 was *Achnanthidium minutissimum* (ADMI). Authors refer ADMI amongst the most tolerant diatoms with respect to metals (e.g. Falasco et al. 2009, Morin et al. 2012). It was interesting to check that the highest percentage of teratological valves was found in Group 2, thus not at the sites with the highest metal concentrations (in Group 1) but at those where a decrease in metal gradient was already noted (sites JU, RJ), the pH was not extreme (6.1-6.8), and where development and establishing of more diverse diatom communities was already possible. Teratologies just occurred in April 2008 which was the sampling moment with higher concentration of metals in waters and lower in the sediments. The fact of ADMI being abundant in Group 3 but not with teratological forms, can reinforce the idea that teratologies were caused by metals. Charles et al. (1990) considered it as being able to persist in pH of 6.6, but also capable of withstanding large pH oscillations in some mine-impacted Ohio streams (Verb and Vis 2000) and in an acidic lake with severe metal contamination (Ruggiu et al. 1998). Nevertheless, Van Dam et al. (1981) considered ADMI as a circumneutral taxon. Other physical and chemical parameters were pointed as potential deformation causers such as: drought conditions, light intensity, UV, salinity

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levels, nutrients and other toxic compounds such as cyanide, polycyclic aromatic hydrocarbons (PAH) and pesticides (Falasco et al. 2009).

Whichever the explanation is for morphological alterations, these changes are a symptom of a metabolic disorder that can represent the price for tolerance to some environmental factor, which in the present study we believe to be the high metal concentrations. These type of alterations have been observed frequently in acidic media and/or impacted by heavy metals, suggesting the existence of biological stress (Luís et al. 2011).

6.5 CONCLUSIONS

In this study, the geochemical conditions of the Aljustrel mining area were evaluated and their integration with biological factors related to the diatom communities was made. The selection of AMD impacted and unimpacted sites showed a perfect association with diatom species. These sites were divided in 3 groups: a group corresponding to the most impacted sites; another with the unimpacted sites; and a transition group, characterized by a slight impact.

The high pH range, found along the sampling area, seemed to condition the diatom species in each site, even if there were species with high resistance to pH oscillations. However, the metals appeared to be largely responsible for the reduction in diatom abundances as well as the emergence of teratologies in the communities. Indeed, for similar acidity conditions there were large variations both in abundance as well as in the emergence of diatom deformations, which were explained by metal contents. Given the large amounts of different metals, it was not possible to distinguish clearly which metal had the major impact on diatoms. Other factors, as salinity, affected the appearance of certain species. In this sense, even if environmental conditions for the emergence of teratologies exist in some communities, they will not occur simply because salinity conditions will not allow the emergence of the species that normally show teratologies. This affects the establishment of correlations between the abundance of diatoms or teratologies with metals.

Therefore, in contexts as complex as this, with strong chemical variations in short periods of time, diatoms arise as important bioindicators due to their short generation time to assess these geochemical changes. However, their analysis will be necessarily confronted with several geochemical data in addition to pH and metals. The weighting factors for each component requires detailed evaluation in order to develop a model/index

applicable to these environments as a quality parameter to evaluate the contamination degree, similarly to what is already done in other types of impairments, like organic, and nutrient contamination, and also to understand which factors affect mostly the abundance and occurrence of teratologies in diatom communities.

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CHAPTER 7

Benthic diatom communities in streams from zinc mining areas in continental (Canada) and Mediterranean climates (Portugal)

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Abstract This study compares regional differences in benthic diatom communities exposed to similar stresses in Canada and Portugal. Diatoms were sampled in the Água Forte Stream, Aljustrel (SW Portugal) and in the Little River, New Brunswick (SE Canada), both streams surround the respective zinc mine and are subject to similar metal (e.g. Cd, Cu, Fe, Zn) and acidic (Água Forte pH = 1.9 – 2.9 vs. Little River pH = 2.2 – 5.5) stresses. In this kind of extreme environment, diatoms are frequently the main algae group in the streams, widely used as bioindicators. Diatom communities in the Água Forte Stream were dominated mostly by *Pinnularia aljustrelica* and *Eunotia exigua* (5 % teratological forms), whereas communities in the Little River were more diverse (e.g. *Achnanthidium minutissimum, Nitzschia palea, Eunotia sp.*). Shannon-Wiener Index (*H'*) and percentage of taxa relative abundance were used to characterize the diversity and species composition of the diatom communities. Using canonical correspondence analysis (CCA), it was found that regional variation in acceptable in-stream concentrations of metals, conductivity and pH were the primary drivers of benthic diatom community. Mine remediation to decrease metal concentrations and increase pH in streams will increase diatom diversity even in highly impacted streams such as Água Forte.

Key words: diatoms, geographically distinct environments, low pH, metals, Portugal and Canada mining areas

7.1 INTRODUCTION

Two geographically and climatically distinct rivers in similar mining areas were studied, one located in Aljustrel, Alentejo (south-west Portugal) and a second in Bathurst, New Brunswick (south-east Canada). The mining complex of Aljustrel is located in the Portuguese part of the Iberian Pyrite Belt (IPB). This geological unit corresponds to an area of Devonian-Carboniferous volcanic and sedimentary rocks containing massive sulphide deposits, with Zn-Cu being the primary metals extracted. Some ores and sulphides can be deposited as a result of the extraction process. Oxidation of sulphurous ore bodies can result in acid leakages and highly contaminated acid mine drainage (AMD). AMD formation is due to the oxidation of thiosalts that are formed by the incomplete oxidation of sulphides which can be further oxidized to form sulphate minerals and hydrogen ions in the water. These processes can mobilize trace metals such as As, Cd, Cu, Pb and Zn, and have been shown to affect streams surrounding the Aljustrel-Alentejo mining complex (e.g. Cánovas et al. 2007).

In SE Canada, the Brunswick zinc mine, located near the city of Bathurst in the Canadian province of New Brunswick, is also a massive deposit of sulphide minerals (Zn-Pb-Cu) and this zinc deposit is part of a similar geological formation to that found in Aljustrel (Devonian-Carboniferous). In the Little River, water quality is also periodically characterized by low pH conditions downstream of the mine (pH<4; personal observation), again due to AMD formation. Both mining concerns use in stream lime addition as a remediation procedure for acidity excess. Therefore, both of these stream systems, despite geographic separation, face the same stressors but not to the same extent. Both streams are routinely subject to high concentrations of metals such as As, Cd, Cu, Pb and Zn owing to the increased solubility of these metals in acidic water (e.g. Almer et al. 1978). Thus, these metals pose a threat to the majority of the aquatic organisms because of their high concentrations and solubility in acidic streams.

Benthic diatoms were analysed because changes in community composition have been shown to highlight changes in both habitat and water quality. They are primary producers and, thus, are an important basal resource in aquatic food webs for aquatic invertebrates and fish. Diatoms are widely distributed and rapidly respond to environmental changes. In addition, many indices using diatoms have been successfully developed in Europe; for example, the IPS, Index of Pollution

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Sensitivity (Cemagref 1982) and the IBD, Biological Diatom Index (Prygiel and Coste 2000), both of which are widely thought to be among most sensitive and relevant indicators of contaminants (mostly organic), particularly for risk assessment. Recently, a diatom-based index for assessing stream ecological integrity in eastern Canada has been proposed by Lavoie et al. (2006).

