Universidade de Aveiro Departamento de Biologia
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Tânia Daniela da Silva Vidal

Avaliação Ecológica do Efeito Combinado de Contaminantes Ambientais através do uso de novas metodologias

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Avaliação Ecológica do Efeito Combinado de Contaminantes Ambientais

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ecotoxicologia e Biologia Ambiental, realizada sob a orientação científica do Prof. Doutor Fernando Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Prof. Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho à minha mãe, à minha mãe “adoptiva”-Maria Alice e ao meu pai e ao Zé

o júri

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Ecotoxicologia, directiva quadro da àgua, avaliação do estado ecológico de ecossistemas de água doce, analise de impactos por vários factores de stress, Rio Mau- Sever do Vouga, Minas do Braçal e Palhal, fitobentos, testes de toxicidade com diatomaceas, metais e pesticidas

resumo

A avaliação do estado ecológico de massas de água doce lóticas, baseada em exigentes critérios de classificação, foi definida pela Directiva Quadro da Água (DQA), como resultado da aplicação e otimização de metodologias integradoras de elementos físico-químicos, biológicos e hidromorfológicos. A aplicação destas metodologias não é fácil, já que requer grandes conhecimentos técnicos e científicos e, ao exigir muito tempo na sua aplicação, tem elevados custos financeiros. Assim, o principal objetivo deste estudo foi o desenvolvimento de metodologias complementares que contribuam para uma aplicação técnica, mais barata e mais rápida, dos exigentes critérios de classificação definidos pela DQA, com a obtenção dos mesmos resultados finais de avaliação. Para isso definiu-se o rio Mau, um pequeno rio de montanha sujeito a diferentes agentes de *stress* (*e.g.*, metais, pesticidas), como o local principal de estudo. O presente trabalho é iniciado com uma revisão histórica do desenvolvimento de diversos índices bióticos e da sua aplicação na avaliação da qualidade da água, salientando especialmente a mudança de paradigma, definido pela DQA, e as ações desenvolvidas para otimização e intercalibração de metodologias, na avaliação do estado final das massas de água. O estudo da caracterização espácio-temporal centrou-se na aplicação da metodologia DQA ao rio Mau, usando apenas macroinvertebrados, recolhidos durante quatro estações do ano. Os resultados foram comparados com dados históricos dos últimos três anos e demostraram que o rio se encontra em muito bom estado. No entanto, a qualidade ecológica diminui em determinados locais indicando que os organismos estiveram sujeitos a algum tipo de perturbação. Considerando que a qualidade ecológica pode ser condicionada por episódios cíclicos de contaminação, a partir de sedimentos, em situações ambientais adversas, realizaram-se ensaios com elutriados de sedimentos de rio recolhidos nas imediações do complexo mineiro Braçal-Palhal. Os resultados permitiram concluir que esta metodologia foi eficaz na sinalização de locais negativamente afectados pelos efluentes mineiros do estado de contaminação, sendo importante na priorização de áreas críticas, potencialmente impactadas, na avaliação do estado ecológico. No entanto, esta metodologia implica a recolha de sedimentos, o que pode promover a alteração e/ou perda de contaminantes. Para resolver este potencial problema, pretendeu-se desenvolver uma metodologia que permita obter resultados semelhantes, mas que seja aplicada diretamente no local de estudo (*in situ*). Para isso, recorreu-se a uma microalga bentónica, pertencente à flora Portuguesa, sensível à poluição orgânica e a metais. Esta metodologia foi otimizada, na perspetiva de ser usada como teste padrão *in situ*, recorrendo à imobilização de microalgas bentónicas de água doce em alginato de cálcio. Verificou-se que a sua sensibilidade e o crescimento foram similares aos resultados obtidos em ensaios com células livres. Esta nova metodologia permite obter uma resposta muito rápida sobre o grau de contaminação de um local e poderá constituir uma metodologia complementar à DQA.

keywords

Ecotoxicology, Water Framework Directive, Ecological evaluation of freshwater ecosystems , multi-stressor scenario , Mau river (Sever do Vouga), Mines of Braçal e Palhal, Phytobenthos, Toxicity tests with diatoms, metals and pesticides

abstract

The assessment of ecological status of lotic freshwater bodies, based on stringent criteria of classification, has been defined by the Water Framework Directive (WFD), as a result of the implementation and optimization of methodologies that integrate physico-chemical, biological, and hydromorphological parameters. It is recognized that the application of this methodology is not easy, because it requires deep technical and scientific knowledge; it is time consuming in its application involving high financial costs. Thus, the main objective of this study was the development of cheaper and faster complementary methodologies that may contribute to the technical application of the classification criteria defined by the WFD, achieving the same final results of evaluation. In order to achieve this main goal, the river Mau, a small mountain river subjected to different stressors (*eg*, metals, pesticides), was established as the main sampling area.

This thesis reviewed the historical development of various biotic indexes and its application in assessing water quality, especially highlighting the new paradigm defined by the WFD, and the corresponding actions developed for optimization and intercalibration of methodologies, evaluating the final state of water bodies.

The ecological spatiotemporal characterization of the river Mau focused on the application of the WFD methodology, using at this stage only macroinvertebrates collected during four seasons. Results were compared with historical data of the last three years and they demonstrated that the river is in good condition. However, the ecological quality decreased at certain locations indicating that organisms were subjected to some type of disturbance. As the ecological quality can be conditioned by pulses of contamination from the sediments, in environmental adverse conditions, assays were performed with elutriates, obtained from sediments collected near the mining complex Braçal-Palhal. Results showed that this method was effective achieving the state of contamination, which may be important in prioritizing/scoring of critical areas within river ecosystems potentially impacted, using the WFD methodology.

However, this methodology requires the collection of sediment which can promote the modification and / or loss of contaminants. To solve this potential problem, we developed a new methodology to obtain similar results. For this, we used a benthic microalga, belonging to the Portuguese flora, sensitive to organic pollution and metals. This methodology was optimized for application *in situ*, by immobilization of diatom in calcium alginate beads. The results showed that their sensitivity and normal growth rate are similar to data obtained when used free cells of diatom. This new methodology allowed the achievement of a very quick response on the degree of contamination of a site, providing a complementary methodology to WFD.

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Chapter I

General introduction

General introduction

1.1. Pollution sources and effects on freshwater environments

A significant part of human population inhabits regions in proximity of waterways, estuaries and coastal areas. Aquatic ecosystems are particularly vulnerable to disturbances resulting from anthropogenic activities such as habitat modification, overexploitation of living resources (Diaz et al. 2004), and from mining activities, including metal pollution and acid mine drainage (Malmqvist and Hoffsten 1999). Lotic systems are necessarily dependent on their catchments and land-use changes affect them directly; thus rivers are likely to be subjected to several types of impact. It was predicted that the primary future threats to running waters shall be ecosystem destruction, physical habitat and water chemistry alteration, and the direct addition or removal of species as a result of industrialization, urbanization, land-use change and water-course alterations (Malmqvist and Rundle 2002).

Climate change will also impact running waters through alterations in hydrological and thermal regimes. Effects from climate change may be more extreme over longer time scales (>50 years) (Malmqvist and Rundle 2002). The overriding pressure on future running water ecosystems will stem from the predicted increase in human population, with concomitant increases in urban development, industry, agricultural activities and water abstraction, diversion and damming. Future degradation could be substantial, rapid, and may be concentrated in areas of the world where resources for conservation are most limited and knowledge of lotic ecosystems most incomplete, or, more importantly, ignored.

Due to large impairments observed in freshwater environments, demands regarding conservation issues have led to the improvement of methods delineating and evaluating environmental conditions at national and international levels (Diaz et al. 2004); this can be exemplified by the Water Framework Directive (WFD), published by the European Commission, aiming to establish a legislative framework for protecting European inland surface waters, transitional waters, coastal and ground waters (EU 2000).

1.2. Water Framework Directive

The Water Framework Directive (WFD, 2000/60/CE), establishing a new regulatory framework for all EU Member States, to protect, enhance and restore aquatic environments through the implementation of programs and measures developed as part of River Basin Management Plans (COM 2012) in order to maintain or achieve good water status by 2015 (EU 2000; Ferreira et al. 2011). The WFD changed the classic approach of viewing water as a resource (anthropocentric perspective) into considering water as an ecosystem holder (ecocentric perspective) (Hering et al. 2003; INAG 2008). Although paradigmatic, the WFD approach to monitoring biotic communities is somewhat limited to the use of biotic indices, which consist of numerical expressions used to assess water quality based on the presence and diversity of pollution-tolerant and pollution-intolerant *taxa*. Ultimately, these indices derive from the "Saprobiensystem" of Kolkowitz and Marsson (1908), developed to assess organic pollution, which Washington (1984) considers the first biotic index ever created. More recently, Feld (2004) proposed a more holistic approach using multiple indices capable of assessing the impact of different *habitat* pressures both on instream biota and physical *habitat* (*e.g*. hydromorphological assessment). The most important reason for stressor-specific assessment methods was that individual *taxa* may not be equally sensitive to all types of stressors (Chessman and McEvoy 1997). Multimetric indices (classifying the reference condition based on geographical and physical attributes) and multivariate (use of biological assemblage to establish the variance expected to occur in the reference condition) (Lücke and Johnson 2009) systems were then used to integrate the impact of multi-stressors on freshwater systems and both systems are compared to reference conditions.

The WFD demands that River Basins maintain or achieve good water status for all E.U. member states. Good water status is achieved when both ecological and chemical statuses are at least good. Ecological status is an expression of the quality and function of the aquatic ecosystems and its classification integrates "biological quality elements", hydromorphological, chemical and physico-chemical elements. The application of the WFD implies that all countries use methodologies in assessing the ecological status of streams and rivers in order for the results to be comparable among all E.U. members.

In order to uniform methodologies, processes and data, many European projects (*e.g.*, AQEM, STAR, FAME) were developed in different countries, aiming to define sampling program design according to the stream type, criteria for defining reference sites, and methods used for sampling biological communities.

Hering et al. (2003) published an overview with special focus on the sampling program design, particularly on stream types and sampling site selection, the criteria for defining reference sites and methods used for sampling macroinvertebrates. Establishing reference conditions plays an important role in calculating ecological quality ratios (EQR) and determining the deleterious effects of human-generated stress once the sampled biological communities are compared with the reference data for evaluating the ecological quality of freshwater ecosystem. The EQR is achieved by dividing the value of the observed biological parameter, for a given surface water body, and the expected value under the reference condition. The ratio shall be expressed as a numerical value between 0 and 1, with high ecological status represented by values close to 1 and bad ecological status with values close to 0. Establishing reference conditions nowadays can be very difficult since pristine conditions for hydromorphological, chemical and physical elements are not easily found. The same authors (Hering et al. 2003) claimed that choice of the reference site must have minimal human-generated stress and the most natural variability of the response variable. Hering et al. (2006) also provide practical advice and solutions for many issues associated with the methodologies of the WFD.

In Portugal, and regarding the biological components, the calculation of EQR adopted only the macroinvertebrates and phytobenthos communities (INAG 2008) due to well-known autoecological species information regarding their tolerance to pollution, and also to already complete identification keys and biotic indices used before the implementation of WFD methodologies. The so-called intercalibration process intended to harmonize the values of the boundaries between the classes of high and good status and those between good and moderate status between Member States. It is of great importance that the ecological status has the same meaning within the member states. Harmonized class boundaries are difficult because the process must consider the natural differences between stream types but also eliminate different perceptions of ecological quality (*e.g.* different member states may have different perceptions of the reference situation of what a “slight” or “moderate” deviation from the reference situation is).

1.3. Ecotoxicological evaluation of pollution effects in freshwater ecosystems

The Mau River is a small (13 km length) mountain tributary of the Vouga River, located in the vicinity of Sever do Vouga (40º44'00’’N 8º22'00’’W), flowing from its headwater in Serra do Salgueiro to its mouth in Pessegueiro do Vouga. The Caima River flows from its headwater, in Serra da Freita, into the Vouga River, has several tributaries,

and receives effluent metals from the Palhal mine located on its banks. The Mau River, an important source of water for drinking and irrigation for of the area, is also a recipient of point-source and diffuse contamination by organic compounds and metals from the Braçal Mine. Both mining complexes (Braçal and Palhal) are included in the same sub-watershed of the Vouga River that flows downstream to the shallow Aveiro lagoon (Ria de Aveiro). The Braçal mine (40º 44' 10''N 8º 24' 6.6''W) was an important centre for extraction of galena ore (native lead sulphide), zinc blend ore and iron pyrite ore as described in a broader study about deactivated mines in Portugal (Santos Oliveira et al. 2005). Past mining activities at the Palhal mine (40º 44' 50''N, 8º 27'21.5''W) included the extraction of metals such as Pb, Cu and Ag as pyrrhotite (FeS) ore, chalcopyrite ($CuFeS_2$) ore, galena (PbS) ore, sphalerite (ZnS enriched in cadmium) and pyrite ore (FeS_2) (Nunes et al. 2003). Despite the Braçal and Palhal mines being inactive from the 1950s and 1920s, respectively, the effluent of Braçal mine drains into the Mau River and the effluent of Palhal mine drains into the Caima River. Point source contamination through a metal-rich run-off can be identified in Braçal while in Palhal drainage from tailing accumulation riverside should additionally be considered.

In this sub-section a new methodology was purposed by using a cost-effective ecotoxicological approach to assist the prioritization/scoring of critical areas within river ecosystems potentially impacted, in this case, by deactivated mines. Superficial samples of sediments were collected and were tested by WET (Whole Effluent Toxicity) tests and Elutriate Sediment Toxicity Tests (ESTT), widespread useful tools to address the toxicity of complex environmental samples (USEPA 2001).

The use of elutriates from the river bead sediments approach as the new methodology for testing a sensitive ecotoxicological test battery composed of the bioluminescent bacteria *Vibrio fischeri*, freshwater planktonic microalgae *Pseudokirchneriella subcapitata*, and macrophyte *Lemna minor*. Acute and chronic bioassays used daphnids *D. magna* and *D. longispina*. The results obtained from the several ecotoxicological bioassays performed for each mine (Braçal and Palhal) were, in general, very concordant and allowed to clearly distinguish between their hazardous potential. Indeed, historical ecological assessment of the Mau River suggests that no negative impacts toward the biota are imposed by the effluent from the Braçal mine. On the other hand, our results configure Palhal mine as a priority for further assessment within the scope of the WFD, so that the range of the related ecosystem impacts can be fully recognized and adequate recovery plans can be established. This new methodology had no intention to replace the WFD, but to provide more expedient ecotoxicological

information about the contamination status of the location relative to prioritization/scoring of critical areas.

1.4. Development and optimization of new benthic freshwater microalgae test

Alongside macroinvertebrates, phytobenthos have great importance for river ecological status evaluation in the Portuguese WFD since they are present in abundance from the river spring to the river mouth, but require well trained diatom taxonomists to identify the benthic diatom community increasing the difficulty of phytobenthos. From an ecotoxicological point of view, freshwater microalgae are commonly used as test species due to their ubiquitous, generally sensitive, rapid and cost effective response within the ecotoxicological assessment of several potential toxicants, hence their being considered standard organisms (Michael 1995). They inhabit a very specific habitats and being easily sampled. Regulatory entities recommend algal ecotoxicity tests with planktonic species such as *Pseudokirchneriella subcapitata* in risk assessment frameworks to represent producers of the aquatic food web (EC 2002). Microphytobenthos species, however, have not been included in these recommendations (Ivorra et al. 2000) despite the fact that they are key players in the ecological dynamics of lotic freshwater systems in which planktonic species are almost absent. Diatoms, in general, provide advantages as environmental assessment tools due to their widespread distribution and diversity (Brabec and Szoszkiewicz 2006). In what concerns their bioindicator performance, benthic diatoms are known for their high discriminatory power in assessing acidification, eutrophication, saprobity, nitrogen, salinity and river current velocity (Besse-Lototskaya et al. 2006; Johnson et al. 2006). The regular ecotoxicological tests performed with standard planktonic green microalgae (*P. subcapitata*) evaluate the water column which then should be complemented with phytobenthos ecotoxicological tests in order to evaluate sediment compartments. Sediments in aquatic ecosystems act like a sink by accumulating contaminants (Burton 2002), that were once in the water column, but through action of gravity settle and bind to organic and inorganic particles in the sediment of river beds, and consequently, sediments generally remain contaminated even when water quality has already improved in many aquatic systems. Thus, sediment contamination can be present for very long periods depending on the desorption velocity of contaminants (Burton 2002). Bioassays testing the toxicity of contaminants and sediment toxicity tests are tools with increasing importance from the regulators' and scientists' point of view, but little or no effort has been made in the standardization of testing of bioavailable contaminants on benthic microalgae (Moreno-Garrido et al. 2003; SETAC 1993).

The novelty of this work is related to the development of a new methodology using a single sensitive benthic diatom species from the native Portuguese flora as a reliable tool to assess sediment toxicity. The importance of the species belonging to the Portuguese flora is related to its future application in the field. By choosing a native Portuguese benthic diatom, it was assured that no species would be introduced for sediment toxicity tests purposes. The OECD (2011) guideline for testing chemicals on freshwater algae and cyanobacteria already recommends the use of a planktonic diatom (*Navicula pelliculosa*) among other species considered standard species. Therefore we use a benthic species (*Navicula libonensis*) from the same *genus* (*Navicula*).

This new methodology can be an add-on to complement the complex task the WFD has placed upon the phytobenthos community in their ecotoxicological study and analysis of freshwater river sediments.

1.5 Objective and structure of the thesis

The ecological status evaluation of lotic water bodies, based on the criteria of classification (defined by the WFD as a result of the application and optimization of integrative methodologies of physico-chemical, biological and hydromorphological elements), is not easy and requires deep technical and scientificexpertise— being time consuming and consequently very expensive. Considering this, the main aim of this study was to develop complementary methodologies that can contribute to more cost-effective WFD compliance with the same quality of results. In order to achieve this main goal, the Mau River, a small mountain stream subjected to different stressors (*e.g.*, metals, pesticides), was defined as case study.

This thesis is structured in seven chapters. The first and seventh chapters concern the general introduction and final remarks of the thesis, while the other five chapters are individual research papers, published or submitted to international peer-review journals.

Chapter II – This chapter contributes to the understanding of the major changes that occurred since the creation of the first biotic index to the multivariate index used today to evaluate the water quality. Also, the paradigm changes from anthropocentric to ecocentric and the effort developed for more realistic evaluations of different sources of impact are addressed.

Chapter III – This chapter aims to evaluate the ecological status of the Mau River using the WFD approach based on the macroinvertebrates community, encompassing both spatial and seasonal variations. Our specific goals were: i) to evaluate the sensitivity of the macroinvertebrates community to the multiple stressors along the course of the river; ii) to assess whether the ecological status of the Mau River was impaired by looking at its ecological status during two different periods (2005-2006 and 2009-2010); iii) to compare the WFD approach (based on ecological quality ratios—EQRs—derived from the biotic index IPtIN) with more refined tools in community structure analysis.

Chapter IV – This particular chapter comprises a battery of sediment elutriate toxicity tests allowing the evaluation of historic metal contamination by the effluent that still today drains from the Braçal and Palhal mines into the water flow. The primary aim was to assess whether the ecotoxicological assessment resembled the contamination of the sites following long-term metal input operated by the deactivated mine effluents, hence assisting the establishment of necessary grounds towards the development of adequate early-warning methodology to access water quality.

Chapter V – This chapter describes the optimization of methodologies for culturing single sensitive benthic diatom species, native to Portuguese flora, for ecotoxicological bioassays with the benthic diatom. Furthermore, standard guidelines for testing chemicals already recommend the use of a planktonic diatom from the same *genus* (*Navicula pelliculosa*). The aim of the work was to test the suitability of the native benthonic diatom species to ecotoxicological evaluation of the contamination, in laboratory conditions with reference substances.

Chapter VI – This chapter depicts the optimization procedure, in laboratory, for using the immobilized benthic diatom (*Navicula libonensis*) *in situ* ecotoxicological bioassays. This technique was never before applied to freshwater benthic diatoms to perform water quality evaluation.

The ultimate outcome of this thesis was to generate new information about the ecological status of the sub-watershed of the Vouga River, having performed an ecotoxicological test battery using elutriates from river bed sediment on standard test organisms that cover different functional levels. Furthermore, the novelty of this work was to develop a new technique of using a benthic freshwater diatom in ecotoxicological tests

and also validating their use to *in situ* assessments of ecological status of freshwater environments; this methodology can also assess the contamination bounded to sediment particles and organic matter without the usual community analysis of phytobenthos.

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Chapter II

Evaluation of the Ecological Status of freshwater ecosystems using macroinvertebrates: a review

Evaluation of the Ecological Status of freshwater ecosystems using macroinvertebrates: a review

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Em preparação

Abstract

Nowadays, freshwater environments are exposed to several types of pollution as a result of human activities, like nutrients enrichment, organic upload, metals, industrial wastes, pesticides, fertilizers and other agrochemicals.

The first approach (Saprobien System- 1908), created to evaluate the water systems, was focused on organic pollution. More complex biotic indices have been created since then to face the several pressures posed by increasing human pressure in aquatic freshwater ecosystems. Multimetric and multivariate biotic indices (that address food and feeding habitats of macroinvertebrates), biological traits such as body size, fecundity, voltinism and functional community structure of macroinvertebrates, fish, phytobenthos and macrophytes were reliable tools providing a holistic picture of the ecological status of the freshwater environments.

This work intended to describe the evolution of biotic index use since the Saprobiensystem in 1908 until present time and to show all the controversies related with advantages and disadvantages of their applicability. On the other hand, this review discusses several issues related to the Water Framework Directive (WFD) such as intercalibration, ecological reference conditions, metrics used; seasonal variability, sub-sampling and sampling size, taxonomic resolution, in pursuit of achieving in a more realistic fashion the impacts of degradation on freshwater ecosystems.

Key words: stream assessment, Water Framework Directive, pollution, ecological status, biotic indices

2.1. A brief history of water quality assessment

Ecology has long addressed much research for the understanding of organisms' assemblages and dynamics of species. Early, ecologists understand that stress and pollution produce changes in ecosystems. In order to quantify such changes, they used two main approaches: community structure (by means of ordination according to the overall similarities) and on measures based on indicator organisms (Washington 1984).

"Biotic indices" are an approach to water pollution making use of indicator organism concept; as such they do not represent community structure. An indicator organism can indicate either clean or polluted conditions. Therefore, it cannot be equally sensitive to all types of pollution, (e.g. organic, oil, metals, detergents, pesticides), however, a biotic index is likely to be specific for one (or two) particular types of pollution. The biotic index has been referred, in the literature, in such a vague and wide manner that it could also cover diversity and similarity indices. The concept of biotic index was used for water pollution assessment and adapted further to air pollution, plants and so on (Washington 1984). A biotic index is not universally applicable because indicator organisms' taxonomy will vary greatly among geographic regions and consequently the biotic index. Due to this specificity, EPA, in 1972, did not commonly accept the use of biotic indices while some authors (e.g., King and Ball 1961) already generally accepted biological techniques using indicator organisms. A biotic index (e.g. macroinvertebrates) is obtained from sampling a location to be evaluated, according to standard procedures. Then the organisms are sorted, identified and counted. Each group is given a score according to its abundance of pollution tolerance and pollution intolerance taxa. The total score represents the index and the higher the score the better conditions of the ecological status of the system analyzed. Since 1908, when appears the Saprobiensystem, several other biotic index, developed to calculate the impact of pollution in the freshwater systems based on macroinvertebrates, were created or adapted [see Washington (1984), for a detailed description].

All the indices were described and their advantages and disadvantages were widely discussed by several authors. However, no consensus was reached and some indices showed stronger advantages than others, some were applied and others were never put into practice (Washington 1984).

According to Washington (1984), the Beck's biotic index (1955) was considered undoubtedly, at that time, the first true "biotic index" based on macroinvertebrates and used only for organic pollution assessment. This biotic index considered that the final value should be based on the combination of the number of species of clean water

organisms (Class I organism) and the total number of species comprising the fauna of the section of the river to be evaluated (Class II organisms). Meanwhile Beck's biotic index also suffered from many criticisms, according to Washington (1984), such as: lack of statistical calculation to keep it simple, ignores other forms of pollution, the classes of organism established (Classes I and II) are subjective because the organisms' behavior depends on environmental conditions.

Latter in 1976, in Great Britain, was set up by the Department of the Environment Standing Technical Advisory Committee on Water Quality (STACWQ) a group called Biological Monitoring Working Party (BMWP) with a specific goal: to recommend a system which could be used to assess the biological condition of rivers by identifying the natural communities of river macroinvertebrates and their relationship with environmental factors. It was recognized that chemical and biological data were complementary and provided different kinds of information for the assessment of river water quality. The final version of such index (BMWP index) was published by Chesters (1980). Although not included in the original BMWP, the score system which was very influenced by the sampling size and its correction was purposed dividing the score by the total number of scoring forming the ASPTindex (Average Score per Taxon) which was preferred, among others, by Armitage et al. (1983). The individual family scores were reduced to a range of quality:1(bad) to 10 (good).

BMWP and ASPT indexes suffered adaptations and was used in several European countries (Lorenz et al. 2004; Orwin and Glazaczow 2009; Sandin and Hering 2004). A few years later, BMWPindexwas adapted to Iberian Peninsula by Alba-Tercedor and Sánchez-Ortega (1988). Those adaptations encompass the addition of new macroinvertebrate families, changes in some scores and correlations of particularly significant values representing degrees of pollution. Five levels of water quality were thereby established (class 1 – blue - very clean waters; class 2 – green – some contamination; class 3 – yellow-some contamination effects are observed; class 4 – orange- contaminated; class 5 – purple – heavily contaminated). The adaptation of BMWP index to Iberian Peninsula was considered very successful due to the good, fast and easy results obtained and made it an excellent working device in evaluation of water quality of streams and rivers. The IBMWP index was adapted by the Spanish Society of Limnology (Zamora-Muñoz et al. 1995).

Until the early nineties of the last century, water quality monitoring in EU member states was mainly been based on chemical and physical parameters (paradigm of analytical determination of contaminants in water). IBMWP index (Great Britain and

Iberian Peninsula version) introduces another concept to evaluate the quality of water in rivers and streams (biological quality paradigm). Under the “analytical paradigm” work, the sampling of water, for physic-chemical analyses, provides eventually erroneous information about the contamination of the moment before the collection (snap shot). In the contrary, under the biological quality paradigm, the biological data can integrate information of the water quality of the stream or river months before the data collection.This new paradigm also shifts the concept of indicator organism, to indicator community since it evaluates the community structure of macroinvertebrates present in a stream. Therefore, the new biologic quality paradigm enables the interpretation of all information before the moment of sampling, reflecting the presence of potential contaminations.

During the last decades of the 20th century, the impacts of organic pollution decreases as a result of enhancement of wastewater treatment techniques and facilities and other types of human (increasing industrialization) pressures inducing several impacts in freshwater ecosystems. The traditional water quality assessment approaches failed after the impairment of rivers became impacted with other environmental disturbances (e.g.acidification (Callanan et al. 2008); morphological degradation (Feld, 2004, Lorenz et al. 2004), channel alteration (Ofenböck et al. 2004) and introduction of metals (Dolédec and Statzner 2008).

2.2. Water Framework Directive

The Water Framework Directive (WFD) (EU, 2000) outlined a legal structure for the assessment of all types of water bodies in Europe. WFD established a new regulatory framework for the management, usage, protection, and restoration of surface water and groundwater resources within river basins and catchments, as well as in the transitional (lagoons and estuaries) and coastal waters of the European Union. One of the aims of the WFD is that all water masses achieve good ecological status by the end of 2015. The WFD was a very important step in water quality assessment that benefits the European Union partner countries since the rivers can then be seen and managed as ecosystems by a single legislative framework that sets uniform standards in water policy, respecting the different ecoregions (Moog et al. 2004) and stream typology (Verdonschot and Nijboer 2004). The assessment methods with one biotic index, due to their restricted approach to one or a few aspects of the aquatic ecosystem, no longer provided an adequate tool for integrated water management (Verdonschot and Moog 2006).

2.2.1 Metrics for ecological quality assessment

The metrics used before the WFD were mainly focused on organic pollution but modern times brought new challenges and other human stressors (eutrophication, acidification, salinization and deterioration of stream morphology) and their impacts needed to be evaluated. In the beginning, most of the systems were limited to a single factor and were only applicable in a restricted geographic range or for a certain stream type. Thus, there was a strong demand for systems considering different impact factors to enable an integrated assessment of streams considering multimetric score indices sensible to flow velocity, saprobity, trophy, substrate types and hydromorphological parameters) (Hering et al. 2003).The multimetric index concept was first developed by Karr (1981) as an index of Biotic Integrity (IBI) to assess stream quality using fish assemblages. Therefore, ecological information like how aquatic organisms feed, reproduce, exploit their habitats is used in multimetric indices construction and incorporated in water quality assessment. Dutch EBEOSWA (ecological assessment of running water) was the first approach assessing more than one impact on streams using benthic macroinvertebrates and use metrics related to current velocity, saprobity, trophy and substrate types (Dahl et al. 2004). Another approach was the British RIVPACS system which attempts to integrate all factors affecting the biocoenosis, based on site comparisons with reference conditions, within a habitat classification framework (Hering et al. 2003).

Recently, the WFD brought another demand to the ecological quality of all water bodies in EU members using different water quality elements: benthic invertebrate fauna, phytoplankton, fish fauna and aquatic flora that should be compared to reference conditions based on stream type-specific approach. Innumerous papers have been published testing several multimetric indices and metrics at several streams types in several countries and the results show that some fit better than others according to the situation tested (*e.g.*, Sandin and Hering, 2004; Ofenböck et al. 2004; Dolédec and Statzner, 2000; Dahl and Johnson, 2004). However, recently a paper was published which aims to compare the performance of three methods (single metrics, multimetric and multivariate) commonly used in bioassessment to detect effects of human induced nutrient enrichment stress on stream macroinvertebrates assemblages of fifteen streams of Southern Sweden (Dahl et al. 2004). Six single metrics frequently used in European bioassessment programs were selected: Saprobic index used in Austria (Zelinka and Marvan 1961), Belgian Biotic Index (BBI) (DePauw and Vanhooren 1983), Extended Biotic Index (EBI or IBE) used in Italy (Ghetti 1997), Danish Stream Fauna Index (DSFISkriver et

al. 2000), Biological Monitoring Working Party (BMWP) and Average Score per Taxon (ASPT)(Armitage et al. 1983). Two multimetric methods were developed for detecting the effects of organic enrichment in Swedish streams: DJ Index (Dahl and Johnson 2004) and the AQUEM type S05 Index (Consortium 2002). The multivariate approach was represented by Correspondence Analysis (CA), Detrended Correspondence Analysis (DCA, Hill and Gauch, 1980) and the Benthic Assessment of Sediment (BEAST) approach developed by (Reynoldson et al. 1995). The authors conclude that multimetric and multivariate approaches are reliable tools for detecting the effect of nutrient enrichment on stream macroinvertebrates communities better than the single metric approaches, as indeed, expected. The multimetric DJ Index and multivariate CA scores were sensitive to the stressor gradient and had high precision. In what concerns the single metric, Saprobic Index was the best of the six single metrics approach tested. The authors recommend that due to the differences in multimetric and multivariate approaches, both methods should be used in assessing the specific effect of nutrient enrichment of stream ecosystems (Lücke and Johnson 2009). Single metrics are often used aiming at detecting a specific type of degradation, multimetric indices, however, are considered to provide a broader perspective of the disturbance and thus might perform better in situations where more than one stressor is prevalent (Dahl et al. 2004), giving a more complex picture of the ecological system than single biological indicators do.