Algal community structure responds to metal contamination by shifting from sensitive to tolerant species (e.g. Besch et al. 1972, Gustavson and Wängberg 1995), resulting in an increase of the tolerance of the benthic community. These shifts have also been shown to result in diversity loss (Medley and Clements 1998, Luís et al. 2009) and frequently in teratological forms (Morin et al. 2008). Similarly, algal communities have a negative relationship to acidification whereby increased acidity results in decreased taxa richness (e.g. Findlay 2003). Unfortunately, separating the effects of the combined stress of metals and acidity has been difficult to establish in the field because metal contamination is frequently associated with acidic environments (Dixit et al. 1991). The lowering of pH is caused by the oxidation of metallic sulphides and further, late successional species appear to be more sensitive to metals than their upstream counterparts (Medley and Clements 1998).

The objective of this study was to evaluate the effects of AMD on diatom communities in rivers of continental (Canada) and Mediterranean (Portugal) climates, testing the possibility of using the same biological metal assessment method in these two systems. This purpose will be attained by characterizing and comparing the water chemistry in the Água Forte Stream and Little River with communities growing along a metal pollution–pH–conductivity gradient. It might be reasonably expected that AMD effects decline with increasing distance from the mining source.

This type of study has been done in the Água Forte Stream (e.g. Luís et al. 2009, 2012); however, it was a new approach in the Little River where only one previous diatom study has been conducted in New Brunswick (Besch et al. 1972) but not specifically in the streams surrounding Brunswick Mine. To our knowledge, a comparative study of this kind in such different geographic and climatic areas has never been done before. Evaluating the combined effects of different gradients of metals and acidification in the field is critical to the development of pertinent biomonitoring irrespective of regional differences in taxa, climate and environmental regulation.

7.2 STUDY AREA

Aljustrel Mine: Portugal

Located in the Alentejo province (**Fig. 7.1**) in the south of Portugal, the Aljustrel Mine (37º52'37.90″ N, 8º10'54.64'' W) is one of the IPB mining sites, a volcanic-hosted massive sulphides metalogenetic province (Carvalho et al. 1999, Matos and Martins 2006).
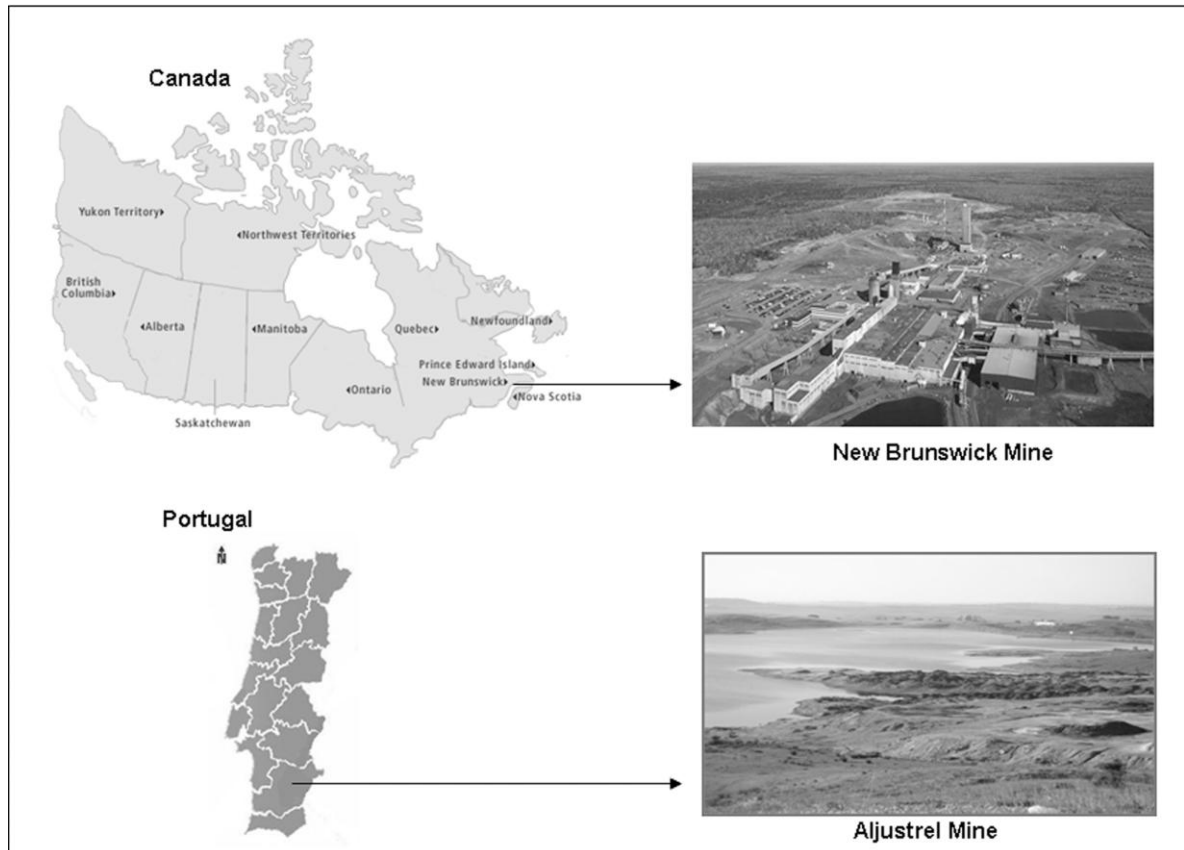
Fig. 7.1 – Geographical location of New Brunswick Mine (Bathurst, New Brunswick, SE Canada) and Aljustrel Mine (Aljustrel, Alentejo, SW Portugal).

Six massive sulphide ore bodies have been identified in the Aljustrel mining site, Moinho, Feitais, Estação, Gavião, Algares and São João, the latter two exploited since Roman times (Silva et al. 1997, Matos and Martins 2006) to a depth of 100 m. Additionally, the Moinho deposit was mined for copper by the public Pirites Alentejanas Company (PA) until 1993. Continuous pyrite ore exploitation in the area since the Roman era has resulted in large areas that are occupied by waste tailings. Tailings were composed of Roman slag, pyrite ore (blocks and brittle massive pyrite ore) and volcano–sedimentary complex host rocks with the Algares industrial area and São João sector representing the highest volumes of mine waste in Portugal. The waste rock varies in composition, although high concentrations of Fe, Pb and Zn are routinely detected. More

specifically, petrographic study of ore waste samples identified interstitial chalcopyrite, sphalerite, galena, arsenopyrite and minor sulpho-salts in the massive pyrite ore. The pyrite ore presents high concentrations of Fe, Cu, Pb, Zn, Ag, Sb, Hg, Se, Co, Au and Cd while the roasted pyrite ore shows high concentrations of Au, Pb, Ag, Fe, Sb, Bi, Se, Cu, Zn and Mo. Roman slag contained high concentrations of Pb, Cu, Zn, Fe, As and Sb elements. Currently, the EDM public company (the owner of the Portuguese mines rehabilitation programme) is developing local rehabilitation strategies for the Aljustrel area (Matos and Martins 2006).

Brunswick Mine: Canada

Brunswick Mine is located 32 km southwest of Bathurst, New Brunswick (**Fig. 7.1**) and the mine site (47º35.914' N, 65º40.216' W) makes up 8.5 km^2 of the 141 km^2 Little River watershed (about 6 %). The large ore body was discovered near Bathurst in 1953. The Brunswick ore body is hosted in dipping volcanic and sedimentary rock units. The deposit comprises massive sulphides associated with various iron formation facies with Zn, Pb, Cu and Ag as the principal metals produced. The host rocks and the mineralization have undergone four significant deformation events, resulting in intense folding and faulting. Most deposits are zoned vertically and laterally from a high-temperature, vent-proximal, Cu-Co-Bi-rich veined and brecciated core to vent-distal Zn-Pb-Ag-rich hydrothermal sediments. The vent complex is commonly underlain by a highly deformed sulphide stringer zone that extends hundreds of metres beneath deposits and consists of veins and impregnations of sulphides, silicates and carbonates that cut chloritized and sericitized volcanic and sedimentary rocks (Mine Sites 1990). Brunswick Mine opened in 1964 and continues to be one of the world's largest zinc producers (on average milling 10,500 tonnes of ore per day). Prior to 1993, all mine water and process water was treated by lime addition followed by precipitation in sludge ponds. In 1993, a state-of-the-art high density sludge (HDS) ETP (effluent treatment plant) was constructed, using a series of surface ponds to increase retention time of water, allowing for more thiosulphate degradation and improved metal removal. However, current pH levels in the Little River system continue to be influenced by the oxidation of thiosalts, which are frequently present in the treated effluent (Mine Sites 1990).

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7.3 METHODS

Sampling methods

Surficial waters

Sampling was carried out in April 2008 in Água Forte Stream (Portugal) and in August 2008 in Little River (Canada). Samples AF1 to AF4 were collected at Água Forte Stream and samples LR1 to LR4 at Little River, with numbers 1 to 4 corresponding to minimum and maximum distance from the mine, respectively. These sampling dates were selected because of the climatic differences between continental and Mediterranean climates, for overlapping seasonal relevance. Specifically, spring in Portugal and late summer in Atlantic Canada were selected, given that summer in Portugal's Mediterranean climate has higher temperatures than the same season in SE Canada's continental climate. Water samples were collected to assess the physical and chemical characteristics of surficial water samples and they were collected in the centre of each river in acid-rinsed polyethylene bottles. Temperature (^{o}C), pH and conductivity (μS cm^{-1}, at 25 ^{o}C) were recorded on-site, using a multiprobe WTW Multiline P4 SET. Water samples were stored at 4 ^{o}C, prior to laboratory analyses. Samples analysed for dissolved metals, cations and anions (dissolved phase), were first filtered using a 0.45 μm Millipore membrane filter then were preserved in ultra-pure nitric acid (samples acidified to pH < 2) to avoid metals precipitation (dissolved phase).

Diatom communities

The available substrates for sampling differed between Portugal and Canada. Epilithic (in Little River) and epipsammic (in Água Forte) diatom communities were sampled at the same four selected water sampling points and during the selected seasonal periods, according to national (INAG 2008) and international (European Committee for Standardization 2003) norms. Five boulders/pebbles were chosen, avoiding shaded zones, in 10–30 cm of water depth from which the epilithic samples were obtained by scraping the upper surface of the boulders/pebbles with a toothbrush. The epipsammic samples were removed from the top layer of the sediment surface with a syringe. Two samples were collected, one kept alive (without any preservation) and the other preserved with formalin solution (5 %), for taxonomic study.

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Analytical techniques

Chemical analyses of surficial waters

The chemical analysis of surface water samples was carried out using inductively coupled plasma-mass spectrometry (ICP-MS) for As, Cd, Cu, Fe, Mn, Ni, Pb and Zn and ion chromatography for SO_4^{2-}. A rigorous quality control programme was implemented during water chemical analysis which included reagent blanks, duplicate samples and certified reference materials (STANDARD WASTWATRA6). The samples from Água Forte Stream were tested at ACME (ACME Analytical ISO 9002 Accredited Lab, Canada) and those from Little River were done at the National Laboratory of Environmental Testing (NLET). Both laboratories are located in Canada.

Diatom treatment

The live samples were examined in each set in order to exclude dead diatoms to avoid errors in the estimation of abundances. From the second sample, an aliquot was chemically treated, using HNO_3 (65 %) and potassium dichromate ($K_2Cr_2O_7$) at room temperature for 24h, to remove the organic portions of the diatoms in order to improve the optical resolution of valves by light microscopy. Samples were repeatedly centrifuged (at 1500 rpm) to remove the excess of acid. Permanent slides were made by mounting air-dried samples on a cover slip with Naphrax®.

Diatoms were identified to species level and quantified under a light microscope (Leitz Biomed 20 EB) using a 100x immersion objective (N.A. 1.32). A total of about 400 valves were counted in each sample. Taxonomy was mainly based on Krammer and Lange-Bertalot (1986, 1988, 1991 a, b) and Prygiel and Coste (2000).

Data analysis

The compiled species database was analysed using canonical correspondence analysis (CCA) to detect changes in diatom community structure and correlations between dia- toms and environmental variables. CCA was performed using the computer program Canoco (version 4.5) (Ter Braak and Šmilauer 2002). The matrix used for CCA was composed of 13 diatom taxa – ACOF cf.: *Amphora* cf. *coffeaeformis* (Agardh) Kützing; ADMI: *Achnanthidium minutissimum* (Kützing) Czarnecki; BVIT: *Brachysira vitrea* (Grunow) Ross in Hartley; EARL: *Eunotia arculus* (Grunow) Lange-Bertalot & Nörpell; EEXI: *Eunotia exigua* (Brébisson ex Kützing) Rabenhorst; ERHO: *Eunotia rhomboidea* Hustedt; EBIL: *Eunotia bilunaris* (Ehrenberg) Mills var. *bilunaris*; FCGR: *Fragilaria capucina* Desmazières var. *gracilis* (Oestrup) Hustedt; FSAX: *Frustulia saxonica* Rabenhorst; GPAR: *Gomphonema parvulum* (Kützing); NPAL: *Nitzschia palea* Kützing W.

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Smith; PALJ: *Pinnularia aljustrelica* Luís, Almeida et Ector; TFLO: *Tabellaria flocculosa* Roth (Kützing) – and 12 environmental variables (conductivity, pH, temperature, As, Cd, Cu, Fe, Mn, Ni, Pb, Zn, SO_4^{2-}). However, this first CCA showed that some of the metals were highly autocorrelated. Spearman correlation matrix ($p < 0.05$) with the metals As, Cd, Cu, Fe, Mn, Ni, Zn and the SO_4^{2-} was done to determine which were auto-correlated ($p < 0.05$) and could be merged into a single cofactor, as well as to reduce the number of environmental variables, according to the number of samples. This correlation showed that only Pb was not correlated with the other metals and was treated as an independent variable. Shapiro-Wilks and Q-Q plots tested whether the metals and SO_4^{2-} values (with no transformation and log transformed) were normally distributed. Log-transformed data were the most suitable for the CCA analysis, avoiding the extremely high metal values of Água Forte which would otherwise dominate the CCA analysis. So, a second CCA analysis was performed with the same 13 diatom taxa and the six environmental variables found, after the correlation analysis, for conductivity, pH, temperature, metal (surrogate cofactor), Pb and SO_4^{2-}.

The input diatom data were composed of percentage of taxa relative abundance over 0.5 % to minimize the influence of rare taxa. A square root transformation was applied to the diatom data rather than a log transformation in order to retain zero values. Transformations are needed to achieve homogeneity of variances. The statistical meaning of each variable was tested with a Monte Carlo permutation test. The power of this test increases with the number of permutations, so the maximum number possible (999) in the program was chosen. Only significant variables ($p \leq 0.05$) were included in the analysis (Ter Braak and Šmilauer 2002). Statistical differences between diatom communities of Canada and Portugal were tested with a distance-based permutational multivariate analysis of variance, PERMANOVA test (Primer 6; Primer-E Ltd, Plymouth, UK).