2.2.2 Bioindicators of disturbance and human impacts

In general, macroinvertebrates are particularly well fitted for assessment and quality indicator of aquatic systems. Macroinvertebrates was, comparatively, the most used group of indicators for several reasons: large amount of data exists, the identification is relatively simple and they occur in large numbers in all stream types (Hering et al., 2003). In some European countries, environmental agencies have been using benthic macroinvertebrates in bioassessment programs for water quality for many decades. The WFD introduced new biological indicators communities to evaluate the ecological status of streams, besides benthic invertebrates, like macrophytes (Brabec and Szoszkiewicz 2006), benthic algae (Brabec and Szoszkiewicz 2006) and fish (Pinto et al. 2006). Hering et al. (2004), aiming to assess the water quality of streams in Europe, from 2000-2002, used only benthic macroinvertebrates. These authors supported that benthic macroinvertebrates reflecting different anthropogenic perturbations through changes in structure or function in the assemblages, enabling an overall assessment of streams.

Furthermore, these organisms can also be used to detect acid stress, habitat loss and overall stream degradation and, indeed, the use of benthic invertebrates constitutes the basis for most biomonitoring programs, currently in use, in Europe (Lücke and Johnson 2009). Some characteristics justify their use such as: different sensitivities to pollutants of various types and react to them quickly; provide a grade response to a broad spectrum of kind and degrees of stress; ubiquity, abundant and easy to collect, relatively sedentary and therefore representative of local conditions (Metcalfe 1989). The other biological communities were addressed by Furse et al. (2006) despite the argument of Hering et al. (2006) that is not necessary to monitor all groups simultaneously. The use of multiple organism groups/assemblages can help to distinguish the effects of human induced stress more efficiently and effectively for several reasons: complex, multicellular, organisms such as fish may be better indicators of change in environment temperature than single-celled organisms like algae (Hering et al. 2006). Short generation organisms (algae and invertebrates) may respond more quickly to environmental changes than organisms with longer generation times (months to years); organisms that obtain nutrients directly (diatoms) from the surrounding may be better indicators of nutrient enrichment; large and mobile organisms that use a wide range of habitats (e.g., fish) may be more influenced by factors acting at spatial scales rather than small and sessile organisms (benthic algae or invertebrates). Therefore, organism's differences can be used to select complementary indicators (Hering et al. 2006).

Regarding the analysis performed on the communities of bioindicators suggested by WFD, Hering et al. (2006) suggest that there is no perfect bioindicators group and so the choice of the combination of groups of organisms used must be adapted to the type of human induced stress.

2.2.3 Design and optimization of water quality monitoring programs

The introduction of the WFD into the European legislation leads to the establishment of many working and investigation groups regarding their field appliance in all European countries using the same criteria and establishing and optimizing their knowledge in several issues like: hydromorphological stream assessment; establishing reference conditions; sub-sampling implications, seasonal variability of the bioindicators community sampled and the influence of the taxonomic resolution used by biotic indices.

2.2.3.1. Hydromorphological assessment in streams and rivers

The complexity of interactions between terrestrial and aquatic environments involving the structure and function of river ecosystems in catchments with undisturbed vegetation are controlled by floodplain forests. Due to complex relationship between hydromorphological attributes and instream biota it still remains controversial how to define habitat degradation and on what spatial scale(s). Hydromorphological degradation has become an important stressor affecting the instream biota and therefore the WFD must recommend that hydromorphological assessment of streams and rivers should be part of monitoring programs of EU members and the hydromorphological assessment methodologies must be in international standardized protocol. The WFD also recognizes that hydromorphological elements, along with chemical ones, support biological quality elements in the definition of quality status: (i) hydromorphological regime (quantity and dynamics of flow, connection to groundwater bodies), (ii) river continuity, and (iii) morphological conditions such as channel patterns, width and depth variation, flow velocities, substrate conditions and the condition of riparian zone. As part of the selection process for undisturbed reference site or the definition of reference conditions, these hydromorphological elements have to be taken in account also in biotic communities (Balestrini et al. 2004).

Hydromorphological assessment, generally, followed the approach to compare test site characteristics with specific reference characteristics per stream type. Therefore, stream type-specific hydromorphological reference conditions had to be defined '*a priori*' to the assessment. The set of hydromorphological variables to identify hydromorphological degradation strongly depends on the spatial scale. The analysis of hydromorphological variables on stream type scale was mainly oriented by catchment properties; from that only land use characteristics reflect the degree of human impact. Although on reach-and site-scale, several variables, such as, % shoreline covered with wooded vegetation and % of shading shown to be adequate descriptors of hydromorphological impact. In Europe, several methods included this site related evaluation, such as the British River Habitat Survey (RHS), the German-Strukturgütekartierung, and the French SEQ-MP. The last one has been developed by the Agence de l'EauRhin-Meuse for the establishment of a hydromorphological baseline of French rivers (Raven et al. 2002). The GSI (German Structure Index) represents a method to measure hydromorphological degradation based on the objectively recorded hydromorphological attributes providing two advantages over the existing methods: (i) GFI (German Fauna Index) is a continuous measure of hydromorphological quality enabling

simple correlation with biocoenstic metrics and (ii) GFI refers to hydromorphological conditions (Balestrini et al. 2004). According to Lorenz et al. (2004), this methodology suffers from handicap that was related to hydromorphological conditions, specifically, the subjective pre-selection of candidate sites, based on researcher subjective judgment on the stress specific ecological status of a site. The same author also concluded that multimetric approaches work well in detecting the impact of hydromorphological degradation on macroinvertebrate fauna. Furthermore, Buffagni et al. (2004) describe a new assessment module developed for small size rivers in southern Italy mainly affected by organic pollution and habitat degradation. Invertebrate communities are strongly affected by water quality when pollution is severe but apparently unresponsive to river morphology. When, water quality improves, the major discriminating factors turns to be river morphological degradation and habitat quality.

Balestrini et al. (2004) described a preliminary evaluation of suitability of Habitat Modification Score (HMS), Habitat Quality Assessment (HQA), Wild State Index (WSI), Buffer Strip Index (BSI) and Index of Fluvial Functioning (IFF) for assessing river hydromorphology. They concluded that HMS and HQA, widely used in UK rivers, seemed to give confident results while HMS needed further testing validation in order to be applied with confidence in South Europe. BSI showed affinity with HQA and was adequate to assess richness and quality of physical structure, including channel and riparian strips of a site. IFF and WSI showed a weak performance for highlighting a particular aspect of the rivers ecosystem degradation. Otherwise, they were able to measure the global environmental condition. Szoszkiewicz et al. (2006) demonstrated that hydromorphological characteristics of rivers differ considerably across Europe. River Habitat Surveywas used to identify hydromorphological features of rivers in four European regions: lowlands, mountains, the Alps and the Mediterranean area (Szoszkiewicz et al. 2006). These results suggested that streams, with differing hydromorphological characteristics, should have different targets for hydromorphological quality restoration, concerning natural sources of hydromorphological variation.The same authors highlighted that the Mediterranean rivers are distinct from other European rivers and the RHS must be adequately developed for evaluating southern European rivers types (Szoszkiewicz et al. 2006). The authorsstrongly recommend that the attention and care should be given to the accurate recording of all scoring attributes in field surveys and attention to the significance of high-scoring attributes on RHS filed survey training courses (Szoszkiewicz et al. 2006). Otherwise, Erba et al. (2006) search, firstly, the component parts of the RHS system which were most strongly related to the overall assessment of hydromorphological quality.

The most common sources of alteration resulting in reduction of the hydromorphological quality of rivers studied were bank resectioning and reinforcement. In addition, integration of the biological and habitat components into a holistic view enable the direct link between hydromorphological characteristics and macroinvertebrates. The assessment was made by identifying the hydromorphological features that were most strongly correlated with macroinvertebrate communities. Erba et al. (2006) found that bank resectioning and reinforcement were correlated with EPT taxa and mayfly total score suggesting that those metrics can be used as metrics to point out alteration in river morphology.

Davy-Bowker and Furse (2006) obtained that channel geometry modification was negatively related to ASPT and ICM index was found to be a good general indicator of morphological alteration. The results obtained about bank structure on the influence on instream benthic macroinvertebrate community, by these authors, agreed with results obtained by Erba et al. (2006). The biotic indices studied were found to be strongly correlated with HQA score indicating that HQA score but less strongly related to HMS.HQA scores high index values to physically diverse sites suggested that physical habitat diversity is more influential in determining macroinvertebrate community structure than the extent of hydromorphological modification (Davy-Bowker and Furse 2006).

2.2.3.2. Reference conditions

A central feature of the WFD is that deviations in ecological quality have to be established as the difference between expected (reference conditions) and observed condition. Hence, the identification of reference conditions is of major importance in calculating ecological quality ratios and determining the effects of human-generated stress. According to WFD, the EC REFCOND Working Group (2001) defined reference conditions as expected background conditions with no (or minimal) anthropogenic stress and fulfilled the following criteria: (i) it should reflect totally, or nearly, undisturbed conditions for hydromorphological elements, physical, chemical and biological quality elements, (ii) concentrations of the specific pollutants should be close to zero or below the limit of detection in the most advanced analytical techniques in use, (iii) concentrations of specific non-synthetic pollutants, should remain within the range normally associated with background levels. WFD stipulated that reference conditions have to be linked to waterbody types and the population of reference conditions should represent the full range of conditions expected to occur naturally within the water body type. Pristine and untouched areas are more and more difficult to find as a result of industrialization and human activities. Hence, spatially based approaches, for establishing reference

conditions, may not be appropriate and alternative methods, such as the using of historical data, 'borrowed' extant data, modeling and expert judgment, may also be used to establish the reference conditions. Hering et al. (2004) used the typology based approach following the recommendation by WFD and the assessment of the ecological quality should be based on differences between observed conditions and reference conditions. Then, a number of factors were used to partition the natural variability expected to occur at a stream site; stream classification by ecoregion; altitude and size of catchment. In addition, a disturbance gradient (human-generated) was established besides the establishment of reference conditions. Hering et al. (2003) focused on sampling sites on streams likely representing classes 1 to 3, since the goal of water policy was to achieve at least good status in all water bodies. In order to distinguish clearly between degradation classes 2 and 3, at least 11 stream sites were chosen for each stream type, three sites were chosen for high ecological status or reference conditions, three sites of good ecological status, three of moderate ecological status and one site of poor and bad ecological status.Thus, for some stream types the number of sampling sites was increased (Hering et al. 2003).

The identification of reference sites for each stream type within the European countries (Hering et al. 2004) was done onsite by comparing site characteristics with a list of *a priori* exclusion criteria. Besides, some countries were also able to use pre-existing data on site conditions or GIS information to compare with the list of criteria for reference sites. The selection of reference sites using criteria to exclude impacted sites is referred as preclassification. Nowadays, onsite evaluation of stream characteristics revealed that none of the potential reference sites fulfill all criteria. So, the objective was to choose sites within each stream type that met as many as possible of the criteria as reference sites. After sampling, the reference sites were validated in a post-classification step. The process of postclassification differed among European countries. For the majority of countries their postclassification was based on evaluation of abiotic variables measured. Still, others compare the pre and posclassification results for the reference sites. Ideally no difference should exist between pre and posclassification and both should result in selection of the same sites as reference sites. Like mentioned above, when no reference sites were available a theoretical description of the reference conditions of these streams types was produced. Historical data use was searched locally in archives at National Museum of Natural History and National Forestry Service, private collections and libraries. Old reports including macroinvertebrates in streams, springs and rivers were also used. Nijboer et al. (2004) addressed on its research the importance of establishingreference

conditions for European streams concerning its usefulness.When the criteria established were not fulfilled, another option is to survey in the same stream type within another geographical area regarding two criteria: (i) they should be situated in the same region and (ii) catchments should be of similar size (Nijboer et al. 2004). It is known that the stress will affect community and degraded communities of different stream types became more similar. The test performed just using reference sites should give better results. WFD principle requires the confirmation and identification of reference conditions for defining the reference community, setting the upper anchor for quality classification and expressing degradation as deviation from it (Verdonschot 2006).

2.2.3.3. Seasonal variabilityof macroinvertebrates

Community composition of macroinvertebrates is affected by temporal and spatial changes. These two aspects should be taken in consideration when collecting representative macroinvertebrate samples. In order to obtain a more precise categorization of assemblage type, sampling strategy requires both habitat and seasonal data. Many physical-chemical factors and seasonality is known to affect macroinvertebrate assemblages over time such as: hydrological regime, water chemistry, light levels and temperature, stream size, distance to the source, vegetation and substrate. In addition lotic assemblages of invertebrates vary both seasonally and with spatial position within the stream. Setting a suitable time period for sampling a given habitat might be therefore a complex problem. Besides that problem another one emerges regarding the interpretation made by bioassessment metrics of seasonal variability. This last issue generated several discussions over time and several authors showed that seasonal changes in macroinvertebrates have marked effect on many biotic indices. Šporka et al. (2006) studied the effect of seasonality on macroinvertebrate community structure. The natural seasonal community variations on metrics determined adequate sampling period(s) cold and warm seasons for mountainous streams in Slovakia. The results from the study (Šporka et al. 2006) showed that the seasonal changes in macroinvertebrate community composition have effects on many biotic indices. The authors recommend that when using quantitative metrics in bioassessment it is important to realize that the season in which the samples were taken can and often will have a strong influence on the results obtained. Consequently the ecological quality obtained will be affected and, usually, the results obtained in spring were better than in other seasons. The community of macroinvertebrates will benefit of an increase in temperature,

discharge, light and nutrient supply which results in primary production and rapid development of spring forms of macrozoobenthos and emergence of water insects. The other seasons discriminated by multimetric index in the same work mentioned above was the warm season (summer) and cold season (autumn and winter). In Ireland, Callanan et al. (2008) also found, in their study of headwatersin different seasons, that variations in the biotic indices occurred in the proportion of the pollution sensitive groups of macroinvertebrates. The turnovers of species between seasons overcome significant seasonal differences as a good example of pollution–sensitive taxa. The apparent lack of species present during summer (higher temperatures, lower oxygen) in headwaters seems to result in a shift of the ecological quality status assigned by several biotic metrics. Species do not necessarily disappear but may have emerged (life cycle) or being in small larvae status and the sampling methods missed them. So, the several metrics routinely used in ecological and risk assessment of water bodies can result in lower water quality which can be due to life cycles rather than a result of anthropogenic circumstances. The same authors suggested the use of appropriate indices, which interpret the results considering the autoecology of the invertebrate species present. Not surprisingly, spring assessment reflects better the ecological quality (Callanan et al. 2008; Šporka et al. 2006). The appropriate number of sampling occasions during the year and the period of time most suitable for sampling depend on the economic perspective and the metrics used, respectively. From the economic point of view, frequency of sampling must be decreased while the biological results showed that the opposite much happen. Several studies (e.g. Furse et al. 1984; Ormerod et al. 1993) reinforce that combined data better characterize and predict macroinvertebrate communities than single season datasets and that increase the possibility of sampling rare *taxa* than if just one sampling time takes place (Šporka et al. 2006). Otherwise, Reece and Richardson (1999) recommend that the assessment should be restricted to a particular season. Once seasonal changes are a natural phenomenon it is very difficult to give an advice on the sampling time schedule. Callanan et al. (2008) considered that spring would be the most suitable time because the number of species will be higher and might reliably reflect the ecological quality of headwaters. This procedure was already suggested previously by Hering et al. (2004) and Furse et al. (2006) since sampling took place during spring-summer or spring-autumn. The metric used was preponderant in the selection of the sampling season and, it is generally recommended, for metrics showing high seasonal variation, that the best solution would be sampling during the same month at all sites (Šporka et al. 2006).

2.2.3.4. Sub-sampling and sample size of macroinvertebrates

Successful biological monitoring and biological assessment rely on rigorous quality control starting from the design and execution of field studies to proper laboratory procedures and data analyzes (Doberstein et al. 2000). Many of these field and laboratory sorting methods are time consuming, tedious, not suited for extensive application and too expensive. High sampling effort contributes to large number of individuals and species and a high explanatory power. However, reducing sampling or sorting effort should not be synonym of quality loss in the results (Lorenz et al. 2004). As a consequence, computing minimal numbers of individuals, per sample, was a solution to obtain a valid assessment of ecological quality of streams (Lorenz et al. 2004). Hence the intensity and methodology needed for correct bioassessment has been subject of discussion and controversial results (Barbour and Gerritsen 1996; Courtemanch 1996; Doberstein et al. 2000; Karr and Chu 1999; King and Richardson 2002a; Somers et al. 1998; Stroom and Richards 1999; Vinson and Hawkins 1996). Some argue that only complete counting provides full information of each expensive sampling effort (Cao et al. 1997; Courtemanch 1996; Karr and Chu 1999; Stroom and Richards 1999). Others rely on subsampling cautiously (Resh and Jackson 1993; Vinson and Hawkins 1996; Walsh 1997). On the other hand, other authors suggested that samples as small as 100 individuals would be sufficient for obtaining good data quality (Barbour et al. 1996; Barbour and Gerritsen 1996; Somers et al. 1998).

Doberstein et al. (2000) showed that variance increased as sample size decreased reducing the trustability of each metric. They defended that multimetric indices based on sub-samples as small as 100 individuals cannot measure the biological conditions beyond good and bad. Furthermore, the same authors enhanced that water managers will be making decisions on the basis of incomplete information since the monitoring data results could show that the place is bad and in fact is not. It would result in the waste of money to recover the water quality good/moderate and hence the bad decision will potentially outweigh the saving associated with sub-sampling system or vice-versa.

The European countries developed an assessment system based on benthic macroinvertebrate samples resulting from standardized methods of sampling, sorting and identification (Hering et al. 2004). Lorenz et al. (2004) considered that sub-sampling for rapid bioassessment protocols was needed. They searched for the number of individuals that should be analyzed from the sample to achieve a valid assessment result based on sub-sampling of 100, 200, 300 organisms and up to complete sample sorting. The same authors concluded that sub-sampling is a solution that potentially saves money and time if

at least 300 individuals were counted and if the variability of the metrics and their different sensitivity to subsampling was not forgotten.

In their work, Furse et al. (2006) based thefield samplingon 20 sampling units taken in proportion to the estimated percentage cover of each major habitat type at the site. This protocol involved a standardized method of laboratory sub-sampling of the macroinvertebrate field sampling. The sampling material was spread out on a tray marked out with a grid of 6 by 5 grid cells. The same protocol required that 5 to 30 grid cells were randomly selected, identified, and counted all macroinvertebrate species or additional cells, until 700 individuals had been identified (Clarke et al. 2006). The sub-sampling procedure will introduce an additional source of variation in the record of taxonomic composition. Most of the metrics had typical replicate sampling variances of 8-18% of the total variability within a stream type, giving rise to misclassifying estimate rates of ecological status class. The precision of such metrics based on this methodology is only enough to indicate gross changes in the ecological status of sites, but there will be considerable uncertainty in the assignment of sites to adjacent status classes (Clarke et al. 2006).

2.2.3.5. Taxonomic resolution

The ecological assessment of running waters different levels of taxonomic resolution can be used (orders, families, genera, species). Several authors have developed assessment systems based on genera or families (e.g, BMWP/ASPT or RIVPACS). Genus or even family level was enough to detect differences for a rapid bioassessment of water quality or a demonstration of biotic relationship on a broader scale (Schmidt-Kloiber and Nijboer 2004). Then, large differences in environmental conditions can be identified by shifts in the number of individuals identified to the genus or even family level. However, others authors stated that ecological information, provided by using genera or families levels in environmental analyzes, may therefore bias the results and may reflect a poorly defined picture of environmental situation (Schmidt-Kloiber and Nijboer 2004). In addition, several authors claimed the benefits of species level information in ecological studies (Moog et al. 1997; Resh and McElravy 1993; Resh and Unzicker 1975; Verdonschot 2000).

WFD also stressed an accurate classificationand high discriminative power in assessing European rivers (Nijboer et al. 2004; Schmidt-Kloiber and Nijboer 2004). In this context, for example, the last authors preferred species level identification because it is seen as the basic biological unit with the highest information content and its use increases

the sensitivity and detection of small changes in ecological quality assessment. In fact, different taxonomic levels represent different advantages and disadvantages. Species level disadvantages are related with increase cost/efficiency; larger human resources; taxonomic expertise (highly qualified technician or missing identification keys for some taxonomic groups); lack of autoecological information.

The assessment of ASS (AQUEM Assessment Software) was built using species level data and cannot be applied with data on higher taxonomic levels (Schmidt-Kloiber and Nijboer 2004).

Schmidt-Kloiber and Nijboer (2004) devoted their study to finding out the influence of taxonomic resolution in assessing ecological quality for water bodies in Austria and Netherlands. The results suggested that the evaluation of ecological quality classes with ASS software,using higher taxonomic levels, lead to wrong estimations. This achievement is clearly caused by underlying multimetric indices and class boundaries and tuned for species level data. The taxonomic resolution divided the opinion among investigators. Despite the advantages and disadvantages of higher taxonomic resolution, some authors follow that higher taxonomic resolution might be an advantage because it reduces the noise created by environmental heterogeneity, and stochastic events which may shade the effects of human activities (Bailey et al. 2001; Bournaud et al. 1996; Bowman and Bailey 1997; Chessman 1995; Graça et al. 1995; Hewlett 2000; Marchant 1990; Marchant et al. 1995; Metzeling and Miller 2001; Reece et al. 2001; Warwick 1988; Warwick 1993; Zamora-Muñoz and Alba-Tercedor 1996). Otherwise, the ecological information provided by genus/species taxonomical levels can be compromised because rarely all species,within one genus/family, have exactly the same ecological requirements and may differ considerably (Dolédec et al. 2000; Hawkins and Vinson 2000; King and Richardson 2002b; Lenat and Resh 2001; Statzner et al. 1994; Townsend and Hildrew 1994; Usseglio-Polatera et al. 2000).

In order to facilitate the decision, on what taxonomic level of the metrics should be integrated into the index, it should be kept in mind that the design of a multimetric index underlines the idea to cover the whole benthic ecosystem for reliable assessment of ecological quality class (Schmidt-Kloiber and Nijboer2004). Karr and Chu (1999) (and as pointed out by WFD) suggested that the selection of core metrics of all relevant types should be included in the final index. Many of them, especially those based on ecological information, are not applicable to higher taxonomic levels. Therefore, information on the functional dimension of the ecosystem is lost (Schmidt-Kloiber and Nijboer 2004).

The results published by Verdonschot (2006) supported that reference samples performed better with species level data and the use of species *vs* family-level data changed the results. Consequently, the description of reference conditions must be based on species-level data. The same author also pointed out two other improvements in current approaches, one is related with metrics and autoecological information and the other is the improvement of taxonomy in European research.

2.3. Intercalibration of assessment methods of river ecological status

In order to ensure the assessment of ecological quality classes are comparablein different European countries, “intercalibration” process is fundamental. In response to WFD goal, an intercalibration working group was formed as a first attempt for intercalibrating the methodologies, to assess the water quality of streams, used in EU countries. The intercalibration group started the approach by dividing EU into at least three regions: Northern, Central and Southern Europe. At the time (2002) the intercalibration group started to concentrate on few widespread and important human stressors (e.g. organic pollution and degradation of stream morphology) (Sandin and Hering 2004).

Sandin and Hering (2004) discussed the intercalibration methodology adopted and the problems faced. Firstly, different stream types are naturally inhabited by different communities due to differences in biogeography and abiotic conditions. Secondly, different stream types may react differently to the same level of disturbance. Thus, intercalibration has to consider the natural differences between stream types but has to eliminate variation resulting from different interpretations. The approach suggested by the authors, in order to solve the problems created by intercalibration, was the definition of a stress gradient using water chemistry and/or physical variables and create class boundaries for high-good and good-moderate ecological status. Next, it was necessary to define the biological criteria for these boundaries, which can, at this point, be compared. Differences in taxonomic composition between stream types and the different class boundaries have to be compared.

The intercalibration of sampling methods, using benthic macroinvertebrate, has been done before in Northern and Central Europe and such exercise has been extended to whole European Countries (Sandin and Hering 2004). It is also important that the harmonization process of the assessment of ecological water quality and different assessment systems continue among state members. This important aspect was revisited and the same concerns addressed to several other issues (Furse et al. 2006). Buffagni

and Furse (2006) presuppose that in order to achieve intercalibration, it is far easier and faster to harmonize the classification results of national assessments than the assessment systems themselves. Meanwhile, WFD also defined the general principles that have to be taken in account when assessing ecological status. The Member States have the flexibility to outline specific details of their own assessment system. The purpose of the European intercalibration exercise was not to harmonize the assessment systems and methods, but only their results. One of the central questions, addressed by Furse et al. (2006) was "How can data, from different assessment methods, taxonomic groups, be compared and intercalibrated and how can the results assist the WFD intercalibration exercise?". Friberg et al. (2006) recorded several differences in terms of sampling process, differences between methods used at field or laboratory sorting and area or duration of sampling. Comparison of the metric values indicated that there are no consistent pattern of differences between the values obtained by WFD and national methods. Besides the differences observed, most of the metrics analyzed correlated significantly and positively with each other for samples collected by (Friberg et al. 2006) when compared to various national methods. In general, the national methods render relatively easy to intercalibrate, however various methods could be improved, in general aspects, as their revision should be developed (Friberg et al. 2006). Regarding intercalibration, Buffagni and Furse (2006) compared and harmonized class boundaries of three European assessment systems by means of ICMi (Intercalibration Common Metric index) indexapproach. The ICMi values were calculated for test datasets from a single stream type in three European countries. Three different approaches for comparison were used, however only one was considered useful for the harmonization of boundaries. The suitability of the ICMi for the harmonization phase is discussed and the use of external benchmarking datasets is recommended in order to make the European intercalibration process more transparent and objective. Birk and Hering (2006), in a different but complementary approach to the previous one, directly compared the existing river quality assessment methods for two European stream types. Supported on benthic macroinvertebrate data, national class boundaries of eight countries were compared by national methods based on two common scales: (i) the national method with the highest mean correlation of all indices and (ii) IMI-IC, Integrative Multimetric Index for Intercalibration an artificial index based on the mean of all index values calculated with a sample. Using this approach the authors showed that good quality status boundaries of the national methods deviated up to 25%, assuring the need for harmonization of class boundaries. Birk and Hering (2006) and Buffagni and Furse (2006) agreed that some form of benchmarking system can help to overcome

differences in definitions of reference state in different countries. Furthermore, the authors recognized that intercalibration has political interest since the definition of quality boundaries sets the environmental standard to be achieved and implies agreement on a level of anthropogenic degradation acceptable for freshwater systems. Buffagni and Furse (2006) also concluded the huge difficulty in applying the intercalibration approaches, commonly used for invertebrates, to macrophytes assessment. These authors stated further research must be employed to produce suitable common macrophytes assessment metrics. Besides the macrophytes, still no intercalibration was performed for other biotic communities of benthic diatoms and fish, maybe, because the reference condition plays a key role in the European intercalibration process.

2.4. Current status of the river basin management plans in European rivers

The European WFD was one of the most ambitious legislative instruments in the field of water policy to be introduced on an international basis for many years. According to Albrecht (2013), in Germany, the implementation of WFD programs of measures and river basin management plans, in all river basin districts in the end of 2009, has been running but to reach the target of a good water status it is still a long way to go. Despite the measures that are planned, only a few additional surface water bodies and some groundwater bodies are likely to achieve the “good status” by the 2015. The same author stated that the experts speculated that in 2015 the exemptions from good status will constitute the rule rather than the exception.

The challenge of water management authorities to achieve the good water status of aquatic ecosystems takes a long recovery period. Also the high cost of the measures, which have to be undertaken, shows that it will take a continuous effort, of at least a whole generation, to realize the aims of WFD (Albrecht 2013). However, according this author the implementation of the WFD have led to a new impetus to water management in Germany contributing to the identification of deficits, collection of more information, cross-boarding cooperation and public awareness. The coordination of programs of measures and river basin management for the whole country can be treated as a success raisin hope for a systematic improvement of water status in the future years and decades.

Naddeo et al. (2013), referring to implementation of WFD in Italy, pointed out that the cost of sampling and analysis has an important impact in water quality assessment and in their case study, the sampling frequency in river water was optimized without compromising the accuracy of the results, and therefore, reducing the cost that can be redistributed to other projects.

In the last few years, some authors published complementary indicators to the traditional monitoring structural methodologies (water quality or taxonomic composition of aquatic organisms), as suggested by the WFD. Young et al. (2008) suggested that for complete assessment of river heath functional metrics should be included, such as the rate of organic matter decomposition and ecosystem metabolism. Organic matter decomposition can be evaluated using leaf breakdown linking the characteristics of riparian vegetation with the activity of both aquatic invertebrates and microbial organisms. Leaf breakdown is affected by natural and human-induced variation in a wide range of environmental factors and have many advantages such as inexpensive equipment, relative simple to use; the response to natural variation and most stressors can be predicted with confidence; can be measured in all aquatic habitat and criteria linking leaf breakdown rate and ecosystem health already exists (Young et al. 2008). On the other hand, the disadvantages of this methodology were related with wide influence of factors difficulting the interpretation of results and the best feasibility for detecting small-scale effects of stressors (Young and Collier 2009; Young et al. 2008).

Vidal et al. (2012) suggested an ecotoxicological approach using a battery of sediment elutriate toxicity tests allowing the evaluation of the historic contamination. In a river, contamination in the water column is very variable depending, for example, on the river flow, turbulence and dilution rates, hence any assessment of water quality should consider the sediment matrix. Sediments can contain several amounts of organic and inorganic material bounded to particles but when disturbed by stormwater runoff they can turn bioavailable as an important pollution source for both benthic and planktonic organism (Burton 2002). This methodology highlights the usefulness of using a cost-effective ecotoxicological approach to assist the prioritization/scoring of critical areas within river ecosystems potentially impacted, for example, by deactivated mines. In this study, two concerning sites were evaluated using a sensitive ecotoxicological test battery that was found able to clearly distinguish their hazardous potential and the impacted location studied was suggested as a priority for further assessment within the scope of the WFD. Thus, Vidal et al. (2012) considered that, only in this conditions, the range of the related ecosystem impacts can be fully recognized and adequate recovery plans can be established.

Prat et al. (2013) claimed that the biomarkers integrated with macroinvertebrates (trichopteran) structural metrics can be used for establishing the biological condition of river communities revealing as a useful tool for operative monitoring programs of the WFD to detect further impairment or as a measure of future decline if no measures was taken.

The biomarker techniques applied on the trichopteran *Hydropsyche* has been used with success to detect metals and salt mining, pesticides or general degradation due to multiple pressures.

Furthermore, Vidal et al (submitted) suggested the use of benthic diatoms (in this case, *N. libonensis*) as bioindicators within the scope of the WFD regarding river quality assessment. Then, one more step was taken towards the establishment of complementary methodologies to assess the ecological status of freshwater lotic systems focused on the sediments compartment. This work described a toxicity test with a sensitive benthic diatom species representative of the microphytobenthic community. Although further studies are necessary to being done a general sensitivity of *N. libonensis* to metals and organic contaminants was confirmed. Meanwhile, the species was indeed shown to be very sensitive to the respective standard representatives potassium dichromate and 3,5-dichlorophenol. Based on the laboratorial results reported (Vidal et al, submitted), follow-up research has been conducted in order to develop higher tier assessment tools with the diatom *N. libonensis*, namely using *in-situ* testing protocols.

2.5. River restoration and macroinvertebrates

The desire of restore biodiversity in streams and rivers that have been degraded by land use change, agriculture, or other environmental stressors, has emerged. During this period, the emphasis has shifted from restoration of single species to restoration of all stream ecosystems and community services they provided (Palmer et al. 2010). Palmer et al. (2010) questioned if the dominant paradigm of increasing habitat heterogeneity (HH) promotes restoration of biodiversity in ecological restoration that lasted over 30 years. The increase of HH consists in increasing species diversity by enhancing the surface area, refuges, higher or more varied supply of limiting resources consequently promoting diversity.

Otherwise, heterogeneity may act in accord with factors such as disturbance regime, food resources and regional species pools, to influence diversity. However, for macroinvertebrates, little evidence was achieved that HH was a primary determinant of diversity (Palmer et al. 2010). The same authors claimed that, for many restoration sites, water quality might not be enough to restore invertebrate diversity, even if the heterogeneity were restored (*e.g.* heterogeneity may not have the opportunity to play important role in increasing diversity when other pressures exists)(Palmer et al. 2010).In order to be successful in river restoration Roni et al. (2008) emphasized the important role of larger-scale factors and advise the following sequence of actions for rehabilitating

streams and rivers, protect critical habitats, improve water quality, restore watershed processes and then improve in-stream habitat. They suggested that if water quality, flow and riparian vegetation conditions were adequate then biota may, indeed, respond to heterogeneity. Restoration effort must target the most limiting factor, i.e., the factor that must be corrected before biota can return (Palmer et al. 2010). The majority of stream restoration projects are made under the assumption that if structures and processes of ecosystem are restored, organisms will recolonize (Palmer et al. 1997).Stream in urban areas belong to the most degraded situation, often polluted and with hydrology and geomorphology altered as a result of underground piping and rapid runoff from impervious surface in the catchment. Large sums of money were already spent in the restoration of urban streams. Proper management of storm water was a prerequisite for successful restoration of urban streams, since rapid runoff of surface water may fail to reach groundwater, flush on its way pollutants and sewage into streams, and high peak discharges may destroy installed habitat structures. Therefore, urban stream restoration should not be undertaken unless integrated within broader catchment management strategies, otherwise the significantly improvement in ecological condition in stream was unrealistic. Proper assessment of the outcome of the restoration process is needed in order to determine when the target ecosystems had recovered as expected and when the project goal was not succeeded (Jansson et al. 2007). Despite this, according with Jansson et al. (2007), most of the restoration projects are improperly monitored or not monitored at all making the evaluation of the restoration success difficult and suggest a guideline proposed by Woolsey et al. (2007).

2.6. Conclusion

This work intended to provide a picture of the great changes operated with the WFD and relevant working groups created in order to stepwise achieve the major goal. The most important conclusion was no perfect biotic indicator of the water quality exists and a more holistic perspective must be pursuit. Several changes (paradigm and methodological) were performed since the creation of the first biotic index in order to integrate the results of the complexity of the lotic freshwater ecosystems. Huge efforts are made in the intercalibration of methodologies and metrics in Europe, under the scope of the WFD. The new steps arise with the development of new complementary measures in order to approach functional measures (*e.g.*, organic matter decomposition), and biomarkers in macroinvertebrate organisms and ecotoxicological bioassays with/on sediment compartment.

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Chapter III

Spatio-temporal characterization of the macroinvertebrate community of Mau River: a multi-stressor case study

Spatio-temporal assessment of the macroinvertebrate community of Mau River: a multi-stressor case study

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Abstract

Mau River is a small mountain river facing multiple stressor impacts including morphological alterations, deforestation and contamination from agricultural activities and deactivated mines. The present study intended: i) to evaluate the sensitivity of the macroinvertebrate community to the stressors along the course of the river; ii) to assess whether the ecological status of Mau River was impaired in two different sampling periods (2005-2006 and 2009-2010); iii) to compare the WFD approach (ecological quality ratios derived from a multimetric index) with more refined community structure analysis. The river presented very good ecological status in most sites. Despite seasonal and inter-annual fluctuations, no evident stressor-related effects were detected by either the analysis approaches. Indeed, community structure analysis showed considerable variation between sampling periods, and most variation within each period was due to seasonal fluctuations. Even so, benthic invertebrate community was very similar among sites and this resulted in reduced species succession gradients. We hypothesize that the observed patterns are the result of: a) the current dormant state of the abandoned mining areas, which are the most relevant potential source of contamination; b) the river's characteristics, such as its short path and strong current, which promote re-colonization of biotic communities from upstream non-impacted sites and rapid flushing of contaminants bound to fine particles. Such a pattern is generally valid for small mountain rivers, making them therefore more resilient to stressor challenges. Nevertheless, small changes in community structure suggest disturbances in the last portion of Mau River signing the need for environmental managers' intervention.

Keywords: Macroinvertebrate community, multistressors, Mau River, Water Framework Directive, Multivariate analysis

3.1. Introduction

Freshwater habitats, and particularly rivers, are usually subjected to multiple stressor scenarios (Diaz et al. 2004; Doledec and Statzner 2008), as they are end receivers of organic effluents, acidification, morphological degradation, deforestation and contamination resulting from agricultural activities, livestock farming and industry (Olsson et al. 2013; Prat and Munne 2000). Metal pollution, such as mining and resulting acid mine drainage, has a well known negative effect on the aquatic ecosystem and especially on metal-sensitive groups like crustaceans and mayflies (Malmqvist and Hoffsten 1999). However, it is more difficult to establish causality when metals exist at sublethal levels and co-occur with other stressors (organic enrichment, pesticides), particularly because biotic communities seem to respond to stress in a non-specific way (Böhmer et al. 2004; Doledec and Statzner 2008). In addition, the bioavailability of metals in freshwater is affected by several factors (e.g. pH, water hardness and dissolved organic matter), which modify the *in situ* toxicity at the organism level (Girgin et al. 2010). These phenomena introduce uncertainty in the prediction and analysis of effects due to metal contamination. It is desirable that current state-of-the-art methodologies for ecological assessment are able to detect the subtle effects of these multiple impacts on the resident biotic communities.

The Water Framework Directive (WFD) 2000/60/EC established a new regulatory framework for the management, usage, protection, and restoration of surface water and groundwater resources within river basins and catchments, as well as in the transitional (lagoons and estuaries) and coastal waters of the European Union. The WFD abandons the classic approach of viewing water as a resource (anthropocentric perspective) and instead sees it as an ecosystem holder (ecocentric perspective)(Hering et al. 2003; INAG 2008a). Although paradigmatic, the WFD approach to monitoring biotic communities is somewhat limited to the use of biotic indices, which consist of numerical expressions used to assess water quality based on the presence and diversity of pollution-tolerant and pollution-intolerant taxa. Ultimately, these indices derive from the “Saprobiensystem” of Kolkowitz and Marsson (1908), developed to assess organic pollution, which Washington (1984) considers the first biotic index ever created. It is thus questionable whether this type of approach allows the evaluation of contamination scenarios other than organic enrichment, such as metal and multiple-source pollution.

Macroinvertebrates are among the most frequently used bioindicators in water quality assessments, mainly due to their relative large size, ease of sampling, low to moderate identification effort, and relatively long life cycles (Barbour et al. 1999; Hellawell

1986; Metcalfe-Smith 1996). Their low mobility and long life cycles ensure that the presence of a given taxon at the time of sampling reflects past conditions (Blijswijk et al. 2004). As stated previously, the current state of knowledge on the autoecological information of most macroinvertebrate taxa was also mostly derived from their tolerance to organic pollution and subsequent decrease of oxygen, which brings some uncertainty to their indicator role of other types of stress (Dahl et al. 2004; Feld 2004; Lorenz et al. 2004; Sandin and Hering 2004; Vlek et al. 2004).

Mau River (Sever do Vouga, Portugal) was chosen as a case study because it is a recipient of point-source and diffuse contamination by organic compounds and metals. Mau River is a small (13 km length) mountain tributary of the Vouga basin, located in the vicinities of Sever do Vouga (40º44'00''N 8º22'00''W), with a catchment area of 12.4 km^2 (Nunes 2007) flowing from its headwater in Serra do Salgueiro to its mouth in Pessegueiro do Vouga (Fig. 1). It is a tributary of Vouga River, which is a very important source of drinking and irrigation water in the region (Nunes 2007). Mau River passes through urban and agricultural areas and is bordered by two deactivated mining areas, less than 1 km apart – Malhada and Braçal mines. The main extraction in Malhada and Braçal mines were galena ore (native lead sulphide), zinc blende ore and iron pirite ore (Cabral et al. 1989). Most of the tailings from the past mining activities were left without any management plan for requalification and are the main contributors to the metal contamination in all surrounding compartments. Thus, Mau River is potentially affected by i) diffuse organic pollution in the first section of the river; and ii) by metal pollution in its last section. Apart from these contamination sources, Mau River's catchment is mostly embedded in a natural forest or plantation landscape, which produces shade and buffers other external contaminant sources. The constant occurrence of rocky outcrops forces the water flow through a sinuous path, thus creating some riffles and waterfalls which constantly oxygenate the water (Santos 2010).

The present study intended to evaluate the ecological status of Mau River using the WFD approach based on the macroinvertebrate community, encompassing both spatial and seasonal variation. Our specific goals were: i) to evaluate the sensitivity of the macroinvertebrate community to the multiple stressors along the course of the river; ii) to assess whether the ecological status of Mau river is impaired, by looking at its ecological status in two different periods (2005-2006 and 2009-2010); iii) to compare the WFD approach (based on ecological quality ratios – EQRs – derived from the biotic index $IPtI_N$) with more refined tools in community structure analysis. Multivariate analysis was used for

community structure analysis, with the purpose of reducing a species x sample matrix to a few dimensions that explain the highest proportion of the total variation in the data.

3.2. Material and Methods

3.2.1. Study area and sampling strategy

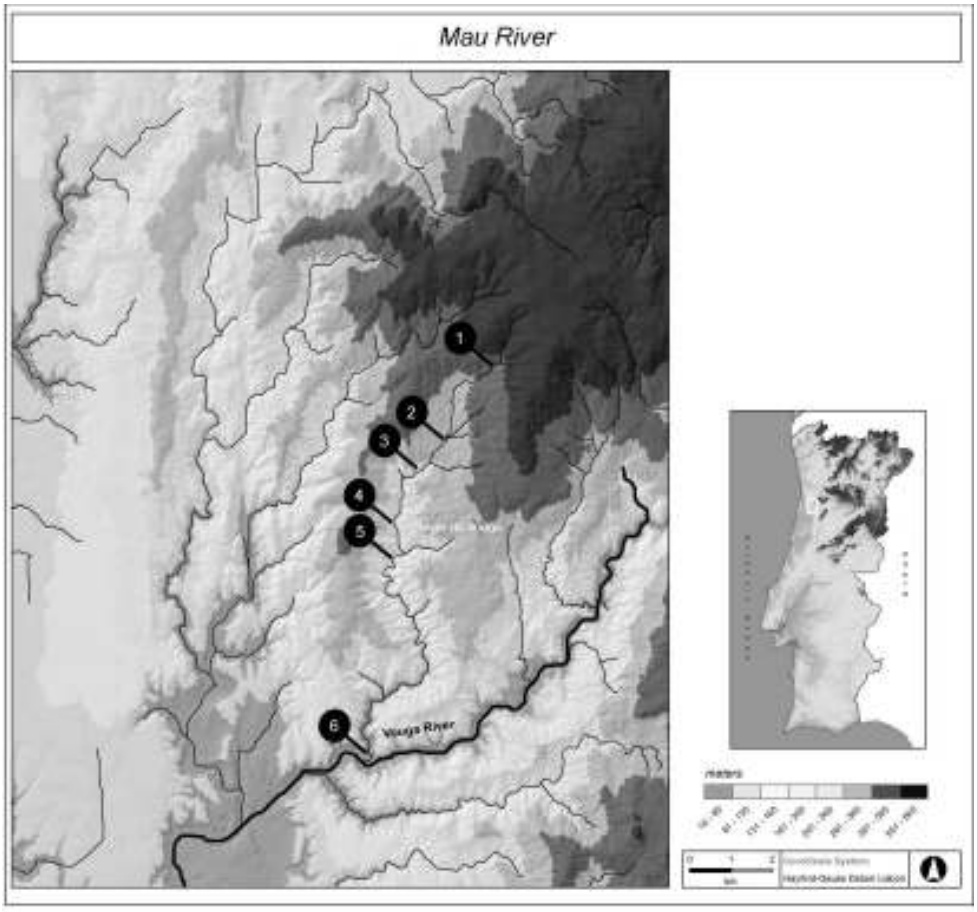

Fig. 1. Geographic location of the studied sampling sites: 1 - river spring; 2 - Silva Escura, where the river crosses a small village and fields used for agriculture; 3 – Cabreia, which is a small forested park following a 25 m waterfall; 4 - deactivated mining area (Malhada); 5 - deactivated mining area (Braçal); 6 - Mau River mouth.

Sampling sites were defined for covering the whole river course, along the river continuum (Fig. 1). Site 1 was located near the river headwater, which faces reduced human impacts. Site 2 was adjacent to the small rural village Silva Escura, where Mau River receives domestic sewage and agricultural runoff, which are likely to provide enhanced input of nutrients and pesticide residues. Site 3 was located further

downstream, following a 25 m high waterfall. There is a recreational area in the river border at this site, producing moderate human pressure, mostly in the summer. Sites 4 and 5 were located downstream two deactivated lead and zinc mining areas - Malhada and Braçal, respectively. Biological effects due to acid mine drainage were expected here. Site 6 was located near the river mouth, before it joins the Vouga River. Riparian vegetation along the river course is generally tall and dense. Some deforestation in the river borders for wood exploitation was carried out before or during the sampling periods, which was particularly extensive near site 6.

Sampling campaigns were carried out in two distinct periods, 2005/06 and 2009/10, covering all the mentioned sampling sites. Sampling was scheduled to capture seasonal variation in the biota: spring (May 2005 and April 2010); summer (August 2005 and July 2010); autumn (November 2005 and October 2009); and winter (February 2006 and January 2010).

3.2.2. Field measurements and sample collection

General chemical and physical characterization was carried out at each sampling site: pH (pH 330 from WTW, Germany), temperature and conductivity (LF 330 WTW, Germany), and dissolved oxygen (Oxi 315i from WTW). A 1.5 L water sample from each location was collected for further characterization (see laboratory analysis). Sediment samples were collected from the river-bed into plastic bags for metal analysis (see laboratory analysis). Water and sediment samples were transported to the laboratory at 4ºC in the dark, and then frozen at -20ºC for immediate preservation until further analysis.

Macroinvertebrate sampling was done according to the proportional presence of microhabitats, as recommended by Portuguese Water Institute (INAG) INAG (2008a). The area sampled was chosen to include both “riffle” and “pool” habitats (Barbour et al. 1999; Hering et al. 2003). Benthic macroinvertebrates were collected at each sampling site by kick-sampling small transects, covering a similar area and sampling effort (in time) across sites. A standard hand net (500 µm pore size; square frame 0.30 x 0.30 m) was used. Collected samples were placed in air-tight plastic containers and preserved with either 4% buffered formalin or 96% ethanol (Barbour et al. 1999). Formalin was progressively replaced by ethanol in our laboratory, due to health concerns (see also Black and Dodson 2003), so no formalin was used in the most recent samples.

3.2.3. Laboratory analysis

The water samples collected at each site were filtered through glass microfiber filters (1.2 µm pore and 47mm Ø); filtrate was used for nutrient analysis and residue was used to quantify total suspended solids (TSS). Nutrients were analysed following widely disseminated protocols (APHA 1995) for the colorimetric quantification of nitrites (NO_2^-), nitrates (NO_3^-), ammonia (NH_4), and orthophosphates (PO_4^{3-}) in water samples. Sediment samples were oven-dried at 40ºC and, later, mixed with distilled water in a proportion of 1:2 (w/v) and left overnight in an orbital shaker at 200 rpm for metal extraction. The resulting elutriates were centrifuged for 15 min at 4000 rpm and the supernatant was filtered with glass microfiber filters (1.2 µm pore and 47mm Ø). This filtrate was acidified to pH < 2 with nitric acid PA 65%. We used this approach as a non-aggressive – and ecologically relevant – metal extraction procedure, simulating natural resuspension phenomena, thus obtaining the mobile fraction of sediment-associated metals. A conceptually similar approach was used for soil samples (using artificial rainwater; Pereira et al. 2008). Common metal extraction procedures from non-aqueous matrices (namely soils and sediments) involve aggressive acid digestions, which tend to overestimate the bioavailable fraction of metal contaminants (see discussion in Pereira et al. 2008). Metal concentrations (Al, As, B, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Sr, V and Zn) were determined in aqueous extracts by inductively coupled plasma mass spectrometry (ICP-MS).

Preserved benthic macroinvertebrate samples were washed through a 500 µm mesh-size sieve and organisms were carefully sorted out. After this, they were stored in 70% ethanol and identified to the lowest practical taxonomical level, generally the family (or genus, when possible) using general and taxon-specific identification keys (Elliott 1977; Macan 1959; Pattée and Gourbault 1981; Richoux 1982; Serra et al. 2009; Sundermann et al. 2007; Tachet et al. 1980).

3.2.4. Data analysis: water quality (WFD) approach

Community metrics were calculated for the macroinvertebrate community samples, using family as the taxonomical/resolution level: total number of families (richness, *S*), diversity and equitability (Shannon's *H'* and Pielou's *J'*). Biotic indices, IASPT (average score per taxon, derived from the biotic index IBMWP Alba-Tercedor and Sánchez-Ortega 1988), log (sel. ETD + 1) (which is the logarithm of the abundance of taxa selected based on their auto-ecology), and number of EPT taxa (taxa belonging to orders Ephemeroptera, Plecoptera, and Trichoptera) were also derived from the species data matrix. Some of these metrics were informative per se, but they were mostly calculated for latter integration in the $IPtI_N$ multimetric index (equivalent to ICM 7/STAR; Munne and Prat

2009), which allows the derivation of Ecological Quality Ratios (EQRs) for the classification of ecological quality for each sample (following the WFD and INAG technical papers - INAG 2009). Reference values for the community metrics and biotic indices were obtained from official guidance documents (INAG 2009), considering Mau River as a type N1 <=100 river – rivers of northern Portugal with a catchment area < 100 km^2 (INAG 2008b).

3.2.5. Data analysis: multivariate approach

Sediment metal concentrations and other physical and chemical parameters were analyzed with Principal Components Analysis (PCA) for assessing the spatial and seasonal variation of the abiotic framework. All environmental data were standardized (by subtracting the mean and dividing by the corresponding standard deviation) prior to analysis.

Redundancy analysis (RDA) was the choice to explore the seasonal and spatial gradients in the benthic invertebrate assemblage. Although species abundances tend to follow a unimodal response to environmental gradients (Jongman et al. 1995), RDA assumes a linear response modal (canonical correspondence analysis or CCA is indicated in the former case). However, theory on gradient analysis (ter Braak and Prentice 1988) states that RDA is best suited to deal with species abundance data when the length of gradient is small (usually below 3-4 SD units), thus approaching a linear response. This can be checked by running a detrended correspondence analysis (DCA, unconstrained ordination technique) on the biotic data matrix and assessing the length of gradient of the first axis of the ordination (ter Braak and Prentice 1988; ter Braak and Smilauer 1998). RDA is a canonical ordination technique that constrains the biotic data matrix relatively to the putative environmental gradients, which makes it a direct gradient analysis technique (ter Braak 1995). As a consequence, it extracts synthetic gradients from the biotic and environmental matrices, which are quantitatively represented as arrows in biplot graphs (ter Braak 1995). The length of the arrow is relative to the importance of the explanatory variable in the ordination, and arrow direction indicates positive or negative correlations with the ordination axes and species or sample scores. To avoid redundancy, an a priori forward selection procedure of environmental variables was carried out, using a cut-off level of 0.10. Biplots (samples x environmental variables and species x environmental variables) used symmetric scaling (Gabriel 2002).

The variation partitioning technique proposed by Borcard et al. (1992) was used to quantify the variation explained by each environmental subset of explanatory variables (see below), using RDA and partial RDA (the latter form allows partialling out the effect of covariables; ter Braak 1998). Two environmental subsets were used: sediment metal concentrations (M) and water physical and chemical (PC) parameters. Thus, five distinct RDA models were built using the macroinvertebrate data set: 1) M dataset as explanatory variables; 2) PC dataset as explanatory variables; 3) global model (M+PC as explanatory variables); 4) M dataset as explanatory variables and PC as covariables (M | PC); 5) PC dataset as explanatory variables and M as covariables (PC | M). Monte Carlo (unrestricted) permutation tests were used to assess the significance of each of the above models. The contribution and overlap of both environmental datasets to the global variation of the abundance data was obtained by comparing the percentage of variance explained (quotient between the sum of all canonical eigenvalues and total inertia) by each RDA model (see Borcard et al. 1992).

Prior to all RDA analyses, we fine-tuned the taxonomical resolution between sampling periods (2005/06 and 2009/10); when in doubt, we used a higher taxonomical level (e.g. family instead of genus) to provide consistency to the final list of taxa. Also, rare taxa (occurring in less than 10% of samples) were removed and abundances were log-transformed prior to analysis.

3.3. Results

3.3.1. Abiotic framework

Overall, oxygen was high, while conductivity and nutrient levels were low, along the river continuum (Table S.1). Except for some values recorded in sampling site 1, low levels of suspended solids (TSS) were recorded. Most fluctuations in the river's physical and chemical characteristics were seasonal (see Fig. 2). Nevertheless, Mau River did show an increasing conductivity gradient from its spring (site 1) to its mouth (site 6) – Table S.2. Differences were also found between sampling periods, with slightly higher temperatures, pH and conductivity being recorded in 2009/2010. Also, nitrate levels were higher in 2009/2010, while nitrites and phosphates were overall higher in 2005/2006.

Sediment metal concentrations were overall heterogeneous (Table S.2), mostly fluctuating seasonally (Fig. 2). Most metal concentrations were low, but Al and Fe were commonly found at concentrations one to two levels of magnitude above all other elements (above 10 mg kg^{-1} or above 100 mg kg^{-1}, in some cases). Some elements were

also commonly found at concentrations above 1 mg kg^{-1} (B, Pb). Elements Zn and Mn were especially relevant (1-10 mg kg^{-1}) in the 2009/10 period (Table S.2).

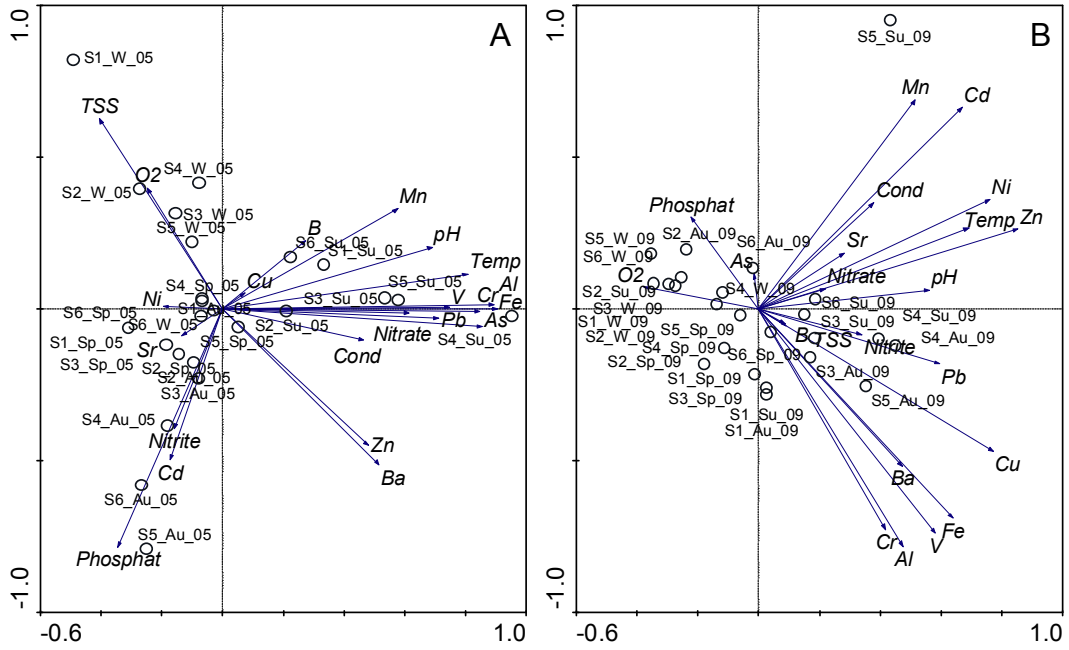

Fig. 2. PCA biplot of samples according to the environmental framework (water physico-chemistry and sediment metal concentrations), in two sampling periods: 2005/06 (A) and 2009/10 (B). Phosphat stands for phosphates, Temp stands for temperature, O2 stand for oxygen, Cond stands for conductivity, TSS stands for total suspended solids, and nitrite and pH were not abbreviated. Sampling stations are represented by S and the site number (1-6) followed by its corresponding collection date (Sp - spring; Su - summer; Au - Autumn, W - winter).

The first two axes of the PCA accounted for 54.9% and 48.5% of the total variance of the samples distribution, for 2005/2006 and 2009/2010, respectively (Fig. 2). Both ordination diagrams display a strong seasonal component in the data variation, with sample scores grouping according to season (W, Au, Su or Sp). Temperature, pH, nitrate and conductivity were the main drivers of this seasonal gradient, with the highest values of these parameters being recorded in the summer. Several metals were also responsible for this segregation, but there was no consistent pattern in the two sampling periods. Some samples, where very high values of one or more parameters were observed, were located apart from the other samples (e.g. high values of Mn were observed in S5_Su_09).

3.3.2. Water quality approach

A total number of 70 536 individuals were identified. They were distributed over 162 different *taxa*. In most samples (> 50%), the number of families and EPT taxa were above the reference value for the corresponding river typology (Fig. 3A and 3B). An overall diverse community, comprehending sensitive taxa, was therefore found in all sites, suggesting no apparent impacts from putative contamination sources. Ecological quality ratios (EQRs) confirm this, with 47 out of 48 samples attaining a “good” (6) or “high” (41) ecological status, respectively. In 2005/06, good quality was achieved by site 5, in the winter, and site 1 was classified as moderate, in the summer. Concerning 2009/10, results below “high” were only found in the lower half of the river, at sites 4 (autumn, winter), 5 (summer, winter), and 6 (winter).

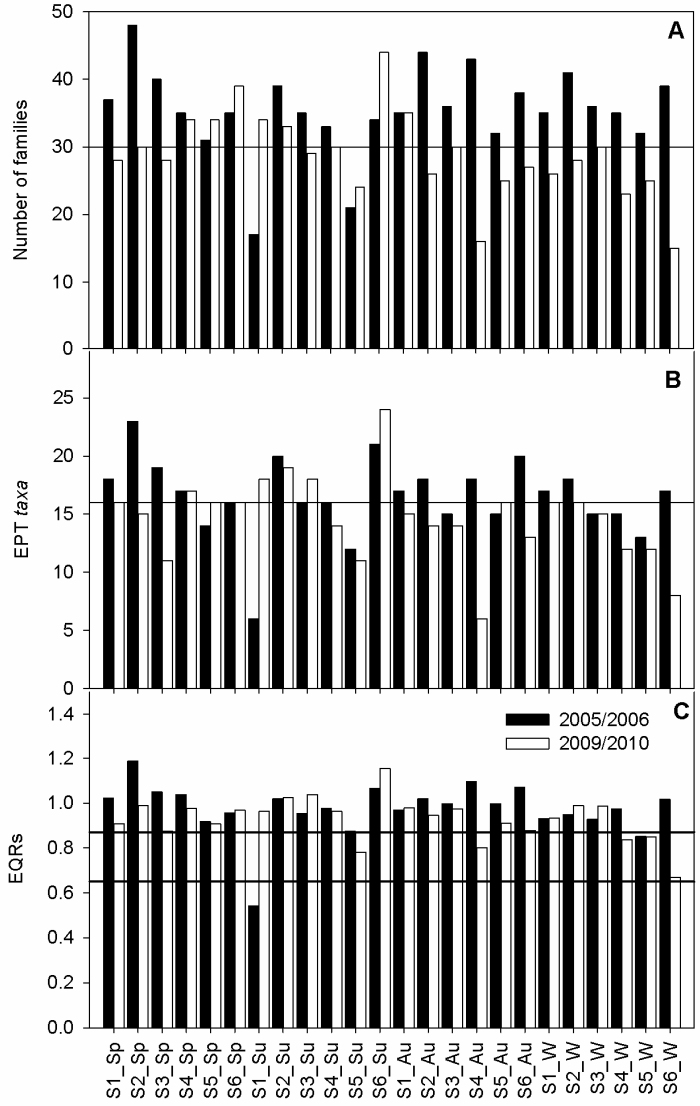

Fig. 3. Macroinvertebrate community metrics and ecological quality ratios (EQRs) for each sampling site (S1 to S6) and season (Sp, Su, Au, W), for both sampling periods (black and white bars). **A**. Number of macroinvertebrate families; horizontal line represents the reference value, according to INAG (2009). **B**. Number of sensitive (Ephemeroptera-Plecoptera-Trichoptera) *taxa*; horizontal line represents the reference value for EPT *taxa*, according to INAG (2009). **C**. EQRs for the classification of ecological quality for each sample; horizontal lines represent high/good (upper line) and good/moderate (lower line) quality thresholds, according to INAG (2009).

3.3.3. Community structure analysis

Preliminary multivariate analyses revealed a clear separation based on period (2005-2006 *versus* 2009-2010; see Fig. 4), thus revealing strong inter-annual differences (see also abiotic framework). These differences were mainly due to unknown factors: a global RDA, integrating both years, explained 35.3% of the data variation (Fig. 4), while individual RDAs for each year explained over 55% of the data variation (see below), thus showing an overall gain in variance explanation when years were analysed separately. Because of this, all the following analyses considered each period separately. Low lengths of gradient were found for the first axis in preliminary DCA analyses (1.888 SD for 2005/06, and 1.398 SD for 2009/10, respectively), thus justifying the use of RDA (see methods).

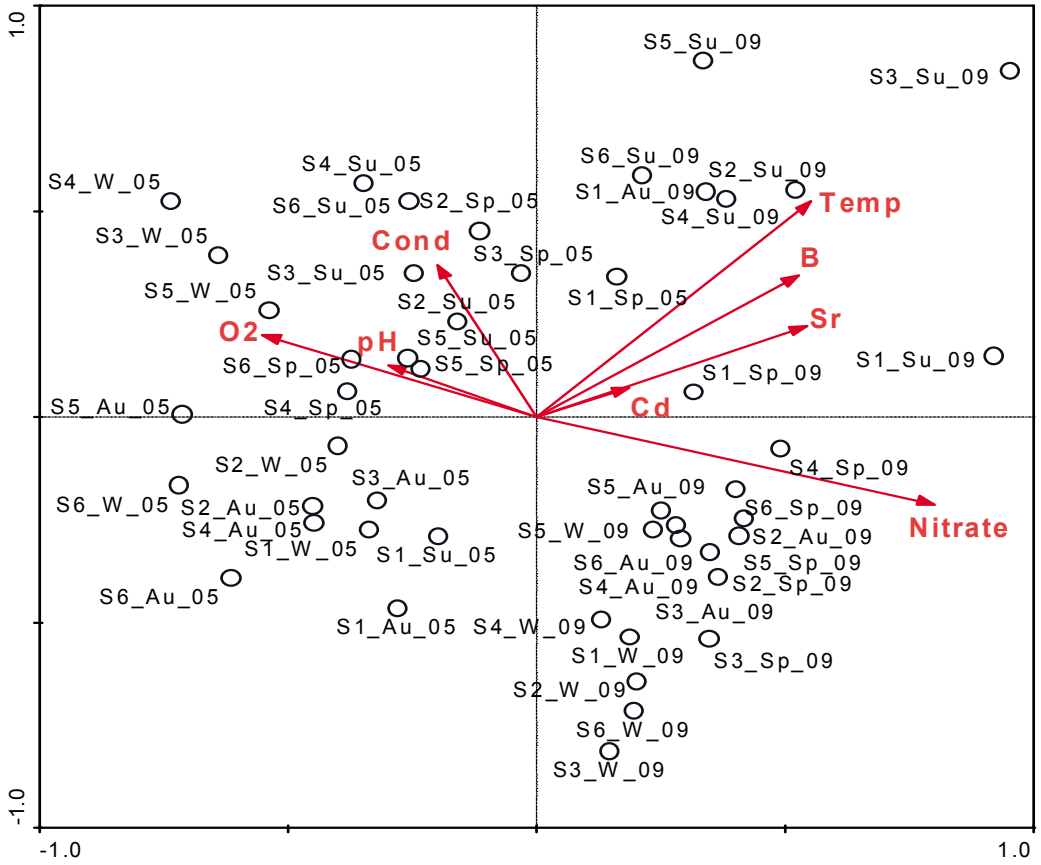

Fig. 4. RDA biplot for the global model (metal + physical and chemical explanatory variables) integrating both years (2005/06 and 2009/10). Sample scores (circles) and environmental gradients (arrows) are represented: Temp stands for temperature, O2 stand for oxygen, Cond stands for conductivity, and nitrate and pH were not abbreviated.