Changes in diversity were evaluated by Shannon-Wiener (*H'*) Index, with OMNIDIA (version 5.2). *H'* is widely used (e.g. Reiss and Kröncke 2005) and was defined by Shannon (1948) in Washington (1984) as:

$$H' = -\sum_{i=1}^{s} \frac{n_i}{N} \log_2 \frac{n_i}{N}$$

where *s* is the number of species, n_i is the number of specimens of species *i*, and *N* is the total number of specimens.

7.4 RESULTS

Surficial waters

According to the results shown in **Table 7.1**, both streams were acidic, but pH was lower in the Água Forte Stream, varying from 1.9 (AF1) to 2.9 (AF3). In the Little River, pH varied from 2.2 (LR3) to 5.5 (LR4). Sites AF1, AF2 (Aljustrel) and LR3 (New Brunswick) had the highest metal concentrations and the lowest pH of either the Água Forte or the Little River (**Table 7.1**).

Table 7.1 – Physicochemical parameters of downstream surficial waters collected at downstream sampling sites of zinc mining activity in Portugal (AF1-AF4) and Canada (LR1-LR4). Conductivity: µS cm^{-1} at 25 ºC; temperature: ºC; SO_4^{2-} in mg L^{-1} and trace metals in µg L^{-1}.

	Água Forte stream (AF), Portugal				Little River (LR), Canadá			
	AF1	AF2	AF3	AF4	LR1	LR2	LR3	LR4
Conductivity	17140	12670	3130	3370	448	563	39	636
pH	1.9	2.2	2.9	2.6	5.3	5.1	2.2	5.5
Temperature	18.5	21.0	15.0	16.4	17.8	17.6	17.4	17.5
SO_4^{2-}	28239	20169	1638	2064	195	258	7.23	297
As	48507	21794	10	7	1	1	2	1
Cd	2487	1620	77	136	0.406	2.89	0.292	0.619
Cu	347779	248150	8304	16574	2	4	7	7
Fe	6173473	4542639	184693	97730	840	820	1860	661
Mn	203603	141731	11445	15378	370	450	484	452
Ni	2984	2155	126	186	1.72	1.74	1.5	1.79
Pb	<10	<10	15	18	1.2	1.38	2.07	1.62
Zn	1201542	776029	38358	57376	35	45	30	53

Distance from impact is indicated by sample site numbers (1 to 4), where lower numbers (e.g. 1) indicate sites with th closest proximity to the mine and higher numbers indicate downstream distance from impact (e.g. 4).

The drop in pH (2.2) and increase in metal concentrations in site LR3 could be caused by quantities of AMD-contaminated backwater entering the stream. Water temperature was similar in both rivers but conductivity was an order of magnitude higher in the Água Forte Stream than in the Little River. The highest metal concentrations were found for Cu, Fe, Mn and Zn in Água Forte Stream. Comparing the guideline values of trace metals for irrigation waters of Canada (Wateresearch Corporation and Agriculture and Agri-Food Canada Field Irrigation and Water Quality 1999) and Portugal (Decreto-Lei nº 236/98 de 1 de Agosto) it was found that in Água Forte Stream (Portugal) the values of Cd, Cu, Mn and Zn were above the guidelines (Zn presented the highest value in AF1: 1201542 µg L^{-1}). As and Ni exceeded the guidelines in AF1 and AF2. Pb never exceeded the guidelines. In Little River sites the concentrations do not exceed the guideline values for all metals.

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Periphytic diatom communities' analysis: pH classification, diversity (H') and relation with environmental parameters (CCA).

The water classification according to pH was made by Van Dam et al. (1994) based on the ecological preferences of the diatom taxa. Água Forte was characterized by acido-biont (*Eunotia exigua*) and acidophilic (*Pinnularia aljustrelica*) taxa. Regarding teratological forms, 5 % of *E. exigua* deformed valves were found in site AF4.

Little River was dominated by neutrophilic taxa. But in site LR3, acidophilic taxa (as well as in Água Forte Stream) such as *Eunotia rhomboidea*, *Eunotia arculus* and *Eunotia bilunaris* dominated or co-dominated.

Seasonal and spatial variations of *H'* are shown in **Fig. 7.2**. Percentage of relative abundance of dominant taxa is shown in **Fig. 7.3**.
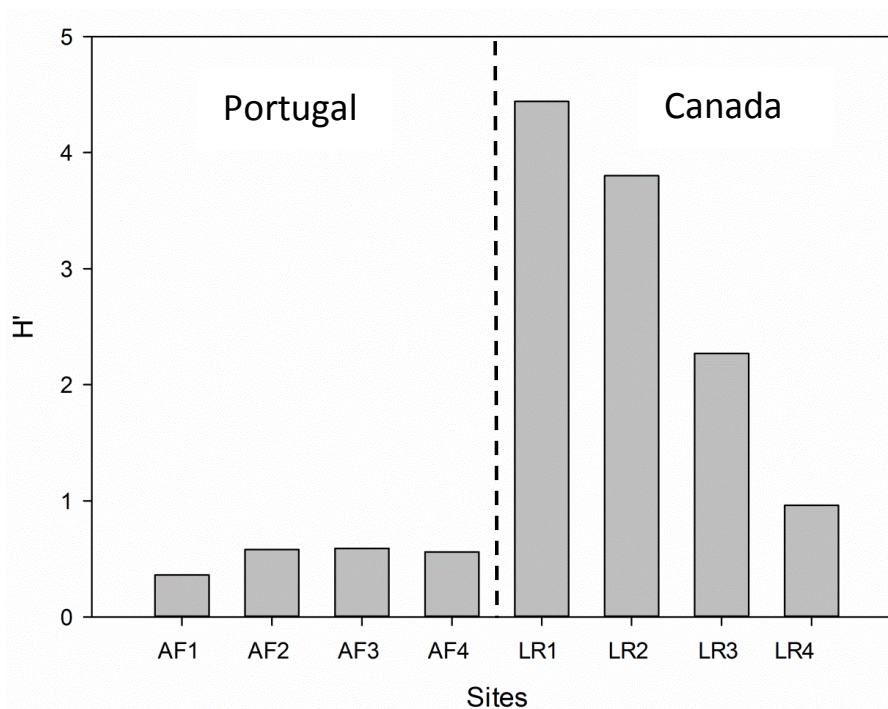
Fig. 7.2 – Spatial variation of Shannon-Wiener Index (*H'*) in Água Forte Stream (sites AF1 to AF4) and in Little River (sites LR1 to LR4). Diversity of diatom taxa in LR1 (closest to the mine) was significantly high.

Diversity responses of diatom communities were significantly different in the Portuguese versus Canadian examined streams. In Água Forte (AF1 to AF4), diatom diversity values were very low (*H'* = 0.36 to 0.59). AF1, the closest to the mine, had the lowest community diversity: *H'* = 0.36). Overall, sites in Little River had higher *H'* values (0.96–4.44), indicating higher diversity compared with Portuguese streams. In Little River, diversity unexpectedly rose in inverse proportion with mine distance. The highest *H'* value was found in LR1 (*H'* = 4.44), the closest site to the mine, which may indicate a sign of recovery due to restoration measurements.

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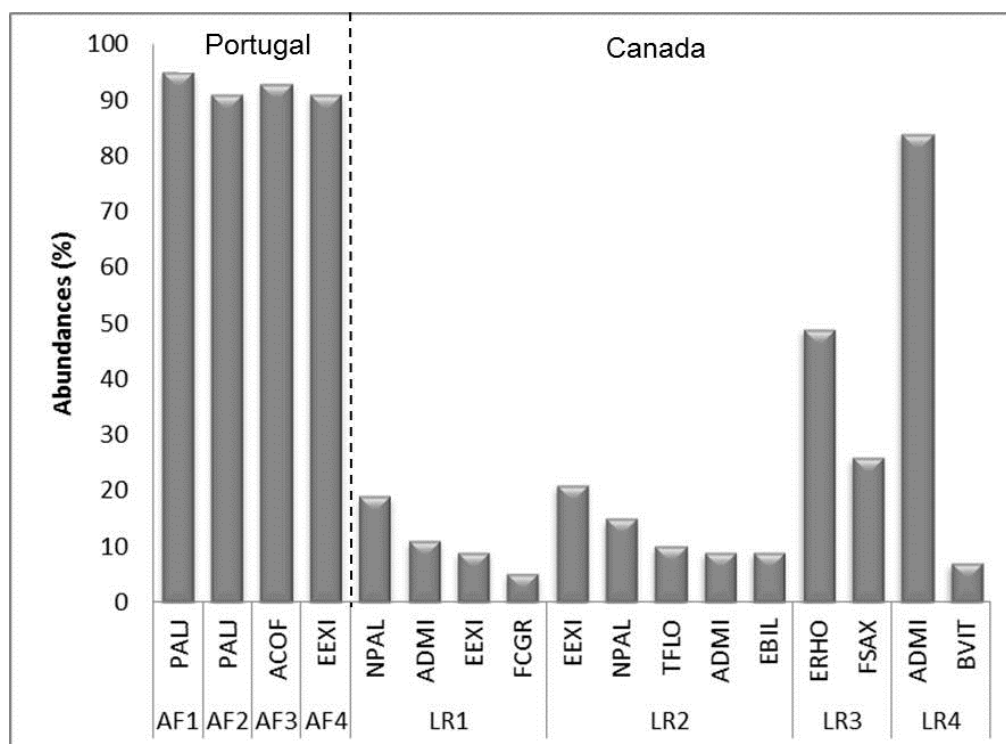
Fig. 7.3 – Percentage of relative abundance of the dominant diatom taxa in Água Forte Stream, Portugal (sites AF1 to AF4) and in Little River, Canada (sites LR1 to LR4). Sampling sites in the Água Forte Stream were typically (> 90 %) composed of a single taxon; for example, AF1 had 95 % of Pinnularia aljustrelica Luís, Almeida et Ector (PALJ). For a full description of taxa see **Fig. 7.4**.

The percentage of relative abundance for the 13 dominant taxa is represented in **Fig. 7.3**. Dominance of a single taxon in each site of Água Forte was evident: *P. aljustrelica* in the highly acidic sites, AF1 and AF2, *Amphora* cf. *coffeaeformis* in site AF3 and *E. exigua* in site AF4. A more diverse community was found in the Canadian sites, with a dominance of two to five taxa: *Achnanthidium minutissimum*, *E. exigua*, *Fragilaria capucina* var. *gracilis* and *Nitzschia palea* at site LR1; *A. minutissimum*, *E. exigua*, *E. bilunaris*, *Tabellaria flocculosa* and *N. palea* at LR2; *E. rhomboidea* and *Frustulia saxonica* at LR3; and dominance of *A. minutissimum* and *Brachysira vitrea* at LR4.

Figure 7.4 and **Fig. 7.5** show the CCA ordination for the first two axes (axis 1/axis 2) and for axes 1 and 3, respectively, considering the environmental variables, sampling points and species data.

The first three CCA axes had eigenvalues of 0.696, 0.540 and 0.402 respectively. Species–environmental correlations were higher than 0.956 for the three axes, and explained collectively 85.4 % of the species–environmental variation. The total inertia was 2.36. A Monte Carlo test revealed metals to be the most significant variables. Correlations between variables and CCA axes can be found in the **Table 7.2**.
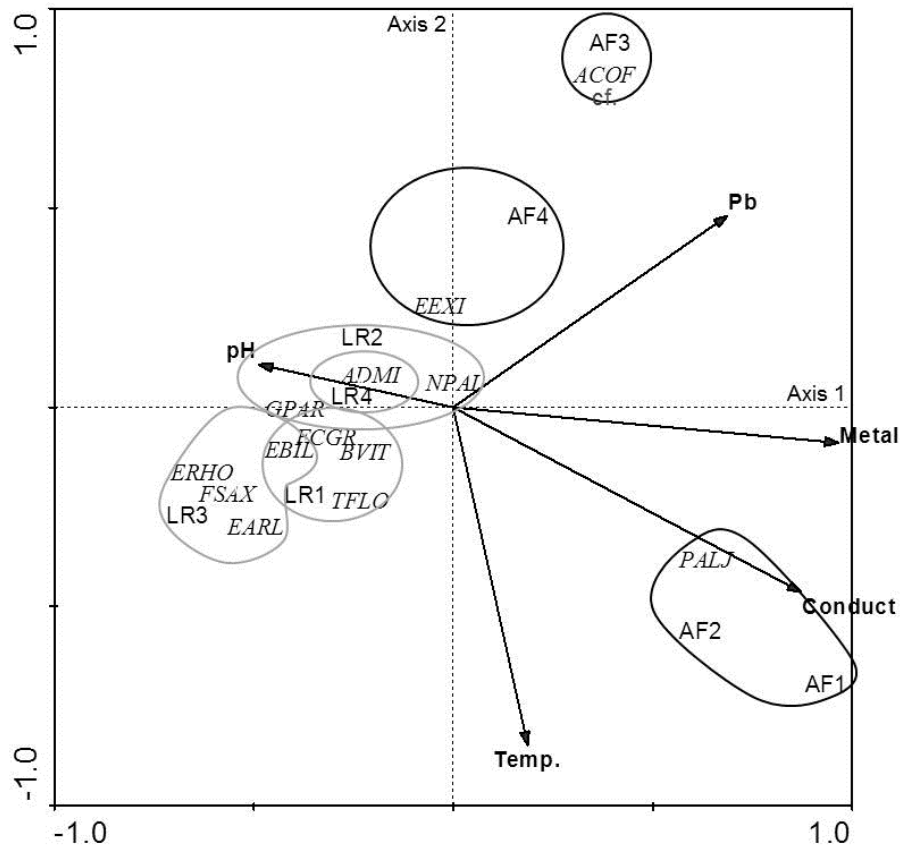
Fig. 7.4 – CCA triplot of the first two axes showing scores for samples, species and environmental variables. Taxa labels consist of a four-letter code (given below and in the text). The six environmental variables examined (Conduct. (conductivity), pH, Temp. (temperature), metal (as surrogate), Pb, SO_4^{2-}) are represented by vectors. Species closest to the tip of an arrow of an environmental variable were highly correlated to it. The 13 most abundant species used in the analysis were ACOF cf.: *Amphora* cf. *coffeaeformis*; ADMI: *Achnanthidium minutissimum*; BVIT: *Brachysira vitrea;* EARL: *Eunotia arculus*; EEXI: *Eunotia exigua*; ERHO: *Eunotia rhomboidea*; EBIL: *Eunotia bilunaris*; FCGR: *Fragilaria capucina* var. *gracilis*; FSAX: *Frustulia saxonica*; GPAR: *Gomphonema parvulum*; NPAL: *Nitzschia palea*; PALJ: *Pinnularia aljustrelica*; TFLO: *Tabellaria flocculosa*. Detailed descriptions of taxonomic sources and variations can be found in the text; grey circles surround a respective Little River site with the respective dominant taxa and black circles do the same for Água Forte Stream sites.