Sampling stations are represented by S and the site number (1-6), followed by sampling season (Sp - spring; Su - summer; Au - Autumn, W - winter) and year (05 or 09).

The global RDA models (PC + M) used to explore the benthic invertebrate data set explained 56.8% and 59.8% of the total variation for 2005/2006 and 2009/10, respectively. Therefore, 43.2% and 40.2% (respectively for 2005/06 and 2009/10) could not be explained by the measured abiotic variables. Variance partitioning revealed a high contribution of the physical and chemical variables, with the PC model explaining 34.7% of variation (in 2005/06) and 35.8% (in 2009/10). The model based on sediment metal concentrations (M) explained 22.9% (in 2005/06) and 29.8% (2009/10) of the total variation. By partialling out each environmental subset (PC and M) at a time, we obtained "pure" PC variation (33.9% for 2005/06; 30.1% for 2009/10) and "pure" M variation (22.1% for 2005/06; 24.0% for 2009/10). This revealed an almost negligible (0.8% and 5.7%, respectively) intersection of PC and M. Therefore, biplots from the PC and M models are shown for each year (Figs. 5-8). All RDA models were significant (Monte Carlo permutations, $p \leq 0.05$).

In 2005/06, segregation of sample scores in two groups was observed (Fig. 5A), suggesting some form of seasonality. One of the groups is composed by spring and summer samples and the other group, in general, is comprised of autumn and winter samples. The main gradients explaining this separation seem to be temperature and nitrate concentration, with higher temperature and nitrate levels in spring and summer and lower temperature and nitrates in autumn and winter samples (Fig. 5A). A mineralization gradient (phosphate and conductivity) is also perceptible in the latter group of samples, observed from the river spring (site 1) to its mouth (site 6). It is also visible that site 1 is partly segregated from the others, at the low end of the mineralization gradient (Fig. 5A). This spatial gradient was not observed in spring and summer samples. Unlike for the RDA PC-model, no spatial or seasonal trends were observed in the M-model biplot, as no evident groups of samples occurred (Fig. 5B). Most of the variation explained by sediment metal concentrations relates to a few off-centre sample scores without an apparent pattern or metal concentration gradient (either spatial or seasonal).

The seasonal and spatial gradients observed in 2005/06 were associated with specific macroinvertebrate taxa. The following taxa were more abundant in spring and summer samples: the plecopteran Leuctridae, the dipterans Chironomidae and Tanypodinae, mites Hydracarina, and adult *Oulimnius* beetles (see respective arrows in Fig. 6). Opposing these, the trichopterans Calamoceratidae, the ephemeropterans

Leptophlebiidae and *Ephemera*, and several oligochaeta taxa had higher abundances in the autumn and winter. In terms of the spatial (mineralization) gradient, RDA grouped site 1 scores apart from the other samples, and this was due to the higher abundance of Chironomidae, Hydrobiidae, Chloroperlidae, Orthocladiinae and Limnephilidae at this location (Fig. 6). For example, Chironomidae had highest abundance in site 1 in summer and throughout the year in sampling stations 1, 2 and 3; at stations 4 and 6, very low abundances were observed. This denotes a spatial gradient. Also, Hydrobiidae (mostly *Potamopyrgus*) was only present in the first third of the river (sites 1, 2 and 3), being entirely absent from sites 4, 5 and 6.

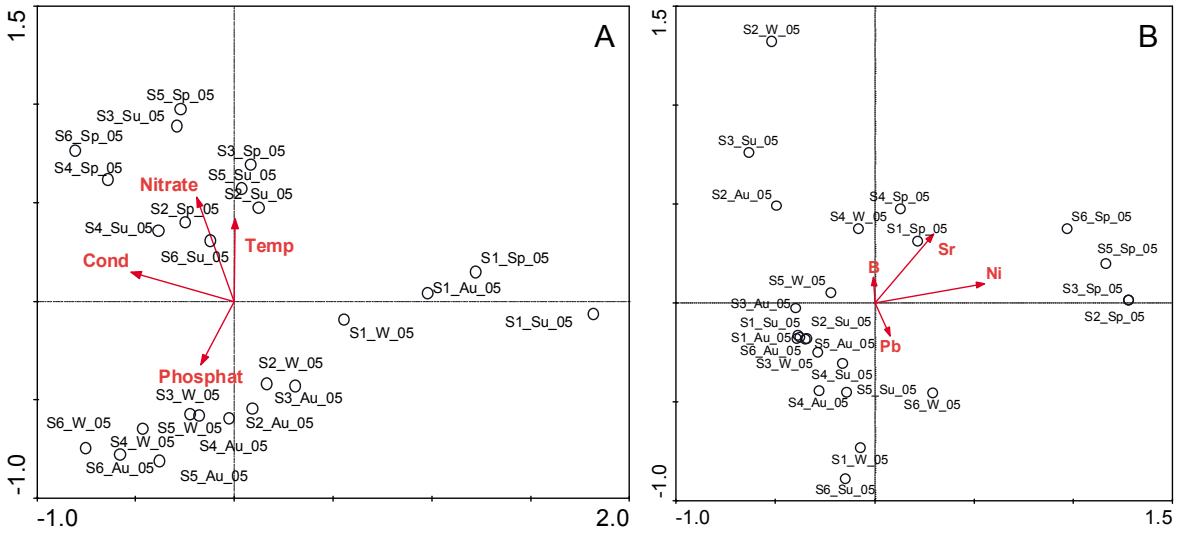

Fig. 5. RDA biplot of the sample scores (circles) and environmental gradients (arrows) obtained from the invertebrate data matrix, for the 2005/06 period. **A**. Physico-chemical explanatory data matrix: Phosphat stands for phosphates, Temp stands for temperature, Cond stands for conductivity and Nitrate was not abbreviated. **B**. Sediment metal explanatory data matrix. Sampling stations are represented by S and the site number (1-6) followed by season of the data collection (Sp - spring; Su - summer; Au - Autumn, W - winter) and finally the year of collection.

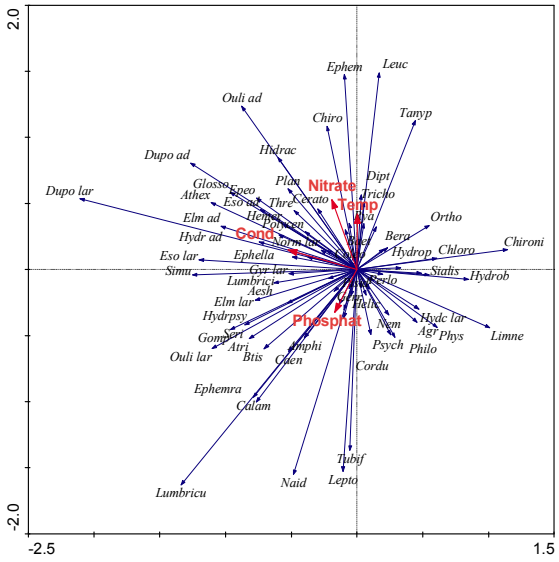

Fig. 6. RDA biplot of species scores (circles) and physico-chemical gradients obtained from the invertebrate data matrix, for the 2005/06 period. Phosphat stands for phosphates, Temp stands for temperature, Cond stands for conductivity and Nitrate was not abbreviated. Some taxa of centre part of the RDA biplot output were excluded for clearance of data presentation. See Table S.3 for taxa abbreviation.

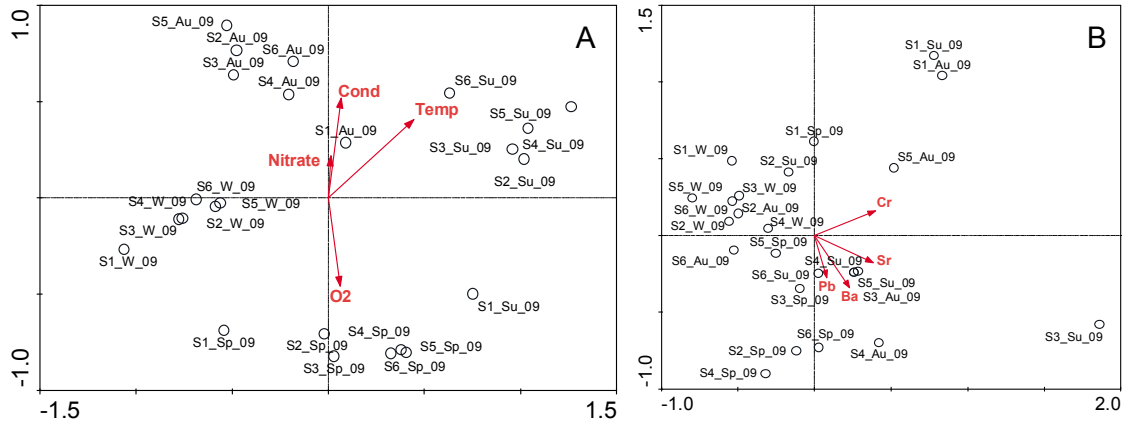

Fig. 7. RDA biplot of the sample scores (circles) and environmental gradients (arrows) obtained from the invertebrate data matrix, for the 2009/10 period. **A**. Physico-chemical explanatory data matrix: Temp stands for temperature, O2 stand for dissolved oxygen, Cond stands for conductivity and Nitrate was not abbreviated. **B**. Sediment metal explanatory data matrix. Sampling stations are represented by S and the site number (1-6) followed by season of the data collection (Sp - spring; Su - summer; Au - Autumn, W - winter) and finally the year of collection.

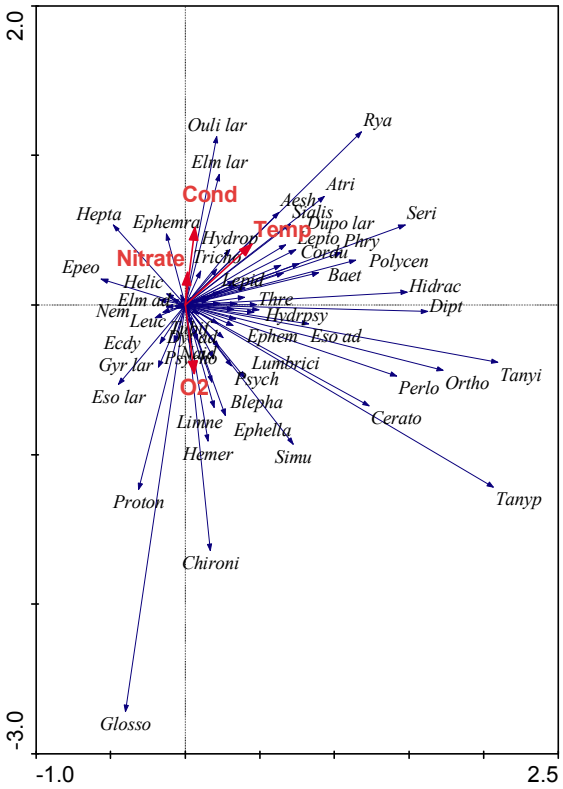

Fig. 8. RDA biplot of species scores (circles) and physico-chemical gradients obtained from the invertebrate data matrix, for the 2009/10 period. Temp stands for temperature, Cond stands for conductivity, O2 stands for oxygen, and Nitrate was not abbreviated. Some taxa of centre part of the RDA biplot output were excluded for clearance of data presentation. See Table S.3 for taxa abbreviation.

In 2009/10, the seasonal gradient was even clearer, with four groups of samples matching the four seasons of the year (Fig. 7A). Temperature was a main driver for this segregation (especially between summer and winter samples), although other variables seem to contribute. Autumn samples were associated with higher mineralization and lower oxygenation, while the opposite pattern was observed in spring samples. Similarly to the 2005/06 period, the RDA biplot of samples and sediment metal concentrations (Fig. 7B) did not reveal a clear pattern, although some seasonality is still apparent (winter samples are grouped all together, at the lowest extreme of sediment metal levels). The RDA biplot of species and physical and chemical variables (Fig. 6) reveals the taxa responsible for the observed seasonal gradient. The association between these taxa and sampling season observed in the RDA was checked and confirmed in the original data matrix. Thus, summer samples were characterized by a higher abundance of *Rhyacophyla*, *Atrichops*, *Sialis*, Sericostomatidae, Phryganeidae, Polycentropodidae, *Atherix*, *Cordulogaster*,

Dupophilus adults. On the other hand, *Epeorus*, Heptageniidae, *Ephemera*, and *Oulimnius* larvae were more abundant in the autumn, while *Esolus* larvae were more abundant in winter samples. In spring, higher abundances of Hemerodrominae, Ephemerella, and Limnephilidae were found.

These patterns were not consistent between 2005/06 and 2009/10, with very few species being characteristic of a certain season or site in a consistent fashion. This natural variability, coupled with the overall occurrence of common species (near the centroid of the biplots), justifies the absence of pronounced gradients in terms of species turnover, and is a consequence of weak environmental gradients (hence the small lengths of gradient observed in the DCAs).

3.4. Discussion

Mau River was expected to suffer the effects of multiple stressors (mine drainage, agricultural runoffs and sewage), either on specific locations or along its extension. For example, contamination caused by mine drainage can persist for centuries after mine closure, contaminating soils and water in the surrounding as well as sites further downstream (Byrne et al. 2013). However, for both years, the river was fairly homogeneous among sampling stations and presented high water quality. Most of the variation observed in its physico-chemistry and macroinvertebrate assemblage was due to seasonality. The good condition of the river contradicts expected impacts of multiple stressors and puts in evidence the idea that the river suffers minor effects from recent and historical pollution. Both the WFD approach and the community structure analysis support this conclusion and confirm the good quality of the Mau River. Therefore, Mau River meets the WFD criteria and requires no intervention in order to fulfill the WFD requirements. Still, small changes in community structure suggest small disturbances in the last portion of the river, which should draw attention to environmental managers in terms of local and regional (downstream) hazards.

The WFD approach, using multimetric biotic indices, concluded that 47 out of 48 samples were classified as good or high. In 2005/06, site 1 scored as moderate in the summer, which can be considered a casual event from being the most upstream site along a subtle conductivity/mineralization gradient (see Fig. 5A). In the 2009/10 period, ecological quality below high was almost always found in the last 2/3 of the river (sites 4, 5 and 6). Although small, this decrease of quality from high to good was due to the decrease in EPT taxa and number of families (Fig. 3), but also other community metrics (e.g. diversity); this is the only evidence that some differences occur among sampling

sites, suggesting minor impacts in the last 2/3 of the river. Still, this is very slight and the WFD approach does not allow any further inference. Contrarily to WFD approach, community structure analysis enables the extraction of gradients that explain much better the proximity or separation between groups of samples, and their relationship with the abiotic parameters measured. This approach showed more clearly that seasonality was a strong driving force for the separation of samples for each year and among them; generally, this was associated with temperature and nutrient (less clear) gradients. Seasonal variations in riverine communities have been thoroughly documented, and this may produce variations in ecological or water quality assessments (Byrne et al. 2013; Rodgher et al. 2013). However, community structure analysis also allowed obtaining some signs of potential impacts in the river (see next paragraph), and it allowed discriminating species that caused some samples to group together or with others. Therefore, community structure analysis provides added-value to the WFD approach. Analysis of impacts in biotic communities clearly benefits from the knowledge that the particular classification (EQRs) of the ecological status matches the spatio-temporal gradients of the biotic community supporting that evaluation, for a particular stream or river.

A few signs of potential impacts in the macroinvertebrate community were found in the last 2/3 of the river. First, ecological status was irregularly lower in these three sites, as previously said. Second, some taxa were absent in the final part of the river, which is most affected by the deactivated mining areas. Unfortunately, inter-annual and seasonal differences masked these patterns and added uncertainty in the assessment of taxa that were eventually more sensitive to environmental changes. The exception was Hydrobiidae (mostly *Potamopyrgus*), which presented a consistent pattern both in 2005/06 and 2009/10, being present in the first third of the river (sites 1, 2 and 3) and absent from sites 4, 5 and 6. This fine analysis reinforces the argument that the river might be under the influence of small metal concentrations. Indeed, Hickey and Golding (2002) found that *Potamopyrgus antipodarum* is markedly more sensitive to chronic metal exposures than would be expected based on laboratory acute and chronic data. This taxa could constitute a bioindicator for the presence of metals, which were found at variable environmental concentrations in mobile form and, therefore, were potentially bioavailable.

Sediment metal concentrations were obtained using a non aggressive technique of metal extraction, which in our perspective helps in increasing the ecological relevance, because it focuses on the bioavailable fraction of these elements (which can be quickly mobilized by resuspension phenomena in the sediment-water interface, unless if present in stable organic or inorganic complexes; Burton 2002). In aquatic systems, metals occur

under a variety of physicochemical forms, or species, as free hydrated metal ions and metal complexes with inorganic and organic ligands in dissolved, colloidal or particulate forms (Pickering 1995). The free ion activity model proposed by Campbell (1995) predicts that biological effects are governed by the activity of the free hydrated metal ions, rather than relating to their total concentration; this is crucial for predicting the biological effects of metals (Barata et al. 1998). Comparison of the metal concentrations obtained in our study was only possible with a few benchmark values available (defined by USEPA, and only for Cu, Zn, Cd and Pb), because sediment quality criteria are rare and they do not exist at all in Portugal. This comparison revealed that Pb concentration was above the reference value of 0.68 μg L^{-1} (according to Hickey and Pyle 2001) in sampling sites 2, 4 and 5 in 2005/06 and sites 3, 4, 5 and 6 in 2009/10. This is particularly relevant given the extraction procedure used, as these Pb concentrations concern the mobile fraction rather than the (pseudo-) total content. Again, this evidences that impacts occur in the last 2/3 of the river; indeed, lead was the main extracted metal in Braçal (S4). Other studies performed in the same area (Nunes et al. 2003; Nunes 2007) or with samples taken there (Vidal et al. 2012) also confirm high values of metals, namely lead, in river water, sediments, and also in plant tissues (leaves, stem, root) and soil. These values (Nunes et al. 2003; Nunes 2007) are not comparable to ours, because the authors used a strong acid mixture (*aqua regia*) for metal extraction, thus focusing on pseudo-total metal concentrations rather than just the mobile fraction.

Despite these cumulative evidences, no evident spatial pattern (i.e. site differentiation) was observed when the macroinvertebrate community was ordinated and constrained to a putative metal gradient (see RDA and partial RDA analyses). This was true even when the influence of potentially confounding physico-chemical variables was removed (since they bear a strong seasonal component). These results contrast with what was a priori expected and with studies using similar approaches (e.g. Beasley and Kneale, 2003). Two possible scenarios can explain why no association could be established between metal concentrations and variation in species composition and ecological status: i) because Mau river is a small mountain stream fed by a permanent spring, its high speed flow rate (especially in the raining season) dilutes the contamination downstream onto Vouga River while, at the same time, prevents settlement of fine sediments and contaminants bound to fine sediments (facilitated by the high surface-volume ratio of finer sediments); in this way, contaminants (sorbed to clay and silt particles) are rapidly flushed downstream and do not have time to affect the biota; ii) local contamination, albeit existent, is too low to produce impacts on resident communities; this

could have to do with resilience or lack of sensitivity from the macroinvertebrate community. Our view is that both scenarios – i) and ii) – are true.

As a support of the second scenario, we did not find acidification of the river or high levels of bioavailable metals, and this suggests that the degree of contamination is, at least currently, low. Another work published by Vidal et al. (2012) concluded that sediments collected from Braçal mine pit were not toxic when tested (WET tests) with a battery of freshwater organisms. The authors claim that these sediments are coated by thick orange precipitates (ferric hydroxides), which form complexes with free metals thus reducing their bioavailability. Vidal et al. (2012) hypothesized that the mine may be in a dormant state (it does not produce an acid effluent), most likely due to a reduced level of oxidation of metal complexes that leads to acid mine drainage (Young 1997). Although metal levels are now safe, the potential release of metals and acidity cannot be discarded in case of disturbance (there are plans for human intervention in the area).

Even if metals are bioavailable, some authors defend that macroinvertebrates, especially insect larvae, may be less sensitive to metals than other organisms (Malaj et al. 2012). We believe this is a biased view, based on comparative laboratory tests. Indeed, field studies document that abundance and diversity of EPT taxa, especially mayflies (Ephemeroptera), are very good indicators of metal pollution (see Beasley and Kneale, 2003; Iwasaki et al., 2009). Therefore, the lack of response from Mau River macroinvertebrate community cannot be attributed to its reduced sensitivity; however, it could be related to the community's resilience. On the one hand, contamination in the river has been probably occurring for a long time, allowing organisms to adapt. On the other hand, upstream (sub)populations may provide a continuous supply of organisms via drift to sites downstream (which are putatively more impacted), especially taking into account the river's high flow. It is likely that such a pattern is generally valid for small mountain rivers, especially due to their small dimension and high flow, making them therefore more resilient to stressor challenges. Flow was considered as master variable regulating the ecological integrity of flowing water systems by Poff et al. (1997) and therefore very important in terms of homogenizing upstream and downstream sites, via intentional or accidental macroinvertebrate drift (Miller et al. 2007). This may contribute to less pronounced community gradients (species turnover) and less divergence between upstream and downstream communities (McIntosh et al. 2002; Miller et al. 2007), even if environmental gradients exist (weak metal and mineralization gradient in the case of Mau River).

This leads us back to the first scenario (see above). Because Mau River is a small mountain stream, with a high speed flow, contaminants are less prone to accumulating in the sediment, as fine particles are flushed downstream (Poff et al. 1997). As previously stated, stream flow quantity and timing are critical components of water supply, water quality and ecological integrity of river systems (Poff et al. 1997). However, high flow regimes in contaminated sites may cause problems downstream. Southworth et al. (2013), for example, described a small river (East Fork Poplar Creek, in Tenesse, USA) that receives inputs of mercury, but whose effects were only observed downstream, due to the high flow of the creek. Indeed, rather than the creek itself, it was the downstream floodplain that was heavily contaminated with mercury. In the case of Mau river, because it is a tributary of Vouga river, the latter will be the first receiver of contaminated sediments flushed downstream. Other Vouga basin tributaries can also contribute; for example, Caima river is affected by contamination from paper mill industries and deactivated mine effluent (Vidal et al. 2012). Vouga river is the main source of drinking and irrigation water in the Aveiro region, so this upstream-downstream contamination scenario should worry local and regional environmental managers, as it poses human health concerns. Furthermore, Vouga river flows into the second potential receiver of sediment-bound contaminants, the Aveiro lagoon (Ria de Aveiro), which works as a transitional floodplain that receives freshwater inputs from several rivers (more than 50% of freshwater inputs come from Vouga river – Dias, 1999). There is a risk that many of these upstream contaminants, which are bound to fine particles, reach the Ria de Aveiro floodplain, endangering an ecologically and economically important area, which already reveals worrying signs of contamination from other sources (Pereira et al. 2009).

3.5. Conclusion

Mau river's benthic invertebrate community was found to be homogeneous, placing Mau river in a good-to-high ecological status. Although the river suffers the influence of multiple stress agents from anthropogenic origin, negative effects in the macroinvertebrate community due to these stressors could not be established, neither by WFD or community structure analysis approaches. We hypothesize that the observed resilience of the invertebrate assemblage is the result of two conditions: a) the current dormant state of the abandoned mining areas, which are the most relevant contamination source and the most potentially damaging; b) the river's characteristics, such as its short path and strong current, which promote re-colonization of biotic communities from

upstream non-impacted sites and rapid flushing of contaminants bound to fine particles. It is likely that such a pattern is generally valid for small mountain rivers, making them therefore more resilient to stressor challenges. Nevertheless, small changes in community structure suggest small disturbances in the last portion of the river, which may extend further downstream. This should draw attention to environmental managers in terms of local and regional (downstream) hazards. A more comprehensive study is, of course, required to fully address potential impacts in the downstream floodplain, including potential risks of "wakening" the dormant mine effluents (extreme weather events or human intervention). In what concerns Mau river itself, we could not tease out the influences of the multiple stressors using this approach. Ideally, an integrated approach including surveys on other biological descriptors (phytobenthos, macrophytes, fish; Hering et al. 2006) and in situ experimental designs, namely directed for the study of river ecological processes(e.g. leaf litter processing Gessner and Chauvet 2002; Pascoal et al. 2003), should be developed in order to solve the remaining uncertainties towards the pollution sources.

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3.7.Supplementary Material

Table S.1. Range (min-max) of physical and chemical parameters measured at Mau River between May 2005 and February 2006 (top) and between July 2009 and April 2010 (bottom), on six sampling sites (1-6).

Parameter	**Sampling sites**					
2005-2006	1	2	3	4	5	6
pH	5.65-8.65	5.91-7.40	5.99-7.83	6.68-7.57	6.85-7.85	6.74-7.61
O_2 (mg L^{-1})	8.4-12.4	8.5-13.1	9.1-18.4	11.1-18.4	8.6-13.7	11.2-11.7
Temperature (ºC)	9.4-14.5	10.8-16.3	10.7-16.3	10.1-16.7	9.5-16.5	9.1-18.0
Conductivity (µS cm^{-1})	23.8-33.2	49.0-64.6	44.6-71.1	49.8-77.8	55.2-66.7	58.3-77.3
TSS (mg L^{-1})	1.34-54.95	2.28-41.24	2.60-5.97	1.38-6.13	0.48-9.89	1.24-4.37
Nitrates (mg L^{-1})	0.07-0.30	0.07-1.20	0.08-1.60	0.08-1.30	0.08-1.4	0.09-1.40
Nitrites (mg L^{-1})	0.00-0.01	0.00-0.01	0.00-0.01	0.00-0.01	0.00-0.01	0.00-0.03
Ammonia (mg L^{-1})	0.00-0.01	0	0.00-0.01	0.00-0.19	0.00-0.28	0.00-0.04
Phosphates (mg L^{-1})	0.12-0.14	0.12-0.37	0.11-0.40	0.15-0.65	0.09-1.06	0.12-0.94
2009-2010	1	2	3	4	5	6
pH	6.05-6.62	6.3-6.79	6.57-6.99	6.56-6.94	6.67-6.97	6.79-7.2
O_2 (mg L^{-1})	7.01-11.10	5.42-11.00	6.48-11.20	7.02-10.80	5.95-10.90	7.18-11.00
Temperature (ºC)	10.5-17.00	11.9-18.80	11.90-18.70	12.60-19.50	12.70-20.50	12.90-18.80
Conductivity (µS cm^{-1})	4.32-37.2	8.34-68.70	9.37-76.20	9.63-76.40	10.20-81.70	10.70-84.80
TSS (mg L^{-1})	0.00-80.71	0.00-16.40	0.00-16.57	0.00-14.57	0.00-17.57	0.00-10.70
Nitrates (mg L^{-1})	0.00-10.23	2.95-6.25	4.82-8.86	5.09-9.52	4.74-7.13	4.97-8.86
Nitrites (mg L^{-1})	0.00-0.00	0.00-0.00	0.00-0.00	0.00-6.56	0.00-0.00	0.00-0.00
Ammonia (mg L^{-1})	*	*	*	*	*	*
Phosphates (mg L^{-1})	0.00-0.09	0.00-0.03	0.00-0.00	0.00-0.00	0.00-0.05	0.00-0.08

*Technical problems compromised ammonia quantification.

Table S.2. Range (min-max) of sediment metal concentrations (mg kg^{-1}) measured at Mau River between May 2005 and February 2006 and between July 2009 and April 2010, on six sampling sites (1-6).

Metal concentration (mg kg^{-1})	**Sampling sites**					
2005-2006	1	2	3	4	5	6
B	0.00-0.48	0.00-0.41	0.26-1.13	0.10-1.22	0.15-1.00	0.04-0.59
Al	0.46-22.92	0.08-16.41	0.59-40.43	0.61-78.20	1.22-27.57	0.4-5.7
V	0.00-0.02	0.00-0.007	0.00-0.06	0.001-0.120	0.000-0.005	0.000-0.005
Cr	0.00-0.02	0.000-0.005	0.00-0.04	0.00-0.09	0.002-0.032	0.000-0.003
Mn	0.06-0.29	0.02-0.14	0.09-0.31	0.033-0.196	0.03-0.65	0.04-0.15
Fe	0.29-17.05	0.04-13.94	0.5-35.4	0.88-75.72	1.94-35.48	0.67-7.86
Ni	0.00-0.01	0.00-0.02	0.000-0.004	0.000-0.006	0.000-0.016	0.005-0.014
Cu	0.00-0.02	0.00-0.01	0.002-0.005	0.000-0.010	0.000-0.007	0.004-0.011
Zn	0.00-0.40	0.00-0.37	0.01-0.20	0.047-0.546	0.06-0.34	0.10-0.17
As	0.001-0.004	0.001-0.004	0.0014-0.0116	0.001-0.030	0.002-0.018	0.001-0.005
Sr	0.00-0.05	0.01-0.08	0.008-0.072	0.014-0.052	0.01-0.06	0.004-0.062
Cd	0.00-0.0003	0.00-0.0001	0.0000-0.0002	0.0001-0.0006	0.0001-0.0011	0.000-0.001
Ba	0.01-0.71	0.03-0.87	0.01-0.90	0.014-1.300	0.05-0.82	0.065-0.628
Pb	0.001-0.02	0.00-0.86	0.008-0.130	0.06-2.60	0.15-2.57	0.005-0.61
2009-2010	1	2	3	4	5	6
B	0.37-2.97	0.16-1.18	0.14-3.91	0.33-1.19	0.43-1.57	0.36-1.05
Al	29.16-150.62	10.93-95.28	41.10-122.38	58.75-119.91	0.00-173.02	26.58-76.25
V	0.03-0.15	0.00-0.09	0.03-0.17	0.06-0.16	0.00-0.20	0.03-0.08
Cr	0.03-0.16	0.00-0.07	0.03-0.10	0.04-0.09	0.00-0.11	0.02-0.06
Mn	1.05-2.54	1.11-5.63	2.08-4.43	1.76-3.56	1.38-26.57	0.38-2.90
Fe	25.72-101.11	19.03-74.57	36.21-143.06	54.57-142.74	3.56-208.70	30.14-129.77
Ni	0.04-0.09	0.02-0.06	0.02-0.07	0.04-0.15	0.01-0.24	0.03-0.11
Cu	0.00-0.10	0.04-0.09	0.05-0.19	0.08-0.24	0.00-0.25	0.04-0.17
Zn	0.64-1.13	0.37-1.08	0.51-1.64	1.15-3.58	0.24-4.45	0.61-3.41
As	0.00-0.00	0.00-0.00	0.00-0.00	0.00-0.00	0.00-0.00	0.00-0.01
Sr	0.05-0.06	0.12-0.20	0.09-1.05	0.08-0.17	0.07-0.34	0.08-0.12
Cd	0.00-0.00	0.00-0.00	0.000-0.003	0.00-0.01	0.00-0.03	0.000-0.007
Ba	0.59-1.21	0.61-1.99	0.78-1.75	0.96-1.86	0.58-1.13	0.74-1.45
Pb	0.05-0.10	0.11-0.39	0.22-1.24	5.00-19.17	0.16-9.98	1.13-17.11

Table S.3. Abbreviation (abbrev.) list of macroinvertebrate taxa collected in Mau river during the study period (2005/06 and 2009/10).

Abbrev.	Taxon (Family)	Group	Abbrev.	Taxon (Family)	Group
Aesh	Aeshnidae	Odonata	Hidrac	Hydracarina	Acari
Agr	*Agrion* (Calopterygidae)	Odonata	Hydc lar	*Hydrocyphon* larvae (Scirtidae)	Coleoptera
Amphi	*Amphinemura* (Nemouridae)	Plecoptera	Hydr ad	*Hydraena* adults (Hydraenidae)	Coleoptera
Athex	*Atherix* (Athericidae)	Diptera	Hydrob	*Potamopyrgus* (Hydrobiidae)	Gastropoda
Atri	*Atrichops* (Athericidae)	Diptera	Hydrop	Hydroptilidae	Trichoptera
Baet	Baetidae	Ephemeroptera	Hydrpsy	Hydropsychidae	Trichoptera
Bera	Beraeidae	Trichoptera	Lepto	Leptophlebiidae	Ephemeroptera
Blepha	Blephariceridae	Diptera	Leuc	Leuctridae	Plecoptera
Btis	*Baetis* (Baetidae)	Ephemeroptera	Limne	Limnephilidae	Trichoptera
Caen	*Caenis* (Caenidae)	Ephemeroptera	Lumbrici	Lumbricidae	Oligochaeta
Calam	Calamoceratidae	Trichoptera	Lumbricu	Lumbriculidae	Oligochaeta
Cerato	Ceratopogonidae	Diptera	Naid	Naididae	Oligochaeta
Chiro	Chironomidae	Diptera	Nem	*Nemoura* (Nemouridae)	Plecoptera
Chironi	Chironomini (Chironomidae)	Diptera	Norm lar	*Normandia* larvae (Elmidae)	Coleoptera
Chloro	Chloroperlidae	Plecoptera	Ortho	Orthocladiinae (Chironomidae)	Diptera
Coleo	unidentified Coleoptera	Coleoptera	Ouli ad	*Oulimnius* adults (Elmidae)	Coleoptera
Cordu	*Cordulegaster* (Cordulegasteridae)	Odonata	Ouli lar	*Oulimnius* larvae (Elmidae)	Coleoptera
Dipt	unidentified Diptera	Diptera	Perlo	Perlodidae	Plecoptera
Dupo ad	*Dupophilus* adults.(Elmidae)	Coleoptera	Philo	Philopotamidae	Trichoptera
Dupo lar	*Dupophilus* larvae (Elmidae)	Coleoptera	Phry	Phryganeidae	Trichoptera
Dyt ad	Dytiscidae adults	Coleoptera	Phys	Physa	Gastropoda
Ecdy	*Ecdyonurus* (Heptageniidae)	Ephemeroptera	Pisid	*Pisidium* (Sphaeriidae)	Bivalvia
Elm ad	*Elmis* adults (Elmidae)	Coleoptera	Plan	*Planaria* (Planariidae)	Hirudinea
Elm lar	*Elmis* larvae (Elmidae)	Coleoptera	Polycen	Polycentropodidae	Trichoptera
Epeo	*Epeorus* (Heptageniidae)	Ephemeroptera	Proton	Protonemura	Plecoptera
Ephella	*Ephemerella* (Ephemerellidae)	Ephemeroptera	Psych	Psychomyiidae	Trichoptera
Ephem	unidentified Ephemeroptera	Ephemeroptera	Psycho	Psychodidae	Diptera
Ephemra	*Ephemera* (Ephemeridae)	Ephemeroptera	Rya	*Ryacophila* (Rhyacophilidae)	Trichoptera
Eso ad	*Esolus* adults (Elmidae)	Coleoptera	Seri	Sericostomatidae	Trichoptera
Eso lar	*Esolus* larvae (Elmidae)	Coleoptera	Sialis	*Sialis* (Sialidae)	Megaloptera
Gerr	*Gerris* (Gerridae)	Heteroptera	Simu	Simuliidae	Diptera
Glosso	Glossosomatidae	Trichoptera	Tanyi	Tanytarsini (Chironomidae)	Diptera
Gomp	Gomphidae	Odonata	Tanyp	Tanypodinae (Chironomidae)	Diptera
Gyr lar	Gyrinidae larvae	Coleoptera	Thre	Thremma (Thremmatidae)	Trichoptera
Helic	Helicopsychidae	Trichoptera	Tricho	unidentified Trichoptera	Trichoptera
Hemer	Hemerodromiinae (Empididae)	Diptera	Tubif	Tubificidae	Oligochaeta
Hepta	Heptageniidae	Ephemeroptera			

Chapter IV

Ecotoxicological assessment of contaminated river sites as a proxy for the Water Framework Directive: an acid mine drainage case study.

Ecotoxicological assessment of contaminated river sites as a proxy for the Water Framework Directive: an acid mine drainage case study.

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Abstract

Metal contamination of freshwater bodies resulting from mining activities or deactivated mines is a common problem worldwide as in Portugal. Braçal (galena ore) and Palhal (pyrrhotite, chalcopyrite, galena, sphalerite and pyrite ore), located in a riverside position, are both examples of deactivated mining areas lacking for implemented recovery plans since their shut-down in the early-mid 1900's. In both mining effluents still flow into two rivers. The purpose of this work was evaluating the potential hazard posed by the mining effluents to freshwater communities. Therefore, short- and long-term ecotoxicological tests were performed on elutriates from river sediments collected at each site using standard test organisms that cover different functional levels (*Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Lemna minor* and *Daphnia* sp.). The results show that elutriates from the sediments of Palhal were very toxic to all tested species while in contrast, elutriates from Braçal showed generally no toxicity for the tested species. Our study highlights the usefulness of using an ecotoxicological approach to help the prioritization/scoring of the most critical areas impacted by deactivated mines. This ecotoxicological test battery can provide important information about the ecological status of each concerning site before investing in the application of time-consuming and costly methods defined by the Water Framework Directive or can stand as a meaningful complementary analysis.

Keywords: deactivated mines; metals; Ecotoxicological test battery; Water Framework Directive

4.1. Introduction

Mining industry is probably one of the anthropic activities causing great impact on the environment since it is responsible for the change in landscape and deforestation (Starnes and Gasper 1995), as well as it constitutes a major source of freshwater contamination by metals along with industrial activities and urban runoff (Hickey and Golding 2002; Yim et al. 2006). Metal pollution of streams and rivers is a major concern within management plans for freshwater worldwide; and mining activities in particular have been shown to promote serious disturbance in trophic chains, which ultimately reflects at the ecosystem level (e.g. Peplow and Edmonds 2005). Deactivated mines essentially pose physical and chemical risks to the ecosystems. Physical risks are related to the collapse of old structure of tunnels and galleries, while the chemical risks refer to the characteristic acidic mine drainage (Pereira et al. 2004b). Portugal, like other countries worldwide, faces a severe environmental problem with deactivated mines. It is relatively frequent to notice piles of mine tailing left exposed to air and water, thus facilitating oxidation and production of acid leachates that are prone to seriously contaminate the surrounding fields and groundwater (Malmqvist and Hoffsten 1999; Pereira et al. 2004b). The present study was focused on two deactivated mines for over 30 years (Nunes et al. 2003; Nunes 2007; Santos 2010), located in the riverside within the watershed of the Vouga river, a major drinking water source in the northern-centre of Portugal.

With the introduction of the EC Water Framework Directive (WFD) in 2000 (2000/60/EC) the assessment of the ecological status of all European water bodies and the comparison between the biological community composition of each site with near-natural reference conditions became mandatory in European countries. Despite its comprehensiveness can be faced as an advantage in environmental monitoring actions, the WFD is very complex and time-consuming in the sense that it requires very specialized work to sample, gather and integrate information from different sources including the biological communities inhabiting each assessed site (e.g. macroinvertebrates, ephytic diatoms, fish and macroalgae). In this way, it is worth investigating potential early-warning methodologies that can provide enough information for early-stage assessment of water quality. As previously suggested (Chovanec et al. 2000; Huschek and Hansen 2006; Marín-Guirao et al. 2005), we used an ecotoxicological test battery to assess the hazardous potential of deactivated mines to the freshwater biota.

WET (Whole Effluent Toxicity) tests and Elutriate Sediment Toxicity Tests (ESTT) are widespread useful tools to address the toxicity of complex environmental samples

(USEPA 2001). Data yield from such assessment techniques provide the necessary grounds for identifying, diagnosing and monitoring the effects of this complex mixtures of contaminants in the environment, thereby assisting the prediction of its potential hazard to the receiving environment (Abrantes et al. 2009; Antunes et al. 2007b; Chapman 2000; Marques et al. 2010; Wharfe 2004). In a river, contamination in the water column is very variable depending e.g. on the river flow and turbulence and dilution rates, hence any assessment of water quality should consider the sediment matrix. Sediments can contain several amounts of organic and inorganic material bounded to particles but when disturbed by stormwater runoff they can turn bioavailable as an important pollution source for both benthic and planktonic organism (Burton 2002).

This study comprises a battery of sediment elutriate toxicity tests allowing to evaluate the historic metal contamination by the effluent that still today drains from the Braçal and Palhal mines into the water flow. We primarily aim to assess whether the ecotoxicological assessment resembles the contamination of the sites following long-term metal input operated by the deactivated mine effluent, hence assisting the establishment of the necessary grounds towards the development of an adequate early-warning methodologies to access water quality. The sediment elutriates were tested using short- and long-term bioassays with bacteria (*Vibrio fischeri),* freshwater microalgae (*Pseudokirchneriella subcapitata)*, macrophytes (*Lemna minor)* and freshwater cladocerans (*Daphnia magna* and *Daphnia longispina*). Provided their high sensitivity to environmental stress and/or their ecological position in the aquatic food web hence functional representativeness, all these species constitute key standard organisms widely used in ecotoxicological assessment of contaminants ((Hanazato 2001; Lewis 1995; Parvez et al. 2006) and are generally included in early stages of environmental risk assessment flowcharts worldwide (Abrantes et al. 2006; Hanazato 2001; Shahidul Islam and Tanaka 2004).

4.2. Material and Methods

4.2.1. Site description

Two deactivated mines, Braçal (inactive since the 1950s) and Palhal (inactive since the 1920s), were considered in this study (Fig. 1). These mines are located, in Sever do Vouga and Águeda, respectively (Aveiro, Northen Portugal), standing in a felsic geological area mainly composed by schist (Braçal) or granite and schist (Palhal). Both mines stand in the riverside of two important Vouga river subsidiaries within rhithron-like

zones (sensu Lampert and Sommer 1997). Point source contamination through a metal-rich run-off can be identified in Braçal while in Palhal drainage from tailing accumulation in the riverside should additionally be considered.

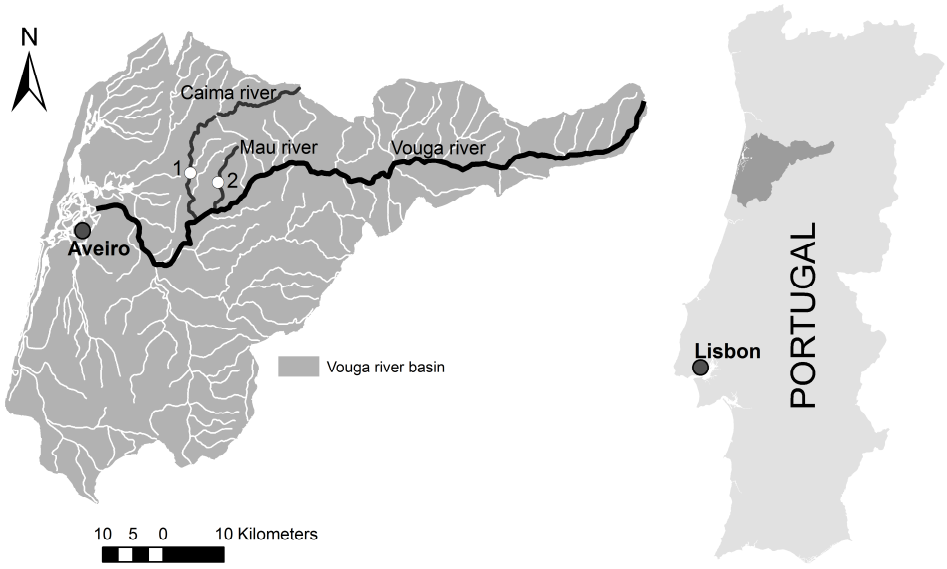

Figure 1. Geographic location of the studied deactivated mines. (1) Palhal mine (40º 44' 50''N, 8º 27'21.5''W), where past mining activities included the extraction of metals such as Pb, Cu and Ag as pyrrhotite (FeS) ore, chalcopyrite (CuFeS2) ore, galena (PbS) ore, sphalerite (ZnS enriched in cadmium) and pyrite ore (FeS2) (Nunes et al. 2003); (2) Braçal mine (40º 44' 1.0''N, 8º 24' 6.6''W), where past mining activities included galena ore (native lead sulphide), zinc blend ore and iron pirite ore (Santos Oliveira et al. 2005).

4.2.2. Sample collection and elutriate preparation

The collection of sediments was performed at the beginning of autumn still during the dry season. For general characterization purposes, water temperature, pH, conductivity and dissolved oxygen were measured at each collection site using a portable multiparametric probe (WTW MULTI 3430). A sampling area of approximately one square meter was established next to the Braçal and Palhal drainage sites and the superficial sediment layer (ca.10 cm depth) was collected with a stainless steel shovel into clean plastic bags, which were airtight sealed for safe transport to the laboratory. Sediment samples were sorted out for removal of large debris and pebbles, and then preserved at -20ºC until further use.

Elutriates were prepared for chemical analysis and toxicity testing within the following 8 weeks after collection, as recommended by USEPA (1998), in erlenmeyer glass vessels according to the procedure described by Ankley et al. (1991) and Nebeker et al. (1984). A 1:4 (v/v) ratio of sediments to each adequate test medium was used. Vessels were shaken for 2 hours at 20 ± 2ºC in an orbital shaker (200 rpm), and left to settle overnight at 4ºC. The overlying layer was centrifuged at 2500 g for 15 minutes. The supernatant (elutriate) was stored at 4º C in dark until further use (1 week maximum holding period).

The elutriate preparation for metal analysis was done by mixing homogenized sediment and distilled water in a proportion of 1:2 (w/v). The mixture was shaken for 24 h at 20 ± 2ºC in the dark and left to settle for a few minutes. The overlying material was then centrifuged at 2500 g for 10 min at 4ºC. The supernatant was filtered through a Whatman GF/C filter (1.2 µm porosity) to remove suspended matter, and the filtrate was immediately acidified with nitric acid (65% PA) to pH < 2. Several metal elements were quantified in the elutriate samples through ICP-MS (APHA 1995).

4.2.3. Test species and culturing conditions

Bioluminescent bacteria (*Vibrio fischeri*) were supplied lyophilized as part of the Microtox® (AE 1998) kit and reconstituted in the corresponding reconstitution solution immediately prior testing. The microalgae *Pseudokirchneriella subcapitata* (Korshikov) Hindak was obtained from unialgal cultures cyclically maintained in 150 ml Erlenmeyer glass vessels filled with 75 ml sterilized Woods Hole MBL culture medium (Stein 1973).The macrophyte *Lemna minor* was collected in a pond and has been maintained in the laboratory as a successful long-term culture in Steinberg culture medium (OECD 2006) once a week. Monoclonal cultures of *Daphnia magna* (clone A, sensu Baird et al. 1989a) and *Daphnia longispina* (clone EM7, sensu Antunes et al. 2003)were continuously reared in the laboratory in synthetic ASTM hardwater medium (ASTM 1980) supplied with organic additive extracted from the algae *Ascophyllum nodosum* (Baird et al. 1989b). Cultures were renewed every other day, and the organisms were fed after renewal with *P. subcapitata* at a rate of 3.0 x 10^5 (*D. magna*) or 1.5 x 10^5 (*D. longispina*) cells mL^{-1}. All cultures were kept under a 16h:8h (light:dark) photoperiod and temperature of 20±2ºC.

4.2.4. Toxicity bioassays

The *Vibrio fischeri* luminescence inhibition test was first done using the sediments as samples through the solid-phase procedure and then using pre-prepared elutriates through the liquid-phase (81.9% basic test for Braçal and basic-test for Palhal) methodology AE (1998).

The growth inhibition of the green microalgae *Pseudokirchneriella subcapitata* following exposure to the sediment elutriates was assessed u sing a static bioassay conducted according to USEPA (2001) and OECD (2011) guidelines, with adaptation to 24-well microplate use (e.g. Geis et al. 2000). The algae were exposed during 72 h under continuous illumination to serial dilutions of the mine sediment elutriate in MBL medium. Three replicates were established per treatment and each replicated well was filled with 990 μl test solution plus 10 μl microalgae inoculums adjusted so that the final nominal cell density at the beginning of the test could be 10^4 cells ml^{-1}. The test microplates were incubated as described above for algal cultures and the contents of each well were thoroughly mixed twice daily by repetitive pipetting to promote active gas exchange and prevent cell clumping. At the end of the bioassay, yield and the daily growth rate were calculated from microscopic cell density measurements.

The growth inhibition tests with *Lemna minor* were performed according to the corresponding OECD (2006) and USEPA (2002) guidelines. Three colonies with 3 visible fronds each were harvested from the inoculum culture and randomly assigned to each test vessel filled with 100 ml Steinberg medium. Three replicates (with 9 fronds each) were established per treatment, and treatments consisted in serial dilutions of elutriate. An additional replicated sample (n = 3) was collected from the inoculum culture and dried at 60ºC overnight to provide the initial dry weight for further growth-related calculations. Incubating conditions were kept as described for cultures during the 7-d exposure period. Growth inhibition was calculated on the basis of the variation in dry weight following exposure.

The 48-h acute exposures of *Daphnia magna* and *Daphnia longispina* to the elutriates followed the recommendations of standard guidelines (OECD 1998; OECD 2004; USEPA 2002). Five newborn daphnids (<24-h old; from the 3rd to the 5th brood) were randomly assigned to each replicate and 4 replicates were established per treatment. The tests were carried out in glass vials filled with 25 ml test solution consisting in clean ASTM for the control and serial elutriate dilutions in ASTM for the remaining test treatments. No food or organic additives were provided during the test and incubation conditions were kept as mentioned for rearing procedures (see above). At the end of the

exposure period (48 h) each vial was checked for immobilised daphnids. Chronic exposure assays with daphnids following corresponding guidelines USEPA (2002) were carried out until 60% of the control females released the third brood of newborns. A semi-static test design was employed with renewal of test solution occurring every other day. Ten individual replicates per treatment (*D. longispina* and *D. magna* newborns from the 3rd to the 5th brood and ageing less than 24-h) were established in glass vials filled with 25 mL test solution consisting in the: blank control (ASTM medium) plus a geometric range of nominal elutriate concentrations from Braçal or Palhal. The test conditions were kept as already described for the maintenance of daphnids. The organisms were fed daily, with the respective *P. subcapitata* ration (see above). The animals were observed daily for mortality and offspring production. The body size of parent females was estimated at the beginning and at the end of the test (Pereira et al. 2004a), allowing the calculation of the somatic growth rate. The population growth was estimated based on *r* (*per capita* rate of population increase), which was derived from the Euler-Lotka equation (Meyer et al. 1986) as follows:

$1 = \sum_{i=1}^{n} e^{-rx} l_x m_x$, where r is expressed in day^{-1}, x is the age class (days; 0…n), l_x is the probability of surviving at age x, and m_x is the fecundity at age x. The corresponding standard deviations were determined according to the Jackknife technique (Meyer et al. 1986).

4.2.5. Statistical analysis

Reference effect concentrations for luminescence inhibition (EC_{50} EC_{20}, EC_{10}) in *V. fischeri* were estimated using Microtox Omni^{TM} Software version 4.3.0.1 (AE 1998). Probit analysis (Finney 1971) was used to estimate the concentration which causes 50%, 20% and 10% immobilization of daphnids in acute toxicity tests (EC_x) and corresponding 95% confidence intervals. EC_{50}, EC_{20} and EC_{10} and their 95% confidence limits were also estimated whenever possible for the continuous variables measured in the bioassays with *P. subcapitata*, *L. minor* and *Daphnia* sp. by non-linear regression using the logistic equation that was fitted to the data through the least squares statistical method. A one-way analysis of variance (ANOVA), followed by a Dunnett test was applied to each endpoint of the chronic assays with daphnids to assign statistical differences between the concentrations tested and the control.

4.3. Results

The physical and chemical parameters recorded *in-situ* on river water at the sampling site, as well as indicative measurements made on elutriates prepared from the sediment samples for further toxicity testing were summarised in Table 1. The water from Braçal and Palhal showed similar records while elutriates prepared from the sediment samples identify distinct pH and conductivity profiles. It is worth noticing the changes observed in pH and conductivity recorded for elutriates prepared from Palhal sediments as compared to the corresponding stream water, with a considerable decrease in pH and increase in conductivity, common to all culture media. The elutriate samples from Palhal show more extreme conditions than those from Braçal. It is worth noticing that these differences were smoothed in the toxicity tests given that much higher dilution was applied to Palhal samples. This is consistent with the metal quantification performed on both elutriate samples (Table 2). A higher metal content was recorded in the elutriate from Palhal sediments, where B, Al, Cr, Mn, Co, Ni, Cu, Zn, As, Sr consistently showed contents more than one order of magnitude higher than in Braçal samples. Iron, lead and zinc were the metal elements found in higher content in the Braçal elutriate samples; sediments from Palhal apparently have a Pb content one order of magnitude lower than those from Braçal. Cadmium was additionally found in concerning concentrations particularly in Palhal.

Table 1. Physical and chemical parameters of stream water recorded in-situ before collecting the sediment samples. For indicative purposes, data are also provided on elutriates prepared from each test sediment with the different media used in further assays.

	Water		**Elutriate**					
	Braçal	**Palhal**	**Braçal**			**Palhal**		
			ASTM	MBL	Steinberg	ASTM	MBL	Steinberg
pH	7.71	7.26	7.60	7.41	7.23	3.50	3.21	3.11
Conductivity (µs cm^{-1})	212	145.9	744	537	720	1069	1242	1632
Temperature (ºC)	14.4	16.6	20.0	20.0	20.0	20.0	20.0	20.0
Dissolved Oxygen (mg L^{-1})	7.68	10.27	8.42	8.38	8.44	8.69	9.13	9.22

All ecotoxicological tests fulfilled the validity requirements established in their respective guidelines. Two different methodologies were employed to test the sediments from Braçal and Palhal with the luminescence bacteria *Vibrio fischeri* so that the likely interference from bacteria adsorption to particulate material in the spectrophotometric readings could be addressed; indeed, Braçal sediments contained finer silt-clay particles, originating a very turbid elutriate before centrifugation while Palhal sediment as sorted for elutriates is mostly composed of sand particles originating a very clear elutriate before centrifugation (see the discussion section for details). Contrasting results were retrieved from solid-phase and liquid-phase testing with Braçal samples, with the solid-phase assay denoting higher toxicity of the sediment (Figure 2 A, B; Table 3).

Controversial results were obtained as to the eventual role of exposure time in modelling toxicity estimations (Table 3; Figure 2): e.g. toxicity increase with time when considering the EC_{50} estimate versus toxicity decrease with time when considering the EC_{20}, EC_{10} values estimated for Palhal; in solid-phase assays, Braçal toxicity decreased through time for all endpoints while for Palhal toxicity increased through time regarding EC_{50} and EC_{20}. These controversial results as the endpoint changes, as well as the overlapping confidence intervals between each ECx value estimated at different exposure periods (see Table 3), indicate that time should not be an important factor constraining toxicity estimates for these samples.

Table 2. Metal concentrations found in the sediments collected in Braçal and Palhal through ICP-MS analysis. Values shown for Braçal and Palhal represent average quantification (n = 3) with standard deviation shown within brackets, both in µg L^{-1} and also converted into mg Kg^{-1} to facilitate direct comparison with the benchmark values below which harmful effects are unlikely found in the literature.

	Braçal		**Palhal**		[3]ANZECC	[4]US EPA	[1]CSQGPAL	[2]TEC	[5]TEL
	µg L^{-1}	mg Kg^{-1}	µg L^{-1}	mg Kg^{-1}	µg.L^{-1}	µg.L^{-1}	mg Kg^{-1}	mg Kg^{-1}	mg Kg^{-1}
B	122.2 (3.2)	0.244 (0.0064)	1094 (0.8)	2.18 (0.0016)	N.a	N.a	N.a	N.a	N.a
Al	124.8 (1.8)	0.250 (0.0036)	58160 (0.5)	116.4 (0.001)	N.a	N.a	N.a	N.a	N.a
V	1.0 (7.5)	0.002 (0.015)	0.7(2.5)	0.0013 (0.005)	N.a	N.a	N.a	N.a	N.a
Cr	0.9 (1.6)	0.00172 (0.0032)	14.4 (0.7)	0.029 (0.0014)	N.a	N.a	37.3	43400	37.3
Mn	10.3 (0.8)	0.0206 (0.0016)	2561 (0.5)	5.12 (0.001)	N.a	N.a	N.a	N.a	N.a
Fe	4028 (1)	8.06 (0.002)	1011 (0.9)	2.02 (0.0018)	N.a	N.a	N.a	N.a	N.a
Co	0.2 (2.0)	0.00034(0.004)	291.5 (0.8)	0.584 (0.0016)	N.a	N.a	N.a	N.a	N.a
Ni	1.6 (2.4)	0.0032 (0.0048)	500.5 (0.9)	1.002 (0.0018)	N.a	N.a	N.a	22700	18

Cu	**13.0 (1.4)**	0.026 (0.0028)	**2963 (0.9)**	5.92 (0.0018)	1.4	3.3	35.7	31600	35.7
Zn	**312.6 (0.9)**	0.626 (0.0018)	**2434 (0.4)**	4.86 (0.0008)	8.0	43	123	121000	123
As	2.6 (1.7)	0.00512 (0.0034)	133.1 (0.7)	0.266 (0.0014)	N.a	N.a	5.9	9790	5.9
Sr	22.6 (0.3)	0.045 (0.0006)	60.0 (0.4)	0.12 (0.0008)	N.a	N.a	N.a	N.a	N.a
Cd	**0.6 (4.9)**	0.114 (0.0098)	**50.1 (0.4)**	0.1002 (0.0008)	0.2	0.96	0.6	990	0.596
Ba	402.3 (0.4)	0.804 (0.0008)	389.9 (0.3)	0.78 (0.0006)	N.a	N.a	N.a	N.a	N.a
Pb	**417.2 (0.7)**	**0.834 (0.0014)**	**10.5 (1.9)**	0.021 (0.285)	3.4	0.68	35.0	35800	0.35

N.a.- Not available; [1]Canadian Sediment Quality Guidelines for the Protection of Aquatic Life (CCME 2002); [2] Threshold effect concentration (NOOA 2008); [3] Australian and New Zealand Environmental and Conservation Council (Hickey and Pyle 2001); [4] USEPA 1996 (*In* Hickey and Pyle 2001); [5]Threshold effect level-concentration level below which adverse effect would be rarely observed (MacDonald et al. 2000).

Table 3. EC_x values (%) and the respective 95% confidence limits (in brackets) estimated for *V. fischeri* (mg L^{-1} or % for the solid- and liquid-phase assays, respectively), *P. subcapitata*, *L. minor,* and also for the mortality, fecundity and *r* (population growth rate) of *D. magna* and *D. longispina* exposed to Braçal and Palhal elutriates.

		Braçal	**Palhal**
***Vibrio fischeri* (solid-phase)**			
Luminescence	5 min EC_{50}	10330 (7643-13960)	5393 (3940- 7381)
	5 min EC_{20}	1507 (993-2287)	781 (482-1267)
	5 min EC_{10}	489 (276-867)	252 (132-484)
	15 min EC_{50}	12890 (9909-16770)	2677 (1444- 4960)
	15 min EC_{20}	1718 (1222 -2417)	674 (270-1682)
	15 min EC_{10}	529 (328-851)	301 (97-938)
	30min EC_{50}	18280 (13440-24880)	2065 (983-4341)
	30 min EC_{20}	2633 (1890-3668)	620 (205-1880)
	30 min EC_{10}	848 (538-1341)	307 (77-1220)
***Vibrio fischeri* (liquid-phase)**			
Luminescence	5 min EC_{50}	n.d.	5.05 (4.31-5.91)
	5 min EC_{20}	n.d.	1.36 (1.01-1.84)
	5 min EC_{10}	n.d.	0.63 (0.43-0.94)
	15 min EC_{50}	n.d.	3.99 (3.20-4.98)
	15 min EC_{20}	n.d.	1.43 (1.03-2.09)
	15 min EC_{10}	n.d.	0.81 (0.52-1.26)

Pseudokirchneriella subcapitata			
	72 h EC_{50}	69.57 (60.50-78.65)	6.65 (6.32-6.99)
Yield	72 h EC_{20}	43.72 (32.25-55.18)	4.85 (4.44-5.26)
	72 h EC_{10}	33.30 (20.93-45.66)	4.03 (3.58-4.48)
	72 h EC_{50}	156.47 (135.15-177.79)	24.63 (17.44-31.83)
Growth Rate	72 h EC_{20}	90.36 (85.94-94.78)	6.41 (2.72-9.76)
	72 h EC_{10}	65.51 (59.09-71.949)	2.79 (0.46-5.11)
Lemna minor			
	7 d EC_{50}	162.40 (nd-386.01)	19.45 (11.69-27.21)
Yield	7 d EC_{20}	98.65 (55.33-141.97)	8.47 (2.87-14.08)
	7 d EC_{10}	73.66 (16.09-131.24)	5.21 (0.71-9.71)
	7 d EC_{50}	250.53 (n.d.-826.18)	18.27 (13.04-23.51)
Growth Rate	7 d EC_{20}	146.72 (n.d.-302.60)	4.80 (2.34-7.26)
	7 d EC_{10}	107.29 (57.73-156.86)	2.19 (0.71-3.68)
D. magna			
	48 h EC_{50}	n.d.	2.91 (1.35-4.96)
Mortality	48 h EC_{20}	n.d.	0.91 (nd-2.21)
	48 h EC_{10}	n.d.	n.d.
	16 d EC_{50}	n.d.	1.77 (1.47-2.07)
Fecundity	16 d EC_{20}	n.d.	1.43 (1.25-1.62)
	16 d EC_{10}	n.d.	1.26 (0.96-1.57)
	16 d EC_{50}	n.d.	2.48 (2.12-2.84)
r	16 d EC_{20}	n.d.	1.91 (1.79-2.04)
	16 d EC_{10}	n.d.	1.65 (1.60-1.69)
D. longispina			
	48 h EC_{50}	n.d.	1.31 (1.11-1.52)
Mortality	48 h EC_{20}	n.d.	0.60 (0.45-0.73)
	48 h EC_{10}	n.d.	0.40 (0.27-0.73)

n.d. – not determined

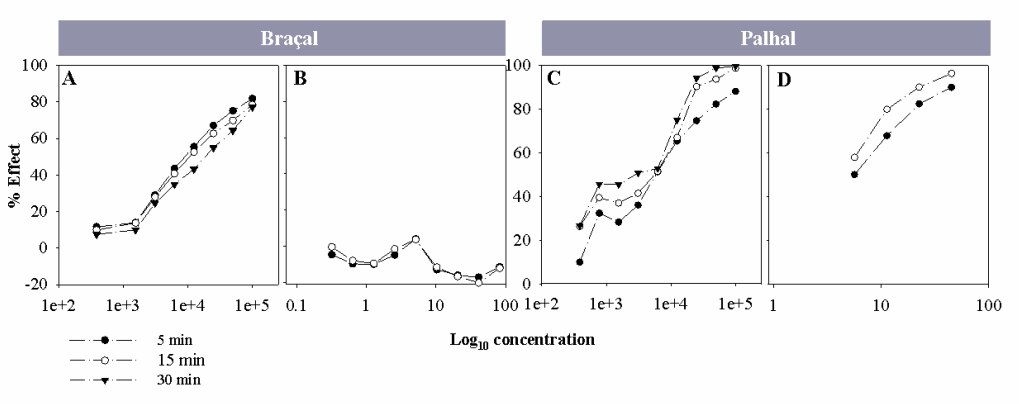

Figure 2. *Vibrio fischeri* luminescence inhibition following exposure to sediments collected at Braçal and Palhal mines (A, C – solid-phase assay) and corresponding elutriates (B, D – liquid-phase assay), expressed as $\log_{10}$ of the concentration tested (mg L^{-1} or % for the solid- and liquid-phase assays, respectively).

The responses of *P. subcapitata* and *L. minor* to Braçal and Palhal elutriates are depicted in Figure 3. Despite yield and growth rate of both species were generally inhibited by elutriates from both Braçal and Palhal, the latter were clearly of higher toxicity. In fact, Braçal showed no appreciable toxicity to *L. minor* regardless the endpoint considered, and slight stimulation of growth was observed at intermediate dilutions of the elutriate (see Yield in Figure 3 B - *L. minor*). *P. subcapitata* generally showed higher sensitivity to both elutriates than *L. minor*, being this trend particularly evidenced when comparing corresponding EC_{50} values (Table 3).