Table 7.2 – Correlations between variables and CCA axes (selected variables with values > |0.5|): eigenvalues, species-environment correlations and cumulative percentage of variance for species-environment relation; values in bold are those more correlated with the respective axis.

Variable	Axis 1	Axis 2	Axis 3
pH	0.4856	0.1045	**- 0.7613**
Conductivity	**0.8685**	- 0.4529	0.1465
Metal	**0.9616**	- 0.0874	- 0.2025
Pb	**0.6846**	0.4688	0.2172
Temperature	0.1857	- 0.8282	- 0.1603
Eigenvalues	0.696	0.540	0.402
Species–environment correlations	0.996	0.976	0.959
Cumulative % variance of species–environment relation	36.3	64.5	85.4

Despite the differences in the collection technique, communities of different

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substrates were highly similar compositionally and placed together in the CCA diagram (i.e. see Luís et al. 2009).

A quantitative analysis of the diatom data using PERMANOVA found the two geographical groups, Canada and Portugal, to be statistically different (Pseudo-F = 3.9134, $p < 0.05$, df = 1).

In **Fig. 7.4** and according to PERMANOVA, the sampling sites are easily discriminated. Água Forte sites are found on the right part of the CCA graph (top and bottom) and Little River sites are found in the left part of the CCA graph (top and bottom). pH was the primary factor separating the Portuguese from the Canadian sites (see also **Fig. 7.5**). Axis 1 had a strong positive correlation with trace metals (As, Cd, Cu, Fe, Mn, Ni, Zn and SO_4^{2-} - metal surrogate). Positive correlations were also found with Pb and conductivity whereas strong negative correlations were found with respect to pH.
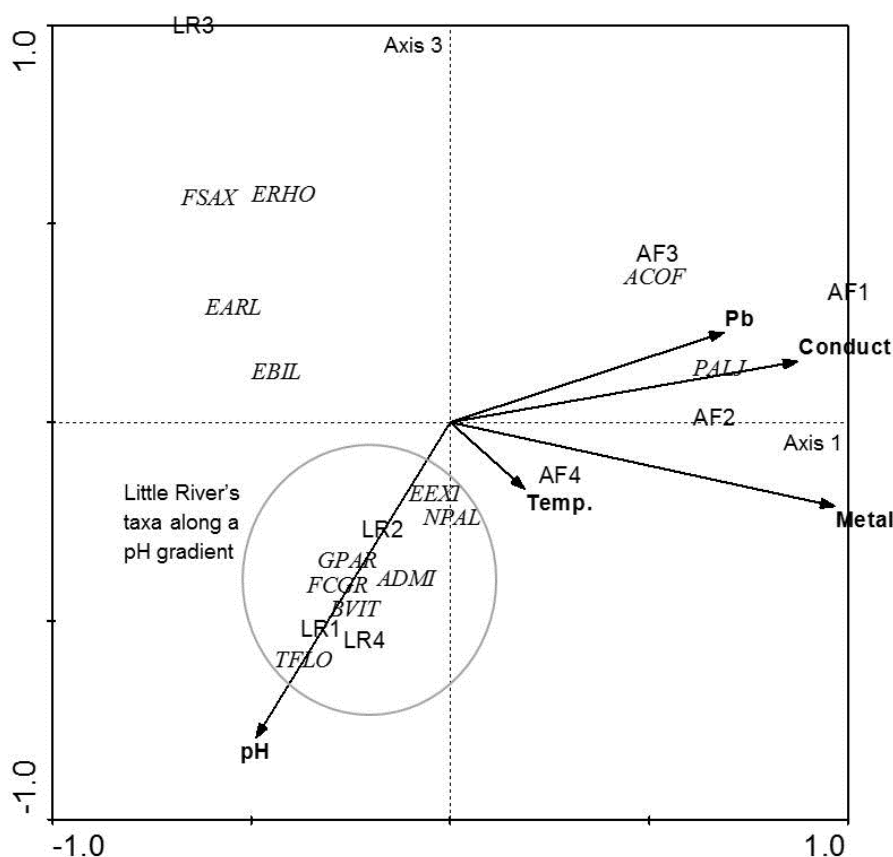
Fig. 7.5 – CCA triplot of axis 1 and axis 3 showing scores for samples, species and environmental variables as in **Fig. 7.4**, but with axis 3 showing evidence for Little River sites along a pH gradient (group surrounded by a grey circle).

The presence of the diatom *P. aljustrelica* was associated with the most acidic sites (AF1 and AF2) in the Água Forte Stream. In **Fig. 7.4**, *P. aljustrelica* is found in the area of low pH (**Figure 7.4**: bottom right) and is also well correlated with the highest metal, SO_4^{2-}

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concentrations and the conductivities measured, this last parameter being the most discriminant for this taxon. Site AF3 was isolated and well correlated with Pb (0.6846) probably because of the dominance of *A.* cf. *coffeaeformis* and it was clear (see pH arrow) that sites from Little River have higher pH.

In **Fig. 7.5** the pH gradient (significant correlation of pH with axis 3: 0.7613 (see also **Table 7.2**)) can be clearly seen along the Little River sites. Água Forte sites are in the opposite direction of the pH arrow.

7.5 DISCUSSION

Low pH values, high metals and SO_4^{2-} concentrations and high conductivities in the Água Forte Stream have been previously shown to result in catastrophic decreases in diatom diversity due to the elimination of sensitive taxa (Luís et al. 2009). Similar patterns have been shown by other authors who have predominantly found that the number of taxa and diversity of diatom assemblages decreases along decreasing pH and increasing metal gradients (Sabater 2000, Bray et al. 2008). In this study, numerous AMD tolerant taxa were present, especially *Eunotia exigua* (Patrick 1977, Verb and Vis 2000, Novis and Harding 2007) and *Pinnularia aljustrelica* (Luís et al. 2012).

Teratological forms of *E. exigua* were found (consistent with previous observations), which may be an indicator of metal stress because frustule deformations (in this study: distortions of cell outline) (e.g. Cattaneo et al. 2004, Morin et al. 2008) have been correlated with high metal concentrations, although other chemical parameters can induce valve abnormalities (Falasco et al. 2009). Recently, in Canada, a study in a mining area – the Montauban Mine (Québec, Canada) – conducted by Lavoie et al. (2012) showed the presence of diatom taxa, known to tolerate high metal concentrations as well as the presence of deformities of their valves. These deformities were found as valuable endpoints for metal toxicity assessment – 1 % of *Achnanthidium minutissimum* deformed valves was observed in the less impacted site (Lavoie et al. 2012) – as well as in this study, where 5 % of *E. exigua* teratological forms was also found in the less contaminated site, AF4 (Água Forte Stream).

Interestingly, *Amphora* cf. *coffeaeformis* dominant in site AF3 appears to be the exception to the acidic specialist trend because ecological information available for *A.* cf. *coffeaeformis* (alkaliphilic taxon from brackish waters (Zlatko 2009)) does not match with our findings (acidic sites). Despite the morphological resemblances of this taxon identified as *A.* cf. *coffeaeformis* (according to the published information on this species), further studies are needed in order to clarify its identification. According to the bibliography, *A.*

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coffeaeformis presented tolerance mechanisms to heavy metals (e.g. Brown et al. 1988). Here, as shown by CCA analysis, it might be adapted and perhaps a suitable indicator for Pb. But, as explained above, whether its identification and its Pb adaptation represent a species-specific or site-specific acclimation should be clarified.

On the other hand, Little River is characterized by somewhat acidic waters with lower sulphate and metal concentrations compared with those found in the Água Forte. In Little River, *E. exigua* was also routinely found; it is an acidobiont taxon according to Van Dam et al. (1994) and Patrick (1977); it is highly tolerant to contamination and is a common species in rivers and lakes influenced by AMD in North America (Besch et al. 1972) and Europe, when pH values are less than 5 (Kwandrans 1993). Sites closest to the mine and furthest downstream from the mine (LR1 and LR4, respectively) had neutrophilic taxa such as *A. minutissimum* and *Nitzschia palea* as dominant species, but as well as in Água Forte Stream, acidobiontic (e.g. *E. exigua*, *Frustulia saxonica*) as well as acidophilic taxa (e.g. *Eunotia arculus*, *Eunotia bilunaris*, *Eunotia rhomboidea*, *Tabellaria flocculosa*) were found in Little River. This finding is in agreement with *H'* values for Little River sites of intermediate closeness to the mine (LR2 and LR3) with the higher diatom diversity found, which was not found in sites of similar proximity in the Água Forte Stream. In particular, diversity of diatom taxa in LR1 was significantly higher, which suggests that the additional remediation procedures were effective immediately downstream of mining activity. In a study by Campeau et al. (2005), the authors determined that the most impacted sites in Canada were rarely attributed the worst quality values, indicating that the level of alteration is lower for Canadian streams than for similar sites in Europe. In response to this finding, Lavoie et al. (2006) created an index to evaluate water quality using diatoms in Eastern Canada (IDEC) based solely on the biota present.

In this study, the CCA analysis determined that pH, conductivity, sulphate concentrations and metals were the predominant factors separating the two sets of sites, despite their geographic separation. Água Forte sites were predominantly responding to acidity and high metals concentrations, as well as Little River sites despite higher pH values and comparatively lower metal concentrations. More than 85 % of the variation in the dataset was captured by the first three CCA axes and Monte Carlo tests indicated that metals were the most significant predictors of diatom community pattern. According to CCA analysis, it is clear that *P. aljustrelica* is highly correlated with high conductivities and to a lesser extent with high metal and sulphates concentrations. Despite geographic isolation, differences in climate and historical impacts (Roman era versus modern day), these two rivers had some common taxa (e.g. acidic diatoms such as *Eunotia* sp.).

However, Little River sites had higher pH and lower metal concentrations which led to more diverse communities whereas in the Água Forte system, taxa were responding to the combined action of increased acidity and metal impacts which indicates the specialization of diatom taxa to multiple stressors. Of particular interest for future studies is response to pH, as there is some evidence that diatom communities recover quickly when acid stress is removed (as in Findlay 2003) although the time to recovery with respect to climatic and regional components has yet to be adequately addressed. Therefore, the positive increase in diversity in the Canadian sampling sites begs the question as to whether (and how quickly) similar remediation approaches could initiate positive responses in the Água Forte. We suggest that in the Água Forte, restoration efforts would lead to a dramatic increase in the abundance of less tolerant taxa, probably increasing the diversity and stability of the diatom community. Similar changes are less likely in New Brunswick where the diatom community is already dominated by numerous taxa in sites closest to the Brunswick Mines; therefore, changes in environmental conditions are unlikely to result in major changes in community structure because diverse diatom communities are more stable and more resistant to changes in the environment.

In Canada, additional remediation measures in the processing of effluent greatly reduced the presence of acidifying thiosalts in downstream reaches. Runoff from the mine site, along with processed effluent, is collected, stored in a buffer pond and treated with lime at a HDS ETP prior to discharge into South Little River (near site LR1). By reducing the release of thiosalts, the principal source of metals and low pH from the tailings ponds, pH conditions were restored to acceptable limits and, as a result, more stable diatom communities persisted. Therefore, non-dominance of acidic taxa in the closest site to the mine is likely to be related to secondary mine effluent treatment.

In contrast, in Portugal, AMD in the Água Forte Stream is more difficult to correct given restraints on space to build treatment ponds as well as cost. Also the ongoing remediation measures in the Água Forte Stream (acid remediation using lime additives or thiosalt removal treatments and water diversion systems to minimize wastewater/rock contact) are thought to be ineffective (Maia et al. 2012) and comparisons to other systems where species diversity has been maintained are warrented.

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7.6 CONCLUSIONS

This is an exploratory study that compares two river systems exposed to the same geological and mining impacts in different geographic, climatic and regulatory contexts. Regional differences in riverine diatom communities exposed to pH/metal stresses in both Canada and Portugal were seen, but with much higher metal concentrations and lower pH found in the Água Forte Stream. Using the same biological metal assessment method in the two systems, regional differences in concentrations of metals, conductivity and pH as the primary drivers of benthic diatom community structure were found, despite the obvious geographic and climatic differences between sites. The most abundant species at any given site (e.g. *Pinnularia aljustrelica*, *Eunotia exigua*) were those whose characteristics made them well-adapted to the surrounding biotic and abiotic factors. As a stress sign, teratological forms (deformed valves) of *Eunotia exigua* were found.

Numerous studies have shown that diatoms are excel- lent indicators of changes in metals (e.g. Morin et al. 2012) and acidity (e.g. Van Dam et al. 1994). This study has shown that diatoms can also be consistent indicators between continents and climatic regions. Therefore, the potential exists for global indicators using diatom diversity and tolerant taxa, respectively, although there are many challenges to consider before this approach is universally applied (e.g. Lavoie et al. 2012). Finally, this floristic survey is a useful basis for future studies on the conditions of geographically and climatically distinct zinc mining areas and suggests that further examination of patterns between regions exposed to similar stressors is warranted.

7.7 ACKNOWLEDGMENTS

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CHAPTER 8

Conclusions and Future Directions

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8.1 GENERAL CONCLUSIONS

The objectives proposed for this PhD study have been achieved. It was possible to predict the geochemical and biological alterations caused by mining activity and its chemical rehabilitation in terms of sediments' and waters' physico-chemical characterization and on the biodiversity of benthic diatom communities. This research highlighted the diatom diversity of impacted and unimpacted streams of the Aljustrel pyritic mining area, belonging to the important geological unit - IBP (Iberian Pyrite Belt). Novel taxonomical, ecological and physiological information on diatoms from AMD (Acid Mine Drainage) environments was retrieved and will aid in future management plans of these extreme areas.

Geochemical and mineralogical conditions of the Aljustrel mining area were analysed and their integration with diatom communities was achieved. Sampling years were climatically atypical (dry years), where the influence of the rainfall was determinant to physico-chemical behavior of waters and sediments. Sampling sites were arranged in three groups taking into account the geochemical conditions, ranging from impacted sites (Group 1) to slightly impacted (Group 2) and to unimpacted sites (Group 3). The mineralogy of the sediments of the unimpacted sites was characterized only by quartz and feldspars, whereas in impacted sites also some clay minerals and sulphates and oxy-hydroxysulphates dominated. These last two are considered as very soluble mineral fractions that precipitate during dry periods, retaining high metal contents. Then with the first rainfall, the sulphates can hydrolyze, which leads to a decrease in pH. These conditions in AMD impacted areas can lead to drastic changes in diatom communities, which can ultimately reach extinction of the entire communities. Through sequential chemical extraction, it was possible to verify that metals are mostly associated to the iron oxy-hydroxide phases and are thus, bioavailable in the impacted sites, whereas in unimpacted sites there was a tendency to the association of the metals to the primary phases of sulphides which is not a bioavailable fraction.