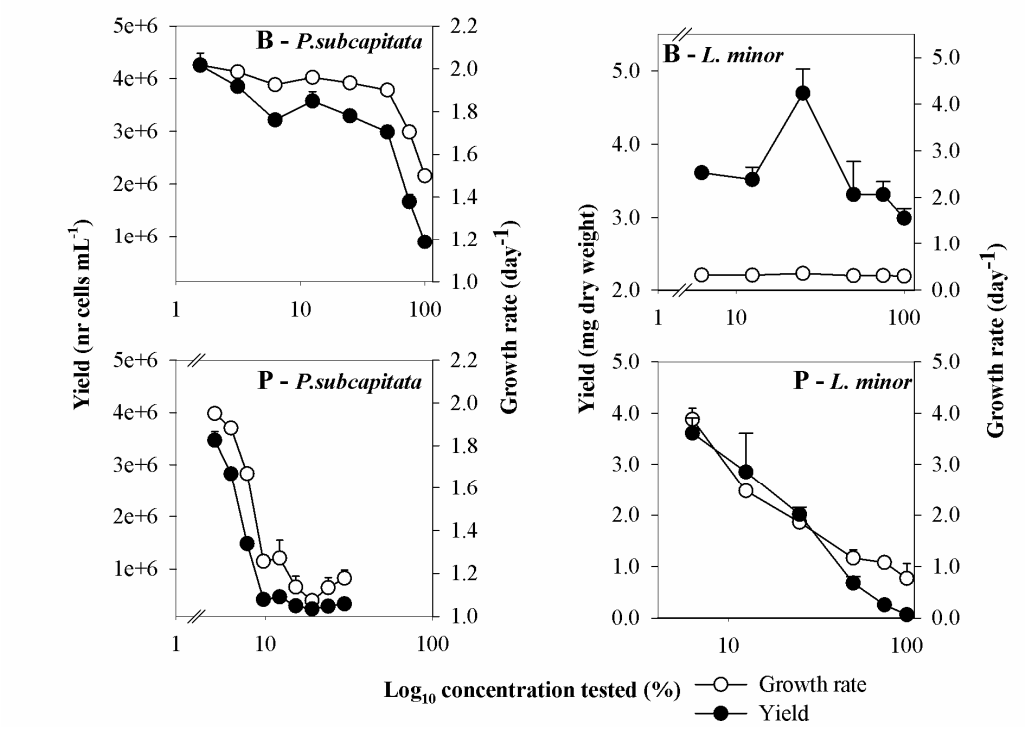

Figure 3. Growth output of *Pseudokirchneriella subcapitata* and *Lemna minor* following exposure to Braçal (B) and Palhal (P) elutriates. Changes in yield (nr cells ml^{-1} or mg dry weight) and growth rate (day^{-1}) are provided for both species as a function of increasing elutriate concentrations (log_{10} %) and error bars indicate standard error.

In line with previous findings for the non-target bacteria and producers Braçal did not represent acute or chronic toxicity either to the standard *D. magna* or the indigenous *D. longispina*. Both reproductive endpoints and growth rates of the chronic ecotoxicity tests with Braçal elutriate denoted stimulatory effects driven by increasing elutriate concentrations. On the other hand, Palhal elutriate was very acutely toxic to both daphnids with slightly higher toxicity shown for *D. longispina* (see immobilisation ECx values in Table 3). In chronic exposures of both daphnids to Braçal elutriate, a stimulatory effect was generally recorded (Figure 4), often confirmed by the statistics (Table 4). Somewhat unexpectedly given the acute toxicity records, *D. longispina* was less sensitive to Palhal elutriates than the standard *D. magna*, and significant adverse effects were only noticed in age at first reproduction of *D. longispina* (Figure 4; LOEC value of 0.533). The bioassays were repeated several times with this species to confirm the results and whenever higher concentrations were tested mortality increased remarkably, which resembles a typical all-or-nothing effect. Because our main interest was to address reproductive effects the present results were preferred for discussion here. Palhal elutriate was very toxic to *D. magna*, impairing significantly fecundity and population growth rate,

with LOEC values of 1.58% and 0.635%, respectively (Figure 4; Table 4). Contrarily to the trend shown by the concentration range-dependent LOEC values, the estimated ECx denote fecundity as a more responsive endpoint than the integrated population growth rate (Table 3).

Table 4. Summary table of the one-way analyses of variance applied to the life history responses of the daphnids. Somatic growth rate (SGR), fecundity (total number of offspring), age at first reproduction (AFR), population growth rate (*r*) were analyzed for each site independently.

		Braçal				Palhal			
	Endpoint	df	$Ms_{residual}$	F_{ratio}	P	df	$Ms_{residual}$	F_{ratio}	P
Daphnia magna	SGR	6, 61	$1.85\ E^{-05}$	1.418	0.222	6,60	$1.15\ E^{-05}$	1.124	0.360
	Fecundity	6, 61	111.4	5.816	<0.001	6,61	54.99	4.455	<0.001
	AFR	6, 61	0.528	1.980	0.082	6, 60	0.753	0.985	0.443
	r	6, 63	$1.48\ E^{-05}$	219.5	<0.001	6, 63	$6.44\ E^{-06}$	227.6	<0.001
Daphnia longispina	SGR	6, 43	$6.35E^{-05}$	2.992	0.016	6, 61	$5.28E^{-05}$	0.717	0.638
	Fecundity	6. 63	157.5	3.401	0.006	6, 61	6.451	18.07	<0.001
	AFR	6, 47	0.118	1.814	0.117	6, 61	0.251	2.362	0.041
	r	6, 70	$3.54E^{-05}$	1231	<0.001	6, 63	$3.07E^{-05}$	167.0	<0.001

4.4. Discussion

This study provides a comprehensive ecotoxicological evaluation of sediments collected at the effluent site from two deactivated mines. The sediments are of clear distinction as to basic physical and chemical properties, being Palhal elutriate very acidic and of high conductivity i.e. the one resembling typical acid mine drainage profile.

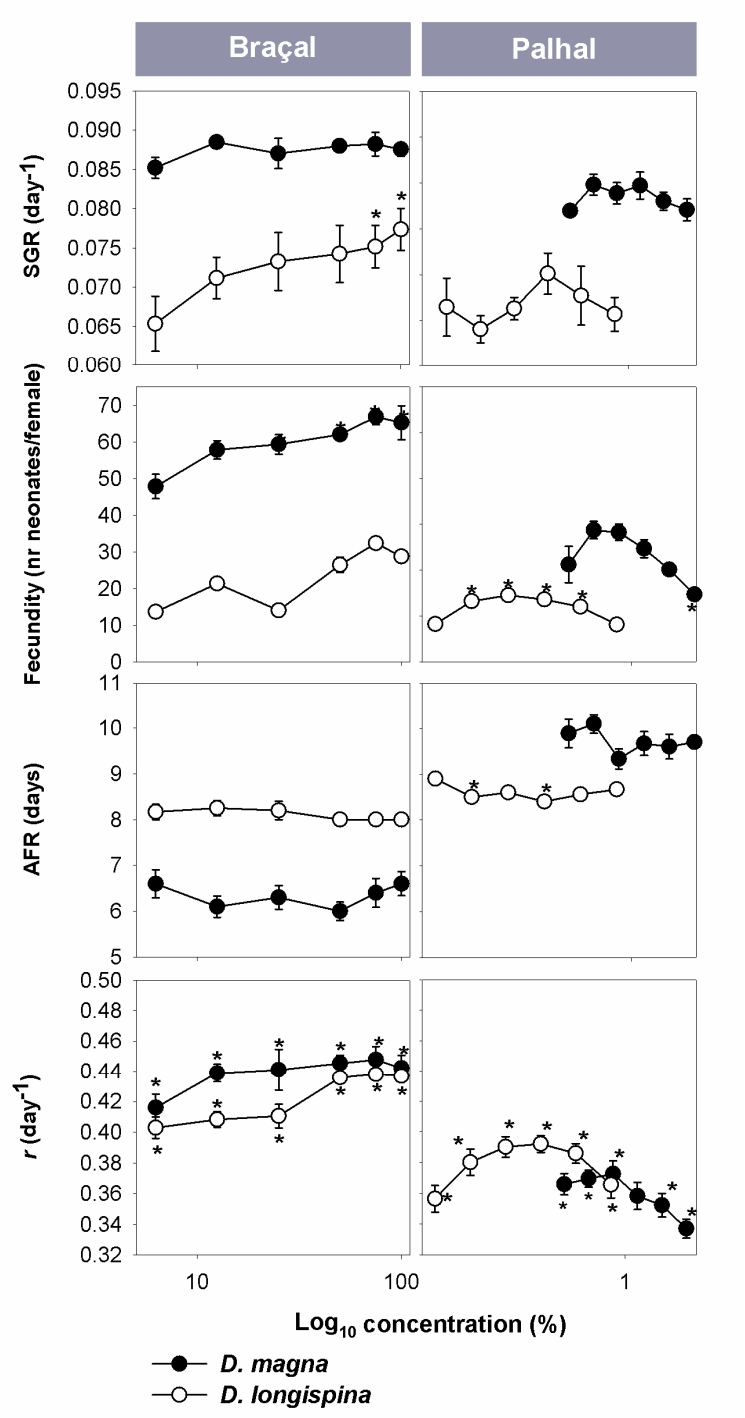

Figure 4. Changes in somatic growth rate (SGR), fecundity (average number of neonates per female), age at first reproduction (AFR) and population growth rate (*r*) are expressed as a function of log $_{10}$ concentration tested**.** Reproductive output of *D. magna* and *D. longispina* exposed to increasing concentrations of Braçal and Palhal elutriates concentration (%) tested. (*) Represent differences between elutriate treatments and the

blank control (One-Way ANOVA followed by Dunnett test; $p < 0.05$) and error bars indicate standard error.

Consistently, Braçal samples showed lower metal content than Palhal samples and such evidence relates to the higher observed ecotoxicity of the latter. Direct comparisons with benchmark values from other countries and studies are generally not straightforward for several reasons. There are no sediment quality criteria values for national freshwater sediments, which constrains comparisons provided that the behaviour of the contaminants and their potential to cause toxic effects depends largely on the properties of sediments (Pereira et al. 2008). Furthermore, large differences in the safety levels for the same metal can generally be identified between references as evidenced in the literature summary of Table 2. Bias in direct comparisons can also relate to methodological details of the extraction step prior quantification, which is not frequently discriminated in the reference documents. Several authors (Ozmen et al. 2004; Peijnenburg and Jager 2003; Pereira et al. 2008) have already proven that low pH increase the desorption of cations and mobilize them to the aqueous phase, which probably motivates the use strong acids or acid combinations in many other studies (Hass and Fine 2010; Liang and Thomson 2010; Tessier et al. 1979) but is likely to overestimate risk whenever environmental assessment is concerned (Pereira et al. 2008). Provided the availability of different extraction methodologies and respective targeted fractions, we followed that showing better adjustment to our aims i.e. that able to mimic natural conditions (using distilled water) and represent mobility and consequent bioavailability of metals in the environment. The texture of the sediments, namely clay relative proportion (which is qualitatively higher in Braçal samples) and the organic matter content promote metal binding thus reducing their bioavailability (Langdon et al. 2001) and consequently lowering their quantification records in the case of this study, where only the liquid-phase of elutriates was analysed.

Even though the above discussed constraints of referring as to general benchmark values for comparative purposes, there are no available alternatives concerning preliminary assessment of risks based on chemical scrutiny. Values obtained for Cu, Zn, Cd and Pb content both in Braçal and Palhal were generally higher than available benchmark values suggesting the hazardous potential of both the sediments to the local biota. Apparently Palhal was the site showing most concerning metal content yet Zn and Pb content in Braçal seems also to be more than one order of magnitude higher than the related references. In spite of the unavailability of benchmark values for Fe, it is worth noticing that Braçal and Palhal seem to deliver high Fe content in nearby river sediments.

The higher ecotoxicity found for Palhal as compared to Braçal samples is likely to reflect the higher metal content, with several metals above regulatory safety levels. Although our experimental evidences support such a link, converse arguments can be found in the literature. Campbell (1995) recognized that the biological uptake and toxicity of metals were poorly correlated with theirs total concentration. In aquatic systems, metals occur under a variety of physico-chemical forms, or species, as free hydrated metal ions and metal complexes with inorganic and organic ligands in dissolved, colloidal or particulate forms (Pickering 1995). The free ion activity model proposed by Campbell (1995) predicts that biological effects are governed by the activity of the free hydrated metal ions, rather than relate to their total concentration, hence understanding metal speciation as crucial for predicting the biological effects of metals (Barata et al. 1998). In fact, poor agreement could generally be found between our species-specific toxicity records and those reported in the literature for single exposures to the concerning metals.

The solid-phase method to test *Vibrio fischeri* luminescence indicated similar toxicity of Braçal and Palhal samples while consistent higher toxicity of the latter was shown by the liquid-phase assay. Ringwood et al. (1997) noticed that high proportion of silt or clay particles in sediment samples promotes the adsorption of bacteria into the sediment thus decreasing suspended bacteria and artificially decreasing luminescence emission i.e. overestimating toxicity. Higher confidence is thus due to the liquid-phase assay for conclusions in this study. Taking into account the metal quantification dataset, e.g. the liquid-phase 15 min-EC_{50} value for Palhal samples translates into Pb, Zn and Cd concentrations of about 0.42, 97.12 and 1.20 µg L^{-1}, respectively. Teodorovic et al. (2009) found conspicuously higher EC_{50} values for these metals using the same *Vibrio fischeri* luminescence endpoint: 35.97, 4.64 and 52.51 mg L^{-1} for Pb, Zn and Cd, respectively.
Similar outcome can be retrieved from comparisons between literature records as to single metal toxicity assays and our results on the toxicity of particular metals within the complex elutriate mixture for other non-target tested species. For *P. subcapitata*, the yield-EC_{50} of Palhal samples translates into Pb, Zn and Cd concentrations of about 0.70, 161.86, 3.33 µg L^{-1}, respectively. Blinova (2004) found higher EC_{50} values for Pb and Cd of 0.05, 0.065 mg L^{-1}, respectively, and Koukal et al. (2003) found a higher EC_{50} value for Zn of 390 µg L^{-1}. Blinova (2004)and Khellaf and Zerdaoui (2009) confirm higher growth-EC50 for when testing single exposures of *L. minor* to Pb, Cd and Zn as compared to our estimated values of about 1.92, 9.15, 444.69 µg L^{-1}. Our results for daphnids were also consistent with this pattern with estimated EC_{50} values lower than the equivalent found for single-metal exposures; see e.g. the immobilisation-EC_{50} of 31.89 µg L^{-1} as compared to

that found by Teodorovic et al. (2009) for exposures of *D. longispina* to Zn. It seems clear that the metal toxicity assessed under a single-chemical exposure scenario provides an underestimation of the actual toxicity of metals as composing complex mixtures, corresponding in this study to elutriates of natural sediment samples. This suggests that metals interact within the mixture following a more than additive or synergistic action (for mixtures toxicity theory see e.g. Jonker et al. 2005). Spehar and Fiandt (1986)got to the same conclusion and argued that for three species studied (rainbow trout, fathead minnows and *Ceriodaphnia dubia*) the water quality criteria concentrations might not be sufficiently protective if the metals tested (Ar, Cd, Cr, Cu, Hg and Pb) were present in the water as mixtures. Furthermore all the chemicals tested in criterion maximum protective concentration caused nearly 100% of mortality in rainbow trout and *Ceriodaphnia dubia* (Spehar and Fiandt 1986). The same study also mentioned that the acute and chronic mixture of the metals tested showed to be strictly additive for the daphnids species tested showing that in long-term the mixture of metals is more severe in daphnids than in fish. These arguments on the unsuitability of single exposures to predict toxicity in natural samples are not straightforward. For example, Dirilgen and Inel (1994) found that duckweed was severely affected by just 2 ppm (mg Kg^{-1}) of Cu and Zn. As eventually expected considering our metal quantification table for Braçal (Cu and Zn below 2 mg Kg^{-1}) and assuming non synergistic (or more than additive) action of the metals within the mixture, no significant *L. minor* growth inhibition was observed; in Palhal, with Zn and Cu content above the 2 mg Kg^{-1}, severe inhibition can be observed in the dry weight and growth rate.

Although sharper effects were noticed following exposures to Palhal elutriate, *P. subcapitata* was negatively affected by Braçal too at concentrations near 100% elutriate. Freshwater microalgae were indeed already shown to be very sensitive to metal exposure (Antunes et al. 2007a; Geis et al. 2000; Janssen et al. 2003; Lewis 1995; Shehata et al. 1999). Considering our specific test battery, *P. subcapitata* seems to be the most protective species when mining effluents with high metal content are concerned. While other authors found duckweed more sensitive than *P. subcapitata* to wastewater Blinova (2004) and herbicides (Fairchild et al. 1997), our results evidence the opposite pattern. Duckweed has been successfully used in wastewater treatment plants to remove contaminants and nutrients (Lewis 1995) which suggests higher degree of tolerance to unspecific toxicants than e.g. microalgae. Two different daphnid species were tested here so that the value of the use of a standard species versus an indigenous species under a retrospective risk assessment point of view could be assessed. *D. longispina* was more

sensitive than D*. magna* in acute exposures to Palhal elutriate, but the opposite was found in the consequent chronic exposures. The higher surface-to-volume ratio of *D. longispina* should result in its higher relative sensitivity as already demonstrated in other studies with diverse chemicals (Lilius et al. 1995) and confirmed in the acute exposures to Palhal elutriate. In chronic exposures, the animals were fed every other day and additional metal uptake is very likely to occur adsorbed to food particles (Declerck et al. 1997). As a large-bodied species, *D. magna* is likely to have higher filtering rates than the smaller *D. longispina* (Tessier et al. 2001); furthermore the former was fed higher food ration in the tests as recommended by the standards. Both the trait and the differential test condition have probably contributed to the observed shift in the tolerance order. Slightly higher sensitivity to the Palhal elutriate was shown by daphnids relatively to bacteria and producers (slightly lower EC_{50} values). This may eventually relate to the additional uptake (via ingestion routes) of metal contaminants adsorbed to the particulate organic matter remaining suspended in the sample after centrifugation.

Sediments at the outflow of Braçal mine exhibit typical thick coat of orange or red ochre (ferric hydroxide) precipitates, which melts and becomes unrecognisable as discharged in the river flow. A different picture is generally reported, where large coats of orange precipitate actually deposit on the streambed, smothering the benthos and completely blocking the light with the consequent disabling of the benthic photosynthesis; this triggers the impoverishing of the biotic community as an indirect effect added to the toxicity of metals themselves (Young 1997). An exponential improvement in quality until a safe asymptote of around 10 mg L^{-1} iron can be expected to be reached following approximately 40 years of the mine shutdown, with these iron levels yet being able to cause significant staining in all but large streams (Young 1997). More than 40 years passed since Braçal shutdown in the 1950s, and our results indicate that its effluent should no longer be hazardous to the biota. Otherwise, although no typical coloration can be identified and more than 40 years had passed over Palhal shutdown high toxicity was denoted from effluent sediments, thus raising concerns on the hazardous potential of this deactivated mining complex. While Braçal was left to flood long time ago to prevent unsafe attempts to explore the ruins, Palhal is apparently not flooded, which should lead to increased oxidation of metal complexes with the consequent release of H^+ ions in water and pH lowering (Young 1997).

This study highlights the usefulness of using a cost-effective ecotoxicological approach to assist the prioritization/scoring of critical areas within river ecosystems potentially impacted by deactivated mines. Two concerning sites were evaluated using a

sensitive ecotoxicological test battery that was found able to clearly distinguish their hazardous potential. Indeed, historical ecological assessment of the Mau river, which will be provided elsewhere, suggests that no negative impacts in the biota are imposed by the effluent from the Braçal mine. On the other hand, our results configure Palhal mine as a priority for further assessment within the scope of the WFD so that the range of the related ecosystem impacts can be fully recognised and adequate recovery plans can be established.

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4.5. References

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Chapter V

Toxicity testing with the benthic diatom *Navicula libonensis* (Bacillariophyceae): optimization of procedures for freshwater stream assessment

Toxicity testing with the benthic diatom *Navicula libonensis* (Bacillariophyceae): optimization of procedures for freshwater stream assessment.

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Abstract

Periphytic communities are good indicators of river quality due to its broad sensitivity to several pollutants. This work's primary intent was to develop and optimize a new ecotoxicological testing methodology using a freshwater benthic diatom. *Navicula libonensis* was selected as a suitable test species due to its ubiquity and good attributes for ease of handling in the laboratory. A first tier of the study addressed the diatom culturing on two different synthetic media: Chu 10, commonly used for diatom growth; and the recipe recommended by OECD. Cultures in Chu 10 performed better compared to those reared in the OECD medium, which is likely to be due to the higher silicon content and lower organic load of the former. As a second tier of the study, the toxicity test focused on *N. libonensis*' growth was successfully developed using potassium dichromate and 3,5 – dichlorophenol as model chemicals. The diatom showed similar sensitivity to both chemicals with median effect concentrations being estimated within the same order of magnitude: 6d-E_rC_{50} of 0.119 mg L^{-1} for potassium dichromate and 4d-E_rC_{50} of 0.799 mg L^{-1} for 3,5 – dichlorophenol. Provided the higher sensitivity of this benthic diatom to standard chemicals as compared to planktonic microalgae, and given the success obtained here in establishing rearing and testing procedures, standardisation of a toxicity test protocol with a benthic diatom such as *N. libonensis* should be seriously considered as a reliable tool for river quality assessment or as part of the ecotoxicological test batteries for Environmental Risk Assessment purposes.

Key-words: microphytobenthos, freshwater benthic diatom, *Navicula libonensis*, toxicity testing.

5.1. Introduction

Freshwater planktonic microalgae are commonly used as test species within the ecotoxicological assessment of several potentially hazardous compounds and environmental risk scenarios. In fact, regulatory entities worldwide recommend algal ecotoxicity tests with standard sensitive planktonic species such as *Pseudokirchneriella subcapitata* within risk assessment frameworks to represent producers of the aquatic food webs (e.g. EC 2002). Microphytobenthos species have not been included in these recommendations (EC 2002; Ivorra et al. 2000) despite their importance as key players in the ecological dynamics of lotic freshwater systems where planktonic species do not have significant expression. For example, in 2006, the STAR Project (European Commission Framework V project EVK1-CT-2001-00089) provided solutions regarding many issues within the Water Framework Directive (WFD), including the use of phytobenthic diatoms as valid bioindicators for water quality evaluation purposes. Brabec and Szoszkiewicz (2006) refer to phytobenthos as an early warning indicator with the capacity of sensing the changes in the water quality of the ecosystem. Diatoms, in general, provide advantages as environmental assessment tools due to their widespread distribution and diversity (Brabec and Szoszkiewicz 2006), and are known for their high discriminatory power in assessment studies regarding acidification, eutrophication, saprobity, nitrogen richness, salinization and flow velocity (Besse-Lototskaya et al. 2006; Johnson et al. 2006).

Despite the increasing importance of benthic organisms for assessment of water quality, from the regulatory point of view, little effort has been put in the standardization of tests with benthic producers (Moreno-Garrido et al. 2003; SETAC 1993). In fact, experiments with freshwater benthic diatoms have been conducted but these address differences in the whole community between two sites or changes occurring following translocation of a community inoculum into a given site of interest (Admiraal et al. 1999; Ivorra et al. 1999). Laboratorial studies addressing the responses of fractions of natural freshwater algal communities that include benthic diatoms can be also found in the literature (Munawar and Munawar 1987), but, until recently, monospecific standardised bioassays with diatoms are generally restricted to planktonic species either regarding marine (Matthiessen et al. 1998) or freshwater (Exley et al. 1993; Fezy et al. 1979; Phlips et al. 1992) systems, thus constraining the direct assessment of sediment toxicity (see Serra et al. 2010 for a very directed bioassay with a freshwater diatom). Moreno-Garrido et al. (2003) made the first attempt to develop and standardise an ecotoxicological test with marine benthic diatoms. Since then, several studies have been published that validate and refine the test or used it as an ecotoxicological tool to address marine

contamination, either regarding in particular the sediment compartment or the water column (Adams and Stauber 2004; Araújo et al. 2009; Araújo et al. 2010; Araújo et al. 2008; Moreno-Garrido et al. 2003; Moreno-Garrido et al. 2007a; Moreno-Garrido et al. 2007b). As to our knowledge, and despite the relevance of considering the sediments compartment when assessing contamination in freshwater bodies particularly when lotic systems are concerned (see above), no test procedures have been adapted so far to establish a standardised ecotoxicological test with freshwater primary benthic producer species. In this way, the present study intended to trigger the development of such testing methodologies. While the planktonic diatom *Navicula pelliculosa* is already recommended as a standard test species by OECD (2011) among other species of green microalgae and cyanobacteria, a benthic *Navicula* species, *Navicula libonensis*, was selected here as a model for the technique development. The selection considered an itemised list of meaningful criteria that would be common to species that could be elected test species in standard protocols. This is a ubiquitous benthic diatom that can be found in Europe (Rimet et al. 2007; Souffreau et al. 2010), including in the Iberian Peninsula and specifically Portugal (de Oliveira 2007; Novais 2011), in North America (Sokal et al. 2008; Wilson et al. 1994), and in South America (Hassan et al. 2006; Seeligmann et al. 2008). Such wide distribution range naturally increases the significance of the test results in the risk assessment of different contamination scenarios. The ubiquity criterion was combined with high foreseen sensitivity to contaminants so that test results could be protective as environmental references. In fact, *N. libonensis* has been classified sensitive to non-point source organic pollution by the widespread Specific Pollution Sensitivity Index (SPI) (Cemagref 1982). Finally, *N. libonensis* represents a good handling compromise in the laboratory due to its size, for example as compared to the smaller *N. pelliculosa*. Indeed, diatoms cultured under high nutrient content, such as that provided by synthetic media, and favourable incubation conditions, such as those established in the laboratory, grow very fast through successive asexual divisions. As a consequence, their overall size decreases progressively: one of the daughter cells gets smaller than the parent cell until a minimal size plateau is reached, where sexual reproduction is activated (not common in the laboratory) and the original size is restored (Hoek et al. 1995).

The specific aims of the present study are two-fold, towards the main intent of establishing the grounds for the development of a suitable ecotoxicological testing protocol with the freshwater benthic diatom *N. libonensis*. First, cultures of the diatom in media with different nutritional supply were followed to characterise the species' growth curve and define adequate test periods. Then, the diatom was exposed to an inorganic

(potassium dichromate) and an organic (3,5-dichlorophenol) reference chemical under standardised conditions [generally following those established by (OECD 2011)], in order to assess the species responsiveness to chemical challenges and its relative sensitivity as compared to e.g. planktonic microalgae.

5.2. Material and Methods

5.2.1 Selection, acquisition and culture of the test species

The culture of the diatom was purchased from the UTEX Culture Collection of Algae (University of Texas at Austin, USA; UTEX LB FD183). Since UTEX does not guarantee the accurate classification of species and strains supplied, microscopic confirmation followed the arrival of the cultures. Diatom identification is mainly based on the structure of the siliceous cell wall, therefore, samples were harvested from the bulk culture and cellular content removed chemically. Samples were added 4ml nitric acid 65% SupraPur and a few crystals of potassium dichromate that was completely dissolved with mechanical stirring using a glass rod. The samples were left for oxidation at room temperature for 24 h and then centrifuged for 5 min at 250 g. Repeated washing with distilled water was carried out until no colour could be observed in the suspension. The supernatant was discarded and the pellet was smeared on the coverslip, which was left to dry out at room temperature. The coverslip was mounted with heated Naphrax® (1.74 refraction index) on a glass microscope slide. These slides were observed under an imaging microscope (Zeiss Axioplan 2) with differential interference contrast and the remaining oxidised material was prepared for observation under a scanning electron microscope (Hitachi, SU-70) operated at 10-20 Kv. Detailed examination of the microscopic slides allowed the classification of the culture as *Navicula libonensis* (M. Potapova, person. communication; Figure 1).

Little information is available on the physiological optima of *Navicula* spp. either in general or for *N. libonensis* in particular (see e.g. Kelly et al. 2005; Spaulding et al. 2010), which required additional efforts to establish the most favourable conditions to grow laboratory cultures which a mandatory requirement to define a test species. Several biphasic soil (supplied in the original culture)-water bulk cultures were established from the initial inoculum and progressively substituted by cultures in full-strength artificial media. At a first stage, the diatoms were cultured in Chu 10 medium (Chu 1942), a rather diluted medium, similar to lake water (Fairburn et al. 1987), which is commonly used for freshwater diatom culturing (Nalewajko and Lean 1972; Suzuki and Takahashi 1995; Tang et al. 1997; Watanabe 2005).

Navicula libonensis
Valves are lanceolate with rounded or very slightly drawn-out ends. The axial area is narrow and linear. The central area is transversely elliptical or irregular-rectangular, often slightly asymmetrical. The proximal raphe ends are straight, slightly expanded. Terminal raphe fissures are hooked towards the secondary side. The lineolate striae are radiate, becoming convergent at the valve apices, 12-13 in 10 µm. The areolae are easily observed under LM, 28-32 in 10 µm (Kelly et al. 2005).

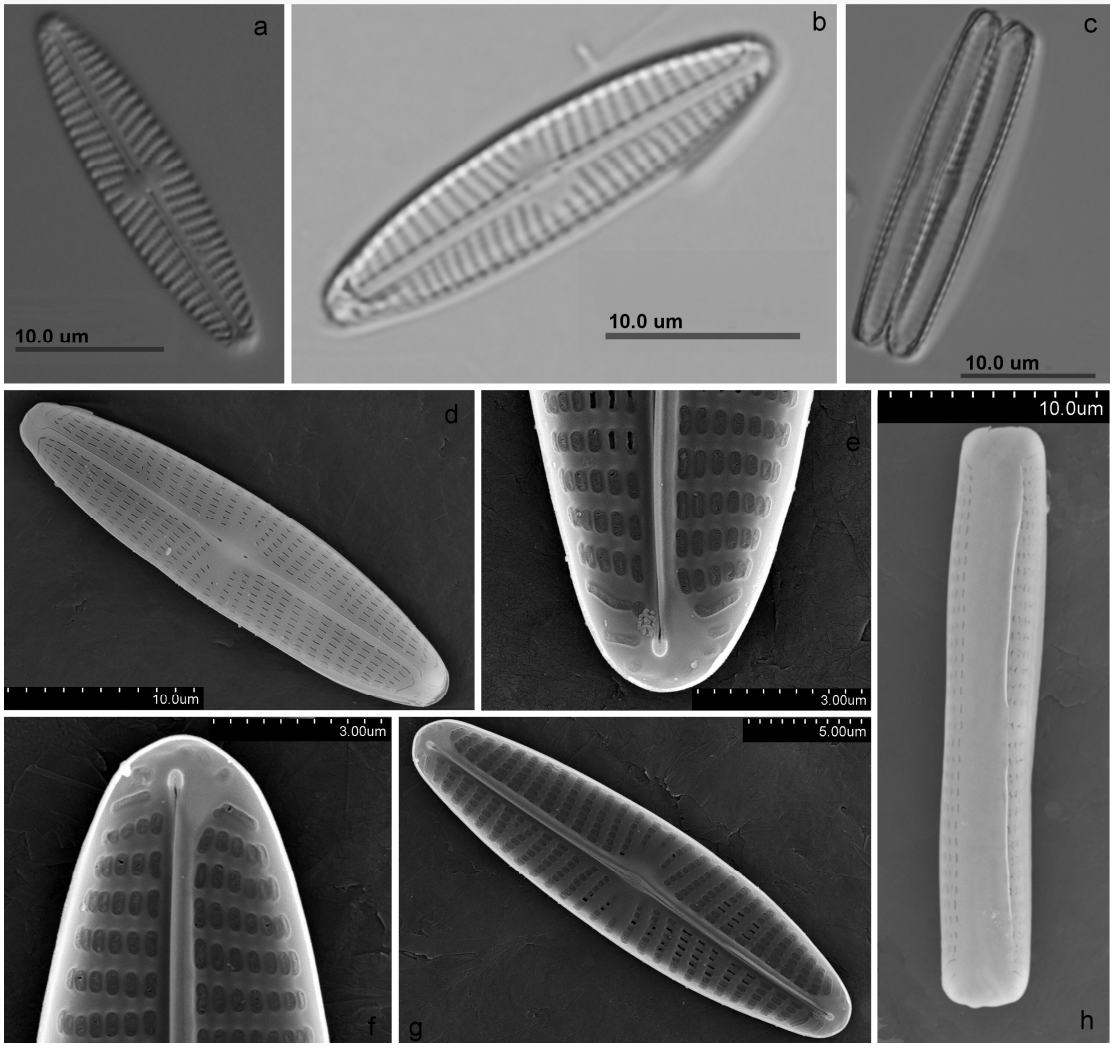

Fig 1. Light microphotographs (a-c) and Scanning Electron microphotographs (d-h) of *Navicula libonensis*. **a b.** Valve views. **c** Girdle view of two diatoms just divided. **d** External view of the valve. **e** Detail of one polar nodule. **f** Detail of the other polar nodule. **g** Internal view of the valve. **h** Girdle view.