The same three Groups already established by geochemical data were also discriminated by diatom assemblages. ***Thus by the integration of geochemical and diatom data***, it was possible to relate metals with teratologies, which is promoted by the occurrence of cationic species of the type Me^{2+}, the most abundant in waters of the sites where diatoms' deformed valves occurred. Thus metal speciation can be important to unravel the causes of valve teratologies of *Eunotia exigua* (Group 1) and *Achnanthidium minutissimum* (Group 2). Based on these results we hypothesized that abnormal valve development was a symptom of metabolic disorder that represented the price for

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tolerance to some environmental factors, which in the present study, can be the high metal concentrations (Luís et al. 2011, 2013). An additional geochemical factor – salinity – can be important to explain many of the anomalies and the low correlations between metals and abundances in teratological forms. In other words, high salinity levels (expressed by Cl⁻) limited the appearance of teratologies once it conditioned the emergence of adapted diatom species.

In summary, by combining geochemical and diatom data it was possible to reveal 3 Groups of sites:

• Group 1: extreme conditions of low pH and high metal contents. The species that survived in this group of sites were perfectly adapted to these extreme conditions (e.g. *Pinnularia aljustrelica*). This adaptation can be due to strong silicification of the diatom frustule, because silica contents were high in this group. This fact can contribute to the non-appearance of teratological (deformed) diatoms. However, teratologies occurred in species of this group (*Eunotia exigua*) but in sites (BM and AF) of Água Forte stream, where pH was around 4 (not as low as in the most acidic sites) and metal contents started to decrease;

• Group 2: included sites with intermediate conditions of pH and metals, which already permitted the existence of typical species of uncontaminated streams. However, in this group, teratological forms also occurred. This can be due to the location of the sites where deformations occurred, which may have been affected by some AMD input, demonstrated by an increase of metal contents in solution, which affected the sensitive species (e.g. *Achnanthidium minutissimum*) presented here;

• Group 3: unimpacted sites, not influenced by AMD, thus no teratological forms appeared; with high contents in Cl⁻ which can justify the presence of brackish to marine species (e.g. *Entomoneis paludosa*) very silicified and not predisposed to deformations.

Regarding taxonomy and ecology of diatoms the discovery of *Pinnularia aljustrelica* sp. nov. Luís, Almeida et Ector was of great importance, making the evaluation of AMD impact on diatoms more precise, once 3 taxa - *Pinnularia aljustrelica*, *Pinnularia acoricola* and *Pinnularia acidophila* – have been mis-identified over time. *P. aljustrelica* has also been observed in samples from other AMD impacted sites of Huelva basin (SW, Spain) (Valente et al. 2013) in the IPB geological unit. This reveals that the taxon commonly called as *P. acoricola* of Río Tinto (Sabater et al. 2003), can be, in fact, *P. aljustrelica*. Particular geochemical conditions could contribute to the natural selection process which led to the appearance of this new species.

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As far as we know, this is the second attempt to determine the **photosynthetic activity** of low pH and metal tolerant phototrophic biofilms. The first experiment was done by Souza-Egipsy et al. (2011) in Río Tinto (SW, Spain) also belonging to IPB, however using a different methodology (rates of dark respiration (DR) and light-saturated net photosynthesis (Pmax) as oxygen exchange using an oxygen electrode). Ours was the first study using PAM fluorometry, a quicker method to determine photosynthetic activity. The information provided by Chl-*a* fluorescence parameters was an early warning signal of biological effects after acute exposure to chemicals. This study confirmed PAM fluorescence as an effective method for the rapid and non-destructive estimation of photosynthetic performance, increasing knowledge on the ecological importance of these biofilms for the maintenance of the primary production in these extreme and unique ecosystems. Nevertheless, PAM fluorometry is currently being used in the laboratory and in the field, for the measurement of impact of toxicants (e.g. metals) on the photosynthesis of autotrophic components of fluvial biofilms (e.g. Corcoll et al. 2012). However, to our knowledge this method was never used before, with both factors together: metals and acidity. The study of the complex geochemistry and interactions with the biota under field conditions makes the interpretation of the species ecological behavior and the isolation of the multiple parameters difficult.

A system of artificial streams in the laboratory was used to perform an experiment which mimicked the complex water chemistry of Água Forte stream, the most impacted stream of Aljustrel area. This experiment contributed to understand the selection pressure exerted by low pH, Fe, Cu and Zn toxicity and how they interact with biofilms of AMD field systems. Fe and acidity effect effects were never tested simultaneously, in laboratory experiments. During chronic exposure, acidic pH affected growth more negatively than high Fe. However, when Cu and Zn were added, in acidic treatments, the photosynthetic parameters increased their values in comparison to controls, showing improvement in the photosynthetic apparatus. The enzymes (e.g. catalase (CAT), glutathione reductase (GR)) didn't increase their activities in acidic treatments, revealing a better adapted community to metals. Thus, in pre-exposure conditions, acidic pH seems to ameliorate metals' effects.

Comparing geographical differences in benthic diatom communities exposed to similar stresses, a combined study in Canada and Portugal was made, reflecting similar diatom communities. It was a new approach in the Little River where only one previous diatom study was done in New Brunswick (Besch et al. 1972). To our knowledge, a study as this one, in such different climatic and geographic areas, was never done before. It

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highlighted the possibility of having a sustainable mining production keeping equilibrated the communities and the water's chemical conditions.

According to this study (especially based in chapters 5, 6 and 7) metals were found as largely responsible for the reduction in diatom abundances as well as the emergence of teratologies in the communities. In a general way, this work contributed to improve the knowledge of biological responses to Acid Mine Drainage (AMD) effects, regarding ecology, taxonomy, physiology and biochemistry of diatoms and their link with physico-chemical characteristics of sediments and waters.

8.2 FUTURE PERSPECTIVES

Three main directions can be followed after this study:

1) The European Water Framework Directive (2000) incorporates diatoms in water quality evaluations, however, AMD contaminated waters cannot be assessed based on the already established diatom indices (e.g. Specific Polluosensitivity Index (SPI) and Biological Diatom Index (BDI)), as already verified in a previous study (Luís 2007). These indices were developed based on diatoms' responses to saprobic and trophic states (organic contamination, dissolved oxygen, ammonia, nitrites, phosphates, BOD – biological oxygen demand). Interference caused by metals and some taxa (e.g. acidic) not included in the index calculation can be some of the reasons that make them unsuitable to determine the water quality in environments with high metal contamination. In a near future, an index should be proposed, based on diatoms (e.g. relative abundances of tolerant and sensitive species, diversity index, absence/presence of deformed forms, population's size reduction, especially the last two, indicators of metal toxicity) and chemical characteristics of water and sediments correlations, using this and previous works (Ferreira da Silva et al. 2009, Luís et al. 2009, 2011) to further extrapolate the index to other similar AMD environments;

2) it is our goal to propose a sustainable, low cost bioremediation technique, with low chemical toxic sludge, based on photosynthetic microorganisms, which produce carbon and generate alkalinity (bicarbonate to produce hydroxyl ions). They can also form an anoxic zone for Sulphate Reducing Bacteria (SRB) activity to generate even more alkalinity;

3) in the last years, the discovery of extremophile organisms able to succeed in past conditions inhospitable for life, showed that life can arise in unfavorable conditions and can adapt to different and extreme situations crucial for understanding the beginning of life on Earth (Amils et al. 2007), making AMD systems biologically and geochemically active.

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Part of these extreme acid environments can be preserved for astrobiological experiments *in situ*: acidophilic microorganisms can survive and adapt to the vast deposits of sulphates and iron oxides of pyritic mines, an analog environment to Mars.

8.3. REFERENCES

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