A second batch of cultures was maintained in OECD medium as recommended in the OECD guidelines for standard toxicity testing with freshwater organisms (OECD 2011) with the necessary adjustment in silicon concentration; the nutrient supply provided by the OECD medium, in general, is much higher than that provided by the Chu 10 medium hence ensuring that the cultures were given unlimited resources at least in one of the

batches. Assuming that unlimited nutritional supply and high water temperature (~23ºC) could be set in the laboratory to provide optimal conditions for the species growth, we found pH a meaningful variable for culturing success since some *Navicula* species have been registered as acidophilic (see e.g. Kelly et al. 2005). In order to assess whether pH can influence the species growth, the pH of both media (Chu 10 and OECD) (originally above 8) was adjusted in a third and fourth batch of cultures so that slightly acidic conditions (pH below 7 adjusted with HCl 1M) could be provided in the long-term. All cultures were grown and cyclically maintained in 100 ml Erlenmeyer glass vessels filled with 50 ml of sterilized culture medium, at 22 ± 2 ºC and continuous light supply (4440-8880 lux), as recommended by OECD (2011).

5.2.2. Growth curve of *N. libonensis* in Chu 10 and OECD media

Four replicated Erlenmeyer vessels filled with 40 ml of Chu 10 medium (pH = 9.5 as prepared) or OECD medium (pH = 9.0-9.5 as prepared) were inoculated with corresponding diatom cultures (acclimated in Chu 10 and OECD media, respectively) during the exponential growth phase, so that an initial cell density of 10^4 cells ml^{-1} could be set. The inocula used were maintained in the same conditions as the original cultures, at 22 ± 2ºC and continuous light supply. Cell counting of a sample taken from each replicate was done daily using a tubular plankton chamber (Hydro-Bios, Germany) and following previously optimized procedures (Hasle 1978). In brief, the plankton chamber was filled with a harvested sample (1 mL) from a homogenized (by short sonication for 1-1.5 min) inoculum, which was added two drops of lugol to preserve the sample and facilitate diatoms settling for further counting. The chambers were left to allow settling of the biological material in the coverslip and then examined under an inverted microscope (Olympus CKX 41; 200x magnification). Several optical fields were examined and the number of diatoms within each field was recorded until reaching a total counting record of ca. 200 diatoms. Cell density was then calculated taking into account the area of the examined optical fields, and the total area and volume held by the tubular chamber. The growth curve continued to build-up until daily counting revealed the achievement of cellular death phase, generally after the 6^{th} day for cultures in Chu 10 medium and after the 4^{th} day for cultures in OECD medium. The procedures described above were further applied to address the diatom growth curve in Chu 10 and OECD medium with pH adjusted below 7. The pH was monitored, e.g. at the beginning, the end and every other day along the experimental period, both in the assessed cultures and in similar vessels

set in parallel containing only the media. In both cases pH oscillated (generally decreased) less than 1.5 units.

5.2.3. *N. libonensis* toxicity testing optimization and exposure to reference substances

A toxicity test aiming to address growth inhibition of *N. libonensis* was developed and optimized for the use of both Chu10 and OECD media, following general indications provided by the OECD guideline 201 OECD (2011), which considers the use of the planktonic diatom *N. pelicullosa* as a testing organism. Potassium dichromate (Panreac, Barcelona, 99.5%) and 3,5-dichlorophenol (Sigma Aldrich, St Lois, 97%) were used as model chemicals. The growth curves formerly assessed allowed the proper adjustments to the standard experimental design used in growth inhibition tests with microalgae. Test duration was differently established depending on the test medium: six and four days were used in tests run with Chu 10 and OECD medium, respectively. Provided earlier evidences on the slower growth observed in diatoms cultured under pH lower than7, no pH adjustment was carried out for the bioassays and the media were used as prepared (pH = 9.0 – 9.5). Temperature and continuous light intensity were kept as described for cultures and growth curve assessment, and 50 mL glass tubes filled with 10 mL test solution were used as test vessels. Particular attention was given to the use of sterilised material and handling cultures and treatments under aseptic conditions.

Four days before starting the bioassay, inoculum cultures were incubated in each medium. The initial cellular concentration used in the bioassays was aimed to approach 10^4 cells mL^{-1} (as recommended by OECD 2011) following microscopic cell counting. The fulfilment of the second validation criterion required by the OECD (2011) guidelines (mean coefficient of variation for section-by-section specific growth rate in the control below 35%) was verified by daily harvesting and cell counting made on additional sets of replicated controls established at the beginning of the test. *N. libonensis* was exposed in Chu 10 and OECD media to geometric concentration ranges of the standard chemicals potassium dichromate 0.036 - 1.235 mg L^{-1} and 0, 0.020 - 0.212 mg L^{-1}, respectively) and 3,5-dichlorophenol (0.527 - 1.778 mg L^{-1} and 0.156 - 1.185 mg L^{-1}, respectively), in triplicate. A blank control also with three replicates was used in each assay and the stock solutions used to establish the exposure concentrations were freshly prepared before the test. After 6 and 4 days of incubation (for Chu 10 and OECD medium, respectively) at 22 ±2 ºC and under continuous light supply, microscopic cell counting was carried out in each replicate (see above for details on the methodology) to determine final cell density for each

treatment. The results were expressed in terms of the yield (increase in cell density; cells mL^{-1}) and daily growth rate (increase in cell density per day; day^{-1}) delivered per treatment in each bioassay. As ecotoxicological references, the EC_{50}, EC_{20} and EC_{10} and corresponding 95% confidence limits were estimated considering both variables by non-linear regression, using the logistic equation that was fitted to the data through the least squares statistical method.

5.3. Results and Discussion

5.3.1 Growth curve of *N. libonensis*

N. libonensis grown in Chu 10 medium at unchanged pH (Fig. 2 a) showed slow growth for the initial 2 days, a period known as lag phase, followed by a fast growth period known as exponential phase and evidenced by higher day-by-day slopes in yield records within days 2-6. Afterwards, the culture immediately declined and cellular death was observed from day 6 until day 9. Maximum cell density was achieved at the sixth day (~80000 cells mL^{-1}). A mean yield of 68490 ± 5758 SD cells mL^{-1} with a mean daily growth rate of 0.576 ± 0.036 SD day^{-1} was achieved. Distinct pattern was retrieved from cultures grown in OECD medium at unchanged pH (Fig. 2 b). The lag phase lasted for the initial three days and the exponential growth phase was observed only for one day (from the 3rd to the 4th day). From the 5th day onwards a decline in cell number similar to that observed after the 6th day in Chu10 was recorded. These records translate into a mean yield of 49835 ± 1383 SD cells mL^{-1} with a mean daily growth rate of 0.319 ± 0.016 SD day^{-1}. Neither in Chu 10 nor in OECD medium *N. libonensis* evidenced the stationary growth phase after exponential growth, where growth decelerates and a plateau is held for a given time-period before culture declining, as traditionally observed in cultures of microorganisms (Zwietering et al. 1990) and other diatoms (e.g. Jiang et al. 2012). Rather than being an abnormal record, such a particular physiological pattern might be characteristic of the species as grown in the laboratory. Indeed, Hoogenhout and Amesz (1965) showed that the growth pattern observed in growth curves of photosynthetic organisms such as diatoms depends on conditions like nutrients supply, temperature, CO_2 supply and light availability. Impoverishment of media in late cultures under growth-favouring conditions could be hypothesised to explain the observed pattern. However,

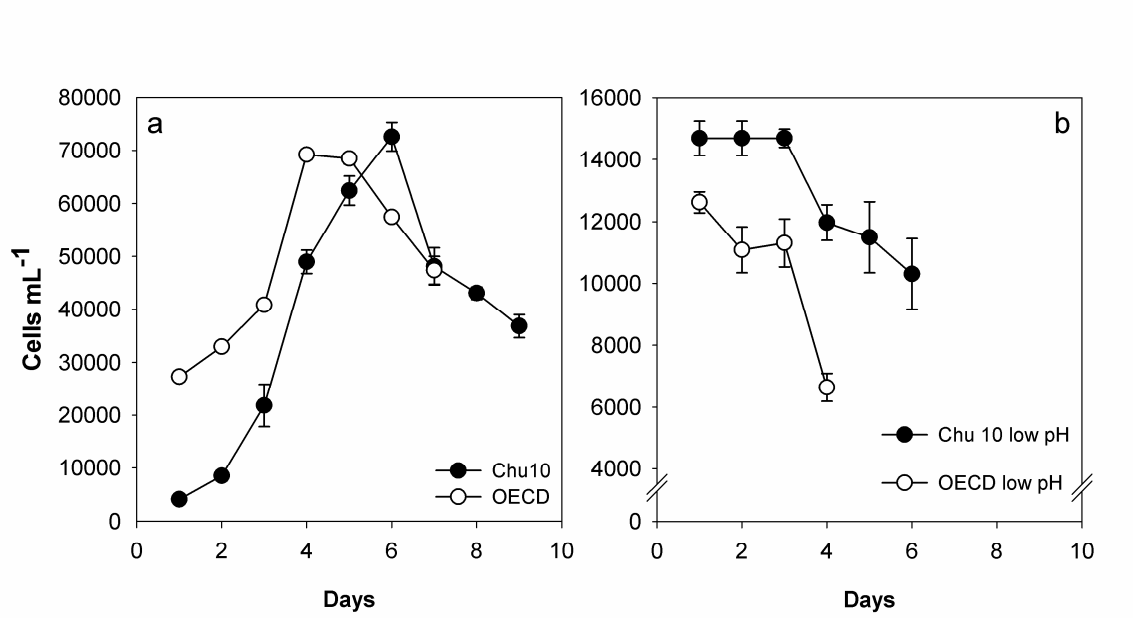

Fig 2**.** Growth curves of the diatom *Navicula libonensis* cultured in Chu 10 and OECD media under controlled laboratory conditions (20º ± 2ºC; continuous light supply). The left hand **a** panel shows the growth of the species in media where no pH adjustment was made while the right-hand **b** panel reports the growth curve of the species in media adjusted to pH below 7. The marks represent means and error bars represent standard deviation regarding four replicates

(i) both media certainly provide unlimited nutrient supply for fast-growing species; and (ii) most microalgae and diatoms with similar or higher requirements have been grown under similar conditions and still evidence a stationary growth phase (Jiang et al. 2012). Therefore, the immediate decline in the culture after the exponential growth could not be clearly explained.

A closer look exclusively at the exponential growth phase of *N. libonensis* confirms lower daily growth rate (doublings per day, K) in OECD medium (0.75 day^{-1}) as compared to that observed in diatoms cultured in Chu10 (1.73 day^{-1}). This suggests that the Chu 10 protocol should be given preference in future studies over the OECD protocol for an optimal test medium for the benthic diatom *N. libonensis*. Although the information on diatom growth rates is scarce in the literature, the Chu 10 K value obtained here can be compared to the records compiled in Hoogenhout and Amesz (1965) denoting its intermediate position within the range of known values for other diatom species: *Stephanodiscus hantzschii*, *Detonula confervacea*, *Navicula minina, Tabellaria flocculosa var. flocculosa* and *Asterionella japonica* registered similar values (K = 1.3-1.7), while

Navicula pelliculosa, *Nitzschia palea*, *Phaeodactylum tricornutum*, *Asterionella formosa*, *Cyclotella nana* and *Skeletonema costatum* double faster (K = 2.0-4.3), and *Cyclotella meneghiniana* (K = 0.34) and *Nitzschia closterium* (K = 0.49) require more time to double their numbers during the exponential phase. It should be noticed that this comparison is certainly constrained by the specific culture conditions used for each species. The initial cell density can also constrain the development of the exponential phase and hence limit comparisons with the above mentioned records for other species. In fact, this is a technical issue that is worth mentioning. While 10^4 cells mL^{-1} was the aimed initial cell density, 4000 and 27000 cells mL^{-1} were actually counted in samples taken at the beginning of the growth curves in Chu10 and OECD medium, respectively. The noticed variability should be due to the mucilage secretion by diatoms for better movement and attachment to sediments and by stalk multilayer's formation (Hoek et al. 1995). This feature makes it hard to obtain homogenous samples even after the best optimization of sonication procedures; the remaining clogs of diatoms, although small, naturally introduce variability into counting. This practical limitation shows that actual microscopic counting rather than using surrogates for cell yield (e.g. spectrophotometric measurements) to correctly estimate initial cell densities is a good practice that should be employed in experiments intended to monitor the growth of benthic diatoms.

Although Chu 10 medium is a rather diluted medium, similar to lake water (Fairburn et al. 1987), it promoted better growth of *N. libonensis* than the generally enriched, but poorer in silicon (1.4 mg Si L^{-1} versus 3.3 mg Si L^{-1} of Chu 10) OECD medium. Such behaviour seems also to indicate the high sensitivity of this particular benthic diatom to the higher organic content of the OECD medium. Sensitivity to organic load is a feature of primary importance in bioindicators used in river quality assessment, as recognised by Dorigo et al. (2010) and Morin et al. (2010) regarding freshwater microphytobenthic communities facing metal and pesticide (organic) contamination. Silicates are the main component of the diatom frustule, being critical in controlling the density of a diatom culture provided that other nutrients are unlimitedly supplied (Lewin 1955); this requirement may also contribute to explain the enhanced growth of *N. libonensis* in Chu 10 medium.

A final note should address the growth curve followed at pH below 7 with both culture media (Figure 2b). *N. libonensis* cell number decreased in both media immediately after initiating the cultures, which reveals that the species is not acidophilic, and rather prefers higher pH ranges within 7-9. This is consistent e.g. with the existent records of *N. libonensis* populations in Portugal that recorded the species in river sites were pH ranges

within 7.1-7.5, and with the typical pH range adopted as optimum for most microalgae cultures (pH = 7-9) (Lebeau and Robert 2003). The preferences of *Navicula* species regarding pH conditions are not straightforward, which justified our option in testing this variable. For example, while *N. angusta* (Kelly et al. 2005) *N. heimansii*, *N. rhyncocephala* and *N. stankovicii* can be found in acidic waters (de Almeida and Gil 2001; Kelly et al. 2005), *N. accomoda*, *N. aquaedurae*, *N. capitata*, *N. decussis*, *N. halophiloides*, *N. lanceolata*, *N. subminuscula* and *N. trivialis* associate preferably with alkaline waters (de Almeida and Gil 2001).

5.3.2. Ecotoxicological sensitivity of *N. libonensis*

Taking the three validation criteria demanded by the OECD guideline 201 (OECD 2011) as a model to follow in the optimization of a standard ecotoxicological test with the freshwater benthic diatom *N. libonensis*, Chu 10 should be elected as the preferable test medium as compared to the OECD medium. The mean coefficient of variation for section-by-section growth rates in the control treatments was lower than 35% as required by the guidelines in both media. However, the biomass (using diatom number as a surrogate measure) increased exponentially by a factor of at least 16 during the established test period and the coefficient of variation of average specific growth rates during the whole test period in the control did not exceed 10% only in Chu 10. Therefore, the response of *N. libonensis* following exposure to standard substances in both media is detailed in figure 3 and table 1 but further discussion will be limited to the results obtained in bioassays performed in Chu 10. In line with the observed by Kusk and Nyholm (1992) on marine diatoms, the growth rate and yield of *N. libonensis* were severely inhibited by both potassium dichromate and 3,5- dichlorophenol denoting the species high sensitivity to the range of concentrations tested. Potassium dichromate is more toxic to the diatom *N. libonensis* than 3,5-dichlorophenol (Figure 3; Table 1) either regarding the more responsive endpoint yield or the less test-specific (Nyholm 1985) but also less responsive growth rate. To our knowledge, there are no studies on the sensitivity of other benthic *Navicula* species to either standard chemicals. As comparison is made with other freshwater microalgae that also tolerate better 3,5-dichlorophenol than potassium dichromate, *N. libonensis* seems to show the highest sensitivity, which indicates that toxicity references taken with this species should be highly protective. EC_{50} values ranging within 0.71-1.38 or 1.79-3.39 were found for standard planktonic microalgae exposed to potassium dichromate or 3,5-dichlorophenol, respectively (Arensberg et al. 1995; Berden-Zrimec et al. 2007; Comber et al. 1995; Mayer et al. 1998; Paixão et al. 2008).

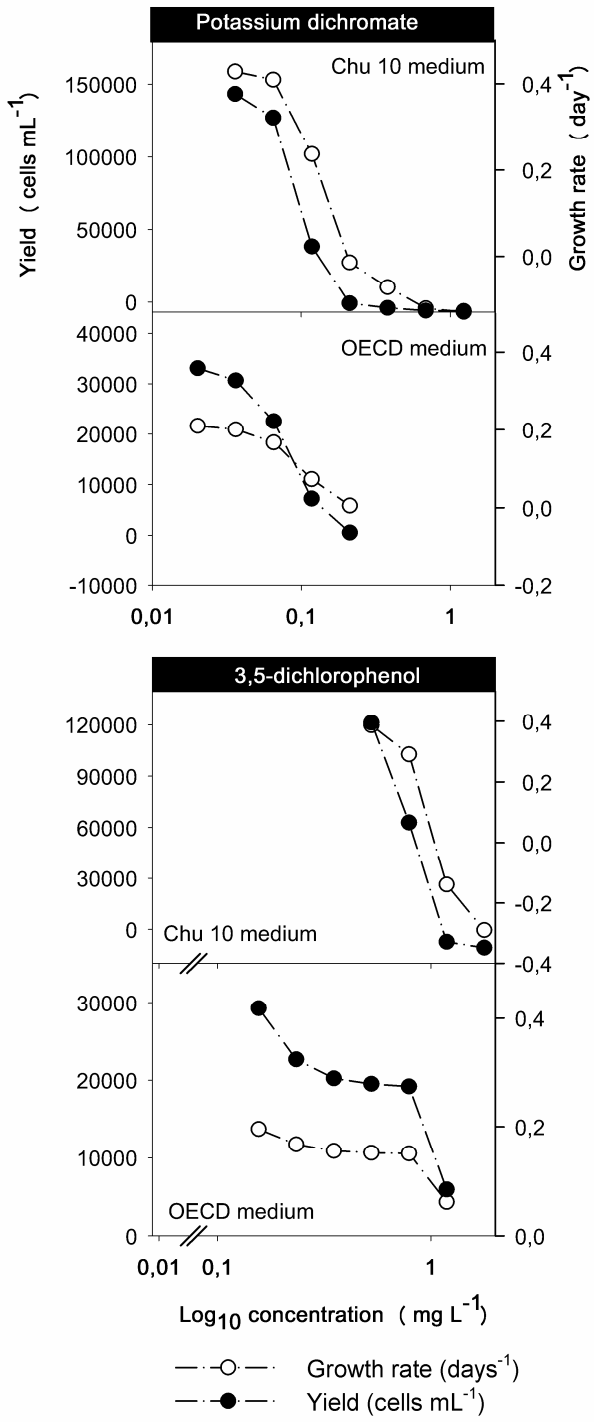

Fig 3. Growth rate (day^{-1}) and yield (cells mL^{-1}) of *Navicula libonensis* following exposure to the standard substances potassium dichromate and 3,5 – dichlorophenol in different culture media (Chu 10 and OECD medium).

Table 1. EC_{50}, EC_{20} and EC_{10} values (mg L^{-1}), and the respective 95% confidence intervals (in brackets), of the standard substances potassium dichromate and 3,5 diclorophenol that were estimated at the end of the exposure periods from the yield and growth rate determined in the bioassays with *N. libonensis* and the standard substances

		$K_2Cr_2O_7$	3,5-dichlorophenol
Chu 10 medium			
	6d E_yC_{50}	0.085 (0.073-0.098)	0.581 (0.521-0.641)
Yield	6d E_yC_{20}	0.058 (0.045-0.072)	0.410 (0.331-0.488)
	6d E_yC_{10}	0.046 (0.032-0.061)	0.334 (0.247-0.420)
	6d E_rC_{50}	0.119 (nd-0.872)	0.799 (0.768-0.830)
Growth Rate	6d E_rC_{20}	0.112 (nd-12.085)	0.777 (nd-1023.055)
	6d E_rC_{10}	0.109 (nd-168.107)	0.771 (0.740-0.802)
OECD medium			
	4d E_yC_{50}	0.080 (0.067-0.092)	0.955 (0.784-1.125)
Yield	4d E_yC_{20}	0.053 (0.040-0.067)	0.759 (0.556-0.961)
	4d E_yC_{10}	0.042 (0.028-0.057)	0.663 (0.428-0.898)
	4d E_rC_{50}	0.097 (0.059-0.134)	1.124 (0.578-1.670)
Growth Rate	4d E_rC_{20}	0.067 (0.023-0.114)	1.013 (nd-2.799)
	4d E_rC_{10}	0.054 (0.007-0.101)	0.948 (nd-3.360)

The mechanism of toxic action of potassium dichromate [Cr (VI)] is only generally established and it is known the metal speciation is responsible for its mobilization and consequently the uptake and its toxicity in photosynthetic organisms consists in yield reduction, leaf and roots effects, inhibition of enzymatic activities and mutagenesis (Shanker et al. 2005). Cervantes et al. (2001) demonstrated that hexavalent chromium severely affects the growth of *Scenedesmus acutus* at concentrations above 15 ppm, and colony growth in *Scenedesmus* and *Selenastrum* at concentrations above 100 ppm, which confirms the higher sensitivity of the benthic diatom *N. libonensis*, assuming that the same mechanism of toxic action operates in both species. Dichlorophenol is known as an inhibitor of respiration, by affecting electron transport in thylakoids and photophospholylation (Berden-Zrimec et al. 2007). It is not readily biodegradable (Zagorc-Koncan et al. 2002) and records log K_{ow} values ranging within 3.62-3.68, indicating potential for bioaccumulation in aquatic organisms.

Two methodological details should be further discussed here. The first regards the counting methodology, which is more time-consuming than that used in other studies where bioassays with diatoms have been developed (e.g. Neubauer haemocytometer Moreno-Garrido et al. 2003). In fact, the tubular plankton chamber and associated counting protocol is more demanding but allows an accurate estimation of cell densities when larger species are being assessed. Alternative techniques should be certainly considered in the future for algae counting in bioassays, such as electronic particle counting and flow cytometry; these will easily be less time-consuming and additionally enable the detection of very low cellular concentrations (Franklin et al. 2002; Moreno-Garrido et al. 2003) but require technology that cannot be always available. A second detail that is worth being mentioned is the potential of this bioassay for adaptation to reduced-size test systems with adaptation to 24-well microplate use (e.g. Geis et al. 2000). This methodology bring some advantages over the regular glass erlenmeyer or glass test tube such as: reducing laboratory resources (time and space) by reducing the sample volume, allowing large number of samples to be tested and generating low volume of waste; avoiding the need to maintain larger cultures to inoculate larger test volumes; and the use of disposable microplate will reduce the risk of contamination by reusing tested vessels (Geis et al. 2000; Paixão et al. 2008). The application of this methodology to our diatoms did not succeed: the test cultures did not grow in control wells, and massive cellular death was observed under the same conditions as used for bulk cultures growth. The coating of the microplates may have interfered with the organisms' mobility function, which is likely to promote the deterioration of the culture via unbalance of increased energy demand for movement with nutrient uptake. Glass labware may constitute an additional source of silicon but this seems to be a controversial argument (see e.g. Lewin 1955). Also, material other than inert glass may release toxic substances into the culture media; in fact, Arensberg et al. (1995) showed that tissue culture vessels treated polystyrene were toxic to the green microalgae *S. capricornutum*. To our knowledge, laboratory experiments with diatoms have always been conducted in glass vessels, suggesting that inert glass should be the most suitable material for the purposes.

This study corroborates the use of benthic diatoms as bioindicators within the scope of the Water Framework Directive regarding river quality assessment. Although further studies are necessary to confirm a general sensitivity of *N. libonensis* to metals and organic contaminants, the species was indeed shown to be very sensitive to the respective standard representatives potassium dichromate and 3,5-dichlorophenol. Furthermore, a step was taken towards the establishment of alternative methodologies to

assess the ecological status of freshwater lotic systems focused on the sediments compartment (see also the rationale by Vidal et al. 2012) through the development of a toxicity test with a sensitive benthic diatom species representative of the microphytobenthic community. Based on the laboratorial results reported here, follow-up research has been conducted in order to develop: (i) testing methodologies that consider sediment rather than water column contaminant spiking, which should involve additional efforts to develop adequate tools to isolate and quantify the diatom cells embedded in the sediment matrix; (ii) higher tier assessment tools with the diatom *N. libonensis*, namely in-situ testing protocols using immobilisation matrices (see e.g. Moreno-Garrido et al. 2005 for similar approaches focusing marine ecosystems).

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Chapter VI

Optimization response of free and immobilized benthic diatom to standard substances

Optimization of growth conditions for laboratory and field assessments using a freshwater benthic diatom

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Em preparação

Abstract

The availability of rapid and effective methodologies for assessing lotic systems with microphytobenthos are still quite scarce, in spite of the requirements set in the Water Framework Directive. Hence, the primary goal of this study was to optimize the growth conditions of the sensitive and ubiquous benthic diatom *Navicula libonensis* for laboratorial and field assessments. The effect of different conditions of temperature (15ºC and 23ºC), photoperiod (24^{L} and 12^{L}:12^{D}), initial cell density (10^{4} and 10^{5} cells mL^{-1}), test duration (6, 9 and 11 days), and cell encapsulation into calcium alginate (1.3 and 1.5%) beads were evaluated in a first set of experiments. There was a slight increase of the growth of free and immobilized cells at 23ºC, at lower initial cell densities and at the shortest experimental period (6 days) in trials run with synthetic medium. Through all the conditions, free cells showed higher, lower or similar growth rates relatively to the immobilized cells. The second experimental trials involved the validation of the selected test conditions (according to the first trials' results), on the ecotoxicological response of *N. libonensis* exposed to two reference chemicals - 3,5-dichlorophenol (DCP) and potassium dichromate (PD). Both chemicals were spiked into a synthetic medium and into a stream water sample from a reference Portuguese stream. In these tests, the variation of temperature and photoperiod did not seem to influence *N. libonensis* sensitivity to the chemicals. A similar response of free and immobilized cells was observed between exposures to the spiked stream water and synthetic medium. Indeed, the sensitivity of free and immobilized cells was overall similar through the treatments tested. This outcome brings up to discussion that *N. libonensis* may provide reliable responses for *in situ*

assessments. Nevertheless, other experimental approaches are yet to be done such as those that involve the testing of sediments as to provide a comprehensive assessment of the quality of lotic systems.

Key-words: *Navicula libonensis*, free versus immobilized cells, environmental factors, 3,5-dichlorophenol, potassium dichromate, microphytobenthos, toxicity tests

6.1. Introduction

In lotic freshwater ecosystems the microphytobenthos communities play an important role as they are in the basis of the trophic chain, are biostabilizers of sediments and regulate benthic-pelagic nutrient cycling (Poulíčková et al. 2008). Among benthic microalgae, diatoms are the most used organisms as indicators of stream quality due to their ubiquity and sensitivity, as well as because their variability spans over most ecological conditions of the aquatic environment (Feio et al. 2009). As a result, worldwide water quality monitoring programs included diatoms as standard bioindicators (Brabec and Szoszkiewicz 2006) and such an option is followed by regulatory legislation, e.g. the European Water Framework Directive (Directive 2000/60/EC 2000). Although international test standards targeting producers of the aquatic food-web are available, the focus is given to macrophytes and planktonic freshwater microalgae, including the planktonic diatom *Navicula pelliculosa* (ISO8692 1989; OECD 2011; USEPA 2002). Notwithstanding, much less interest has been paid to the species that compose the microphytobenthos and the development of methodologies for its use in ecotoxicological testing (Araújo et al. 2010; Moreno-Garrido et al. 2003; SETAC 1993). In fact, planktonic microalgae show high sensitivity to toxicants, often being more sensitive than other planktonic organisms (e.g. Marques et al. 2011; Pereira et al. 2009). In lotic systems, however, the prevalence of planktonic microalgae is negligible, strengthening the need to include sensitive and ecologically relevant benthic microalgae in test batteries for the ecotoxicological assessment of water column and sediment, either in the laboratory or under field conditions as highlighted by (Moreira-Santos et al. 2005).

Under this rationale, the benthic diatom species, *Navicula libonensis*, was herein selected as a potential model species for the development of toxicity testing methodologies, considering several meaningful criteria. This is a ubiquitous benthic diatom that can be found in Europe (Rimet et al. 2007; Souffreau et al. 2010), including in the Iberian Peninsula and specifically in Portugal (de Oliveira 2007; Novais 2011), in North America (Sokal et al. 2008; Wilson et al. 1994), and in South America (Hassan et al. 2006; Seeligmann et al. 2008). From an environmental risk assessment perspective, such wide distribution naturally increases the ecological significance of the results yield in standard laboratorial tests and the suitability of *in situ* assays. Furthermore, *N. libonensis* was classified as sensitive to non-point source organic pollution by the Specific Pollution Sensitivity Index (SPI) (Cemagref 1982), and former studies indicate that the species is significantly more sensitive to the reference chemicals potassium dichromate and dichlorophenol than other microalgae (in some cases the ECx values are one order of

magnitude lower; Vidal et al., submitted). *N. libonensis* also provides a good handling compromise in the laboratory due to its relative larger size as compared to other diatoms.

Laboratorial test procedures for the culturing and toxicity testing with *N. libonensis* were already optimized in a former study (Vidal et al., submitted), but only free cells were considered so far. The immobilization of the diatoms in encapsulation matrices offers many advantages over the use of free cells, especially for *in situ* toxicity assessment as stated by Araújo et al. (2010). The encapsulation of microalgae has been successfully used in toxicity tests developed for application in freshwater (Moreira-Santos et al. 2002; Moreira-Santos et al. 2004a), estuarine and marine ecosystems (Moreno-Garrido et al. 2005; Moreno-Garrido et al. 2003; Moreno-Garrido et al. 2007).

The main aim of this work was the optimization of the conditions for conducting sensitive and cost-effective algal growth bioassays suitable for laboratory and *in situ* assessments, using the freshwater benthic diatom *N. libonensis*. As such, the effect of different conditions on *N. libonensis* growth was assessed by testing: i) different incubation temperatures and photoperiods, which were set considering standard laboratorial conditions and field scenarios; ii) different initial cell density, which was already proven to affect the test outcome in other species (Moreira-Santos et al. 2002; Moreno-Garrido 2008); iii) cell immobilization in matrices with distinct alginate percentages, thus addressing the effect of alginate concentration in exposure and encapsulation efficiency, versus free cells; iv) different test periods, hence evaluating whether longer periods - generally required in tests run in the field due to variation in photoperiod and temperature - suit the species physiology and/or allow better detection of effects. The ecotoxicological response of *N. libonensis* under these conditions was still validated with the testing of reference chemicals in artificial medium and in natural stream water (sample taken from a reference stream in Luso, Portugal; Silva 2008).

6.2. Material and Methods

6.2.1. Test organism

Navicula libonensis (size: length range of 27-35 µm, width range of 5.9-7.0 µm and description; Spaulding et al. 2010) was purchased from the UTEX Culture Collection of Algae (University of Texas at Austin, USA; UTEX LB FD183). The cultures were maintained in 100 ml-Erlenmeyer vessels containing 40 ml of Chu 10 medium (Chu 1942), at 20 ± 2ºC and continuous light supply (4440 – 8880 lux, using cool white lamps). The exponential growth phase of cells starts at the 4th day and the decline phase at the 9th day

according to Vidal et al. (submitted), and inoculation of fresh medium was done during the exponential phase of previous cultures.

6.2.2. First trial – optimization of growth conditions

6.2.2.1. Cell immobilization

The effect of cell immobilization in the growth of *N. libonensis* was assessed by comparing its growth in cultures of free and encapsulated cells. Algal cells were immobilized in beads of calcium alginate 1.3% and 1.5% (w/v) concentrated, following the protocol suggested by Moreira-Santos et al. (2002) and Bozeman et al. (1989). Solutions of 1.3% and 1.5% (w/v) sodium alginate (CAS no.: 9005-38-3) were prepared with sterilized distilled water. Since the initial cell density is critical for the viability of immobilized cells because of nutrient availability, carbon dioxide diffusion and light penetration (Moreira-Santos et al. 2002), we tested two initial cell densities, 10^4 and 10^5 cells mL^{-1}. They were established considering previous studies (e.g. Moreira-Santos et al. 2002; Moreno-Garrido et al. 2003) and the requirements of the OECD guideline for toxicity testing with microalgae (OECD 2011). Following previous optimization procedures, an aliquot of an exponentially growing culture, concentrated by gravity (not by centrifugation), was added to each alginate solution (1.3% and 1.5%) to obtain an alginate-cell suspension with *ca.* 10^4 cells mL^{-1} and *ca.* 10^5 cells mL^{-1}. Beads were then formed by dropwise (using a sterilized needle coupled to a 20-mL syringe) of each alginate-cell suspension into a 2% (w/v) calcium chloride solution. Beads were gently stirred in the $CaCl_2$ solution for approximately 45 min for gel hardening. Afterwards beads were washed with distilled water and stored in dark at 4 °C (in 20x diluted Chu 10 medium). The beads presented a mean ± SD diameter of 3.14 ± 0.07 mm. Cell counting at the beginning and end of each test was carried out after disaggregating beads (in a total of three replicates) in 1 mL of trisodium citrate solution [3% (w/v); CAS no. 6132-04-3] upon smooth shaking. The countings were made in an inverted microscope (Olympus CKX41) using a tubular plankton chamber (Hydro-Bios, Germany) as described by Vidal et al. (submitted).

6.2.2.2. Experimental design and testing conditions

Experiments at different initial cell density and at different incubation conditions (combinations of two temperatures and two photoperiod regimes) were run independently, following a bifactorial design that considered cell immobilization and exposure period as

factors affecting the diatom growth. Table 1 provides an overview of the experimental design, clarifying the treatments set within each experiment. The selected incubation conditions intended to represent the standard conditions described in the guidelines for testing with standard freshwater microalga (OECD 2011) versus field conditions in European temperate regions, including Portugal. The temperature chosen to represent field conditions was based on the average annual water temperatures for Portuguese streams (ca. 15ºC) (INAG 2008).

Table 1. Representation of the experimental design followed to address the effect of different test conditions on the growth of *N. libonensis*. Experimental conditions defining independent trials are clarified as well as the factors involved within each experiment. T6, T9, T11 stand for experimental periods of 6-, 9- and 11-days, respectively.

Experimental conditions			**Factors and factor levels**
Initial cell density	**Temperature**	**Photoperiod**	
10^4 cells mL^{-1}	15ºC	24^L	T6, T9, T11 *vs* free cells, 1.3% or 1.5% alginate beads
		12^L:12^D	T6, T9, T11 *vs* free cells, 1.3% or 1.5% alginate beads
	23ºC	24^L	T6, T9 *vs* free cells, 1.3% or 1.5% alginate beads
		12^L:12^D	T6, T9 *vs* free cells, 1.3% or 1.5% alginate beads
10^5 cells mL^{-1}	15ºC	24^L	T6, T9, T11 *vs* free cells, 1.3% or 1.5% alginate beads
		12^L:12^D	T6, T9, T11 *vs* free cells, 1.3% or 1.5% alginate beads
	23ºC	24^L	T6, T9 *vs* free cells, 1.3% or 1.5% alginate beads
		12^L:12^D	T6, T9 *vs* free cells, 1.3% or 1.5% alginate beads

All experiments were conducted in sterile 50-ml glass test tubes containing 10 ml of Chu 10. Three replicates were considered per test condition. The tubes were covered with perfurated Parafilm®. At the end of the test, the whole suspension was sonicated and agitated in a vortex in order to collect a homogenised sample of 1 mL, which was preserved with 100 µL of a Lugol's solution 3.4% (w/v) of iodine until further cell counting. The growth rates of free and immobilized cells of *N. libonensis* were determined on the basis of the cell density as previously described (cf. sub-section 6.2.2.1). The initial cell density, % of alginate and exposure period delivering higher growth rates within each combination of temperature and photoperiod were selected to the second trial of the study.

6.2.3. Second trial – suitability of cell immobilization to assess the toxicity of chemical substances

The sensitivity of free and immobilized cells of *N. libonensis* to an organic (3,5-dichlorophenol, DCP) and a metallic (potassium dichromate, PD) reference compound was tested under different exposure conditions. The reference compounds were tested at their 6 d - EC_{50} values, according to the data obtained for this species in previous studies (Vidal et al., submitted). In order to evaluate the efficiency of the test apparatus and species to respond in the field, the tests were run under the same combinations of experimental conditions as set in the first test trial, but the chemical spiking was done in a natural stream water sample besides Chu 10. The water sample was collected in a pristine mountain stream (Luso - Northern Portugal). The water samples were characterized by the measurement of different parameters: conductivity, pH, total suspended solids, and dissolved oxygen level, biological oxygen demand (BOD_5) (APHA 1995) and phaeophytin-corrected chlorophyll-*a* (chl-*a*) (Lorenzen 1967). The vacuum filtered sample (1.5 µm mesh pore size) was used for the colorimetric quantification of nitrites (NO_2^-), nitrates (NO_3^-), ammonia (NH_4), and orthophosphates (PO_4^{3-}) (APHA 1995). Ions and metals Mg^{2+}, Ca^{2+}, Si^{2+}, K^+ and Fe^{2+} were analyzed through inductively coupled plasma mass spectrometry (ICP-MS) to verify whether their content was discrepant or not from the levels present in the artificial medium Chu10 (APHA 1995). Prior to the beginning of the test with the diatom, the original water sample was filtered through 0.45 µm mesh pore size filters (USEPA 2002). An extra treatment of stream water enriched with nutrients (at the same ratio used in Chu 10) was considered to prevent nutrient deficiency effects (USEPA 2002). In summary, nine treatments were considered in each bioassay: i) blank Chu 10 medium (Chu10); ii) DCP EC_{50} in Chu 10 (Chu10+DCP); iii) PD EC_{50} in Chu 10 (Chu10+PD); iv) Blank stream water (SW); v) DCP EC_{50} in stream water (SW+DCP); vi) PD EC_{50} in stream water (SW+PD); vii) Nutrient-spiked blank stream water (SW+N); v) DCP EC_{50} in nutrient-spiked stream water (SW+N+DCP); vi) PD EC_{50} in nutrient-spiked stream water (SW+N+PD). Cell immobilization was the second factor considered within each bioassay with two levels set: free cells and immobilized cells in 1.3% alginate beads. Following previous optimization (see above), the bioassays were carried out for 6 days, starting from an initial cell density of 10^4 cells mL^{-1} (cf. section 6.3).

6.2.4. Data analysis

Regarding the first set of experiments, the influence of cell immobilization and test duration on the growth rates of *N. libonensis* was statistically analysed through a two-way analysis of variance (two-way ANOVA) run over the dataset of each independent experiment. When no significant interaction was found, the simple main effects of each factor were then scrutinized by one-way ANOVA followed by the Tukey multiple comparison test. Whenever a significant interaction was found, the $MS_{residual}$ of the two-way ANOVA was used as the denominator for calculating the F statistics of the one-way ANOVA over each factor and the q statistics for the Tukey multiple comparison tests (Quinn and Keough 2002).

In the second set of bioassays, the growth rate obtained in each treatment was expressed as a ratio of the respective controls (either free or immobilized cells exposed to Chu 10 medium) within each combination of temperature and photoperiod. The significant effects of cell immobilization and chemical spiked in different media on the diatom growth were assessed using the same approach as employed for the first trials. A significance level (α) of 0.05 was used in all analyses.

6.3. Results and discussion

As a way to meet some evaluation requirements set in the WFD, it is worth investing research efforts in developing new strategies (e.g. using the responses of sensitive organisms) that could provide a valuable assessment under laboratorial and field conditions, by reliably responding to different environmental factors and contaminants. Thereby, this work gathers relevant data concerning the optimization of the growth of the benthic diatom *N. libonensis* for field and laboratorial assessments. This species was never used as a test organism despite its sensitivity to certain contaminants (Vidal et al. submitted).

6.3.1. First trials – optimization of growth conditions

The results of the first set of experiments are shown in Fig. 1. The growth rates of *N. libonensis* were generally higher at 23ºC, although a similar outcome was obtained under 15ºC at a 12^{L}:12^{D} photoperiod, particularly for experiments with initial cell density of 10^4 cells mL^{-1}. A clear response pattern, however, could not be retrieved as different photoperiods were tested (Fig. 1) – for example, if at 23ºC a full light cycle seems to produce better growth than a 12^{L}:12^{D} cycle, at 15ºC the opposite seems to occur. Indeed,

(Mayer et al. 1998) observed a more conspicuous effect of temperature variations on the growth of *Selenastrum capricornutum* than that provoked by light intensity, nitrogen source or pH. Similarly, Lewis et al. (2002) verified improved growth rates of the diatom *Achnanthes longipes* (free cells) as temperature increased from 10ºC up to 26ºC, as well as, Faafeng et al. (1994) obtained higher growth rates of immobilized, and particularly of free cells of *S. capricornutum* at 20ºC compared to 10ºC. Another study concluded that changes in the temperature and photoperiod regimes from field to standard laboratorial experiments significantly influenced the growth of the immobilized marine diatom *Phaeodactylum tricornutum* (Moreira-Santos et al. 2002). The raise of temperature and photoperiod tends to boost the metabolic rate of the microalgae within a certain optimal range, hence leading to the yield of higher biomass levels (Khoyi et al. 2009; Qian et al. 2010).

For both initial cell densities analysed in this study a 16 fold increase on the growth of free or immobilized cells - a validation criterion set in standard procedures - was never attained. Considering that we are dealing with a benthic diatom, there are some constraints yet to be surpassed in what concerns the handling and resuspension of the cells from the mucilaginous aggregates that they form, in order to allow a consistent control of the procedure and achievement the initial densities pretended. In fact, the yields of microalgae at the end of the test trials are greatly influenced by the initial cell density, especially if encapsulated cells are considered (Moreno-Garrido 2008). The tested initial density of 10^4 cells mL^{-1} rather than the higher density tested of 10^5 cells mL^{-1} had apparently driven to improved growth rates of free and immobilized cells. For this reason, the former was selected as the initial cell density for the second test trials with *N. libonensis*. Although it is extensively documented that higher initial cell densities of microalgae usually lead to higher growth rates at the end of an exposure period (e.g. Moreira-Santos et al. 2004b; Moreno-Garrido 2008) found the opposite trend for *C. vulgaris* encapsulated in calcium alginate beads. Similarly, a previous study demonstrated that the cell division of immobilized *P. tricornutum* was accelerated when the initial number of cells per bead was lower (Moreira-Santos et al. 2002). The authors still found that the final growth rates only differed by ≤ 8% among the upper and lower initial cell densities tested. It is commonly accepted that very high densities of encapsulated microalgae may limit the diffusion of light, nutrients and carbon dioxide (Moreira et al. 2006). This is particularly critical if the test organism has a considerable cell size, as it is the case of *N. libonensis* (cf. sub-topic 6.2.2.1).

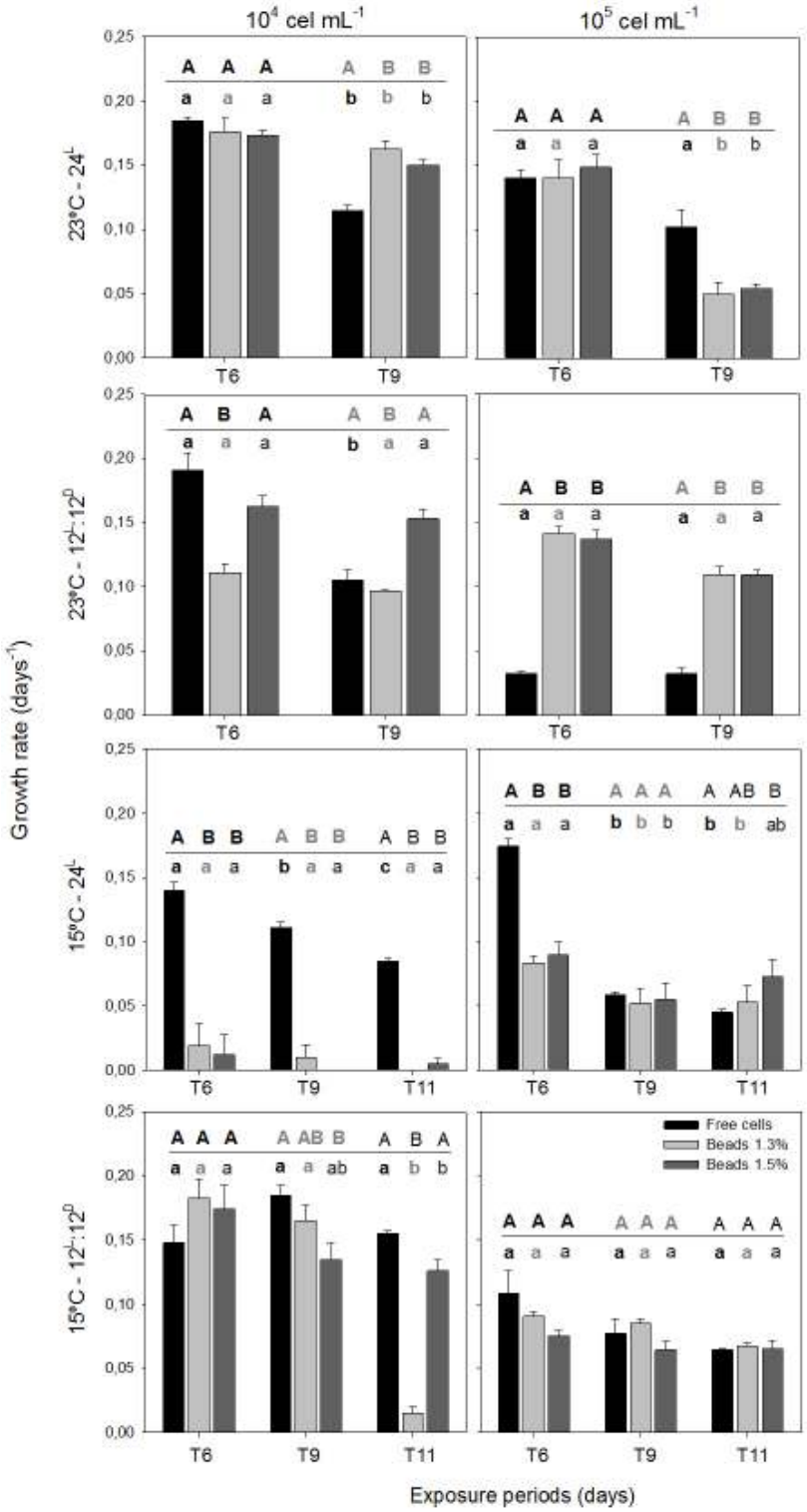

Fig. 1. Growth rates of free and encapsulated (at 1.3 and 1.5% alginate concentration) cells of *N. libonensis* under different experimental conditions of temperature (15 and 23ºC), photoperiod (24^{L} and 12^{L}:12^{D}) and initial cell density (10^4 and 10^5 cells mL^{-1}). Error bars represent standard errors. Upper-case letters indicate significant differences (Tukey test; P<0.05) between the responses of free and immobilized cells within each

experimental period of 6 (T6; black bold letters), 9 (T9; grey bold letters), and 11 (T11; black letters). Lower-case letters indicate significant differences (Tukey test; $P<0.05$) between the diatom response to different experimental periods as the growth of free cells (black bold letters), cells encapsulated at 1.3% alginate (grey bold letters), and cell encapsulated at 1.5% alginate (black letters) were assessed.

The testing of each combination of temperature, photoperiod and cell density conditions led to the overall conclusion that cell encapsulation and test duration influenced significantly the growth rate of *N. libonensis* (Tables 2, Fig. 1). Moreover, a significant interaction between these factors was frequently detected (Table 2). The achievement of high cell densities when microalgae are subjected to temperature and light variations under field conditions usually demands longer test periods (Moreira-Santos et al. 2004b). Notwithstanding, the growth rates of *N. libonensis* were kept at similar levels or decreased as the assessment period enlarged (from 6 to 9 or 11 days), either for free or immobilized cells, irrespectively of the experimental conditions assessed (Fig. 1, Table 2-3). Bearing on this outcome a test duration of 6 days was set for the second stage trials, this being also in agreement with the growth curve previously determined for the species under standard conditions (i.e., 22±2ºC and 24^{L}, Vidal et al. submitted).

Comparing the growth rates of free vs. immobilized cells, it can be concluded that the growth of *N. libonensis* as free cells was either not significantly different, or was significantly higher or lower than that observed in beads (of 1.3 and/or 1.5% alginate) (Fig. 1, Table 4). The growth profiles of free versus encapsulated cells documented in other studies under different incubation conditions are quite variable as well. Previous works with chlorophyta, cyanobacteria and diatom microalgae, either from freshwater or estuarine/marine environments, demonstrated higher (e.g. Faafeng et al. 1994; Moreira-Santos et al. 2004b; Moreno-Garrido et al. 2007), similar or lower (e.g. Mallick 2002; Rai and Mallick 1992; Twist et al. 1997) growth rates for free cells relatively to the encapsulated cells. Several factors were pointed out as possible explanations to this variation in the responses (Moreno-Garrido 2008). Specifically, Hoogenhout and Amesz (1965) concluded that the culturing conditions can modulate largely the responses of these organisms, possibly by acting through their physiological condition. Indeed, contrary to our expectations, under 15ºC a considerable growth of encapsulated diatoms was noticed (10^4 cells mL^{-1} at 12^{L}:12^{D}, 10^5 cells mL^{-1} for both photoperiods), which was frequently statistically similar to the growth of free cells (Fig. 1, Table 4). Regarding the two percentages of alginate used, 1.3% of alginate allowed, in general, slightly higher growth rates, and that was the reason for using this alginate concentration rather than

1.5% in the second experimental trials (Fig. 1). In fact, tightened matrices resulting from a higher percentage of alginate (Gombotz and Wee 1998) may limit the uptake of resources by the diatom hence constraining its growth. In any case, the matrix of encapsulation neither was toxic for the diatom nor affected its morphological integrity, and even promoted the growth of *N. libonensis*, seemingly to act as a protective barrier under lower temperatures. Different studies brought up to discussion the influence of temperature and light intensity on the production, quality, quantity and biological activity of carbohydrate-rich exopolymeric substances (EPS) by benthic diatoms (e.g. Lam et al. 2005). Wolfstein and Stal (2002) observed a reduced production of EPS by *Cylindrotheca closterium* under lower temperatures and irradiances, though it was indirectly affected by the amount of algal cells that could produce EPS. Since the sodium alginate is a polysaccharide, it is likely that the immobilized *N.libonensis* cells, under stress conditions (e.g., low temperature), may take advantage of the alginate matrix to cope with an inhibited ability to produce EPS for their adhesion

Table 2. Two-way ANOVA summary relative to the growth rate of *N. libonensis* at different exposure periods (T6, T9 and T11) and cell encapsulation (i.e., free cells and % alginate in beads), under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom; CD_i – initial cell density.

CD_i (cells mL^{-1})	Test condition	Source of variation	df	$MS_{residual}$	F ratio	*P* value
10^4	23ºC, $24h^L$	Test duration	1	0.006	51.74	**<0.001**
		Free /immobilized	2	6.060 E-4	5.53	**0.020**
		Interaction	2	0.001	12.69	**0.001**
		Residual	12	1.100 E-4	-	-
	23ºC $12H^L$:$12H^D$	Test duration	1	0.006	29.21	**<0.001**
		Free /immobilized	2	0.005	24.35	**<0.001**
		Interaction	2	0.003	13.24	**<0.001**
		Residual	12	2.030 E-4	-	-
	15ºC, $24h^L$	Test duration	2	0.002	17.82	**<0.001**
		Free /immobilized	2	0.037	347.09	**<0.001**
		Interaction	4	1.960 E-3	5.20	**0.006**
		Residual	18	9.420 E-5	-	-
	15ºC, $12h^L$:$12h^D$	Test duration	2	0.013	32.66	**<0.001**
		Free /immobilized	2	0.004	9.74	**0.001**
		Interaction	4	0.008	18.98	**<0.001**
		Residual	18	4.050 E-4	-	-
10^5	23ºC, $24h^L$	Test duration	1	0.025	83.00	**<0.001**
		Free /immobilized	2	0.001	3.67	0.057
		Interaction	2	0.001	4.90	**0.028**
		Residual	12	2.990 E-4	-	-

23ºC $12H^L:12H^D$	Test duration	1	0.002	23.53	**<0.001**
	Free /immobilized	2	0.017	217.89	**<0.001**
	Interaction	2	4.600 E-4	5.90	**0.016**
	Residual	12	7.800 E-5	-	-
15ºC, $24h^L$	Test duration	2	0.011	106.61	**<0.001**
	Free /immobilized	2	0.002	20.49	**<0.001**
	Interaction	4	0.003	30.16	**<0.001**
	Residual	18	1.020 E-4	-	-
15ºC, $12h^L:12h^D$	Test duration	2	0.002	8.08	**0.003**
	Free /immobilized	2	5.840 E-4	3.09	0.070
	Interaction	4	2.870 E-4	1.52	0.239
	Residual	18	1.890 E-4	-	-

Table 3. One-way ANOVA summary regarding the growth rate response of *N. libonensis* to the test duration (T6, T9 and T11) within each level of cell immobilization (i.e., free cells and % alginate in beads), under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom; CD_i – initial cell density.

CD_i (cells mL^{-1})	Test condition	Source of variation	Fixed factor	df	$MS_{residual}$	F ratio	*P* value
10^4	23ºC, 24^L	Test duration	Free cells	1, 4	4.060E-5	67.09	**2.950E-6**
			Beads 1.3%	1, 4	3.950E-4	110.91	**2.041E-7**
			Beads 1.5%	1, 4	1.760E-4	120.91	**1.272E-7**
	23ºC $12^L:12^D$	Test duration	Free cells	1, 4	3.690E-4	53.20	**9.555E-6**
			Beads 1.3%	1, 4	6.210E-5	1.55	0.237
			Beads 1.5%	1, 4	1.770E-4	0.64	0.439
	15ºC, 24^L	Test duration	Free cells	2, 6	6.500E-5	24.20	**7.898E-6**
			Beads 1.3%	2, 6	1.290E-4	2.93	**0.079**
			Beads 1.5%	2, 6	8.900E-5	1.11	0.350
	15ºC, $12^L:12^D$	Test duration	Free cells	2, 6	2.510E-4	2.84	0.085
			Beads 1.3%	2, 6	3.850E-4	62.72	**7.720E-9**
			Beads 1.5%	2, 6	5.790E-4	4.89	**0.020**
10^5	23ºC, 24^L	Test duration	Free cells	1, 4	3.270E-4	7.36	**0.019**
			Beads 1.3%	1, 4	3.950E-4	40.80	**3.465E-5**
			Beads 1.5%	1, 4	1.760E-4	44.48	**2.295E-5**
	23ºC $12^L:12^D$	Test duration	Free cells	1, 4	3.370E-5	2.70E-4	0.987
			Beads 1.3%	1, 4	1.060E-4	20.13	**7.400E-4**
			Beads 1.5%	1, 4	9.410E-5	15.26	**0.002**
	15ºC, 24^L	Test duration	Free cells	2, 6	4.630E-5	149.02	**6.306E-12**
			Beads 1.3%	2, 6	1.070E-4	9.48	**0.002**
			Beads 1.5%	2, 6	1.530E-4	8.99	**0.002**
	15ºC, $12^L:12^D$	Test duration	Free cells	2, 6	4.390E-4	3.51	0.098
			Beads 1.3%	2, 6	3.150E-5	14.09	**0.005**
			Beads 1.5%	2, 6	9.590E-5	1.18	0.369

Table 4. One-way ANOVA summary regarding the growth rate response of the diatom *N. libonensis* towards cell encapsulation (i.e., free cells and % alginate in beads) within each

level of test duration (T6, T9 and T11), under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom; CD_i – initial cell density.

CD_i (cells mL^{-1})	Test condition	Source of variation	Fixed factor	df	$MS_{residual}$	F ratio	*P* value
10^4	23ºC, $24h^L$	Cell encapsulation	T6	2, 6	1.49E-4	0.94	0.419
			T9	2, 6	7.04E-5	1.72	0.221
	23ºC $12H^L$:$12H^D$	Cell encapsulation	T6	2, 6	3.00E-4	23.89	**6.500E-5**
			T9	2, 6	1. 05E-4	13.65	**8.100E-4**
	15ºC, $24h^L$	Cell encapsulation	T6	2, 6	2.19E-4	164.544	**2.71300E-12**
			T9	2, 6	5.27E-5	121.02	**3.649-11**
			T11	2, 6	1.08E-5	70.19	**2.528E-9**
	15ºC, $12h^L$:$12h^D$	Cell encapsulation	T6	2, 6	7.48E-4	2.46	0.113
			T9	2, 6	3.65E-4	4.69	**0.023**
			T11	2, 6	1.01E-4	40.49	**2.200E-7**
10^5	23ºC, $24h^L$	Cell encapsulation	T6	2, 6	3.25E-4	0.22	0.806
			T9	2, 6	2.74E-4	8.36	**0.005**
	23ºC $12H^L$:$12H^D$	Cell encapsulation	T6	2, 6	7.43E-5	147.44	**3.600E-9**
			T9	2, 6	8.16E-5	76.15	**1.500E-7**
	15ºC, $24h^L$	Cell encapsulation	T6	2, 6	8.05E-5	75.88	**1.694E-9**
			T9	2, 6	1.10E-4	0.29	0.751
			T11	2, 6	1.17E-4	4.92	**0.020**
	15ºC, $12h^L$:$12h^D$	Cell encapsulation	T6	2, 6	3.31E-4	2.51	0.161
			T9	2, 6	1.91E-4	1.69	0.261
			T11	2, 6	4.56E-5	0.102	0.905

6.3.2. Second trials – validation of the optimized test procedure

The growth response of *N. libonensis* after exposure to the reference substances spiked into Chu10 and into natural stream water (cf. Table 5 for its physical and chemical characteristics) is illustrated in Fig. 2. Although the relative growth rates and the sensitivity of the diatom to chemical substances under 15ºC were slightly below those observed under 23ºC, no consistent pattern could be clearly defined; the same conclusion can be draw when different photoperiods are compared (Fig. 2). The individual or combined effect of temperature and photoperiod on the accumulation of phenol by *S. capricornutum* (Newsted 2004), and on the transcription of photosynthesis-related genes to Cd in *C. vulgaris* (Qian et al. 2010), has been stressed out. If such or similar negative effects occur in *N. libonensis* exposed to DCP or PD, the growth endpoint assessed does not capture the physiological impairment consistently.

Regardless the test incubation conditions, the overall relative growth rates of free cells in SW and SW+N was, respectively, of more than 41% and 70% of that obtained under Chu10, which was set as the reference rate for calculations; whilst for beads it was

of more than 49% and 89% of the rates obtained for immobilized cells under Chu10 (Fig. 2). When exposed to plain stream water *N. libonensis* evidenced a reduction on its relative growth rates irrespective of the test conditions, what was previously reported by other authors that tested natural waters (e.g. Marques et al. 2011; Moreira-Santos et al. 2002; Moreira-Santos et al. 2004b). Whenever natural samples are being assayed in the laboratory, it is important to discern a toxic effect from that caused by nutrient deprivation, the latter being avoided by adding nutrients to the test water (USEPA 2002). In this case study, the addition of nutrients to SW indeed promoted the diatom growth rates, although these were only significantly higher than in plain SW for immobilized cells at 23ºC, 24^{L} and for free cells at 15ºC, 24^{L} (Fig. 2, Tables 6, 7, 8).

The exposure of free or immobilized cells of *N. libonensis* to SW, SW+N and Chu10 spiked with DCP or PD, generally resulted in growth inhibition. Under some conditions, however, the inhibition of growth rates by DCP or PD was not statistically significant as the chemical treatments were compared to the corresponding natural water controls (i.e., SW and SW+N), particularly considering SW (Fig. 2, Table 8). Such outcome may raise some concerns related to the feasible use of this species to assess the quality of some natural waters. The growth inhibition detected under SW+DCP or SW+PD may actually be an effect of nutrient deficiency in the stream water, hence increasing the probability of accepting false positives. It should be recognised in this context that in a few conditions, the addition of nutrients to SW+DCP (i.e., SW+N+DCP) or SW+PD (i.e., SW+N+PD) led to a significant reduction of the toxicity of both compounds (free cells: 15ºC-12^{L}:12^{D} for PD, beads: 23ºC-24^{L} for PD, 15ºC-12^{L}:12^{D} for DCP and PD). This can relate to a lower bioavailability of the compounds due to complexation or adsorption onto other dissolved chemicals and organic matter present in SW+N (cf. also Table 5) (Moreira-Santos et al. 2002; Newsted 2004). Nevertheless, the growth rates detected under SW+DCP or SW+PD were generally not significantly different from those calculated under Chu10+DCP or Chu10+PD for free and immobilized cells, for all the combinations of temperatures and photoperiods. In fact, the lowest growth rates were normally observed under those four treatments. Hence, this benthic diatom provided similar ecotoxicological response levels to DCP and PD when approaching standard artificial conditions (represented by temperature, photoperiod and Chu10 medium) to field scenarios (represented by temperature, photoperiod and stream water).

The encapsulation of cells, though offering advantages for the *in situ* testing (Moreno-Garrido 2008; Twist et al. 1997), should allow the effective exposure of cells to the surrounding environment and guarantee that reliable responses to the contaminants in

combination with environmental factors are being assessed. Free and immobilized *N. libonensis* cells exposed to natural stream water and artificial medium non-spiked and spiked with DCP or PD generally elicited similar sensitivity, although the statistics found significantly different responses in particular cases (Fig. 2, Table 7). Most of these were associated with SW and/or SW+N treatments, either spiked or non-spiked with chemicals. Except for free cells exposed to SW at 23ºC, 12^{L}:12^{D}, and SW+DCP and SW+N+DCP at 15ºC, 12^{L}:12^{D}, the remaining significant differences between the response of free and immobilized cells resulted from an enhanced growth of encapsulated diatoms. Moreno-Garrido (2008) discussed the protection that the immobilization matrix may give against toxicity. The reason for that could be the partial removing of toxicants and their adsorption to the alginate polymer (Awasthi and Rai 2005), and the lower diffusion of toxicants through the matrix (Jang 1994). Nevertheless, the alginate matrix did not seem to limit the exposure of the diatom, since in Chu10+DCP or Chu10+PD the growth of free and immobilized cells was usually not significantly different. This outcome even suggests that under controlled conditions of temperature and photoperiod, free and immobilized cells respond similarly to the reference chemicals.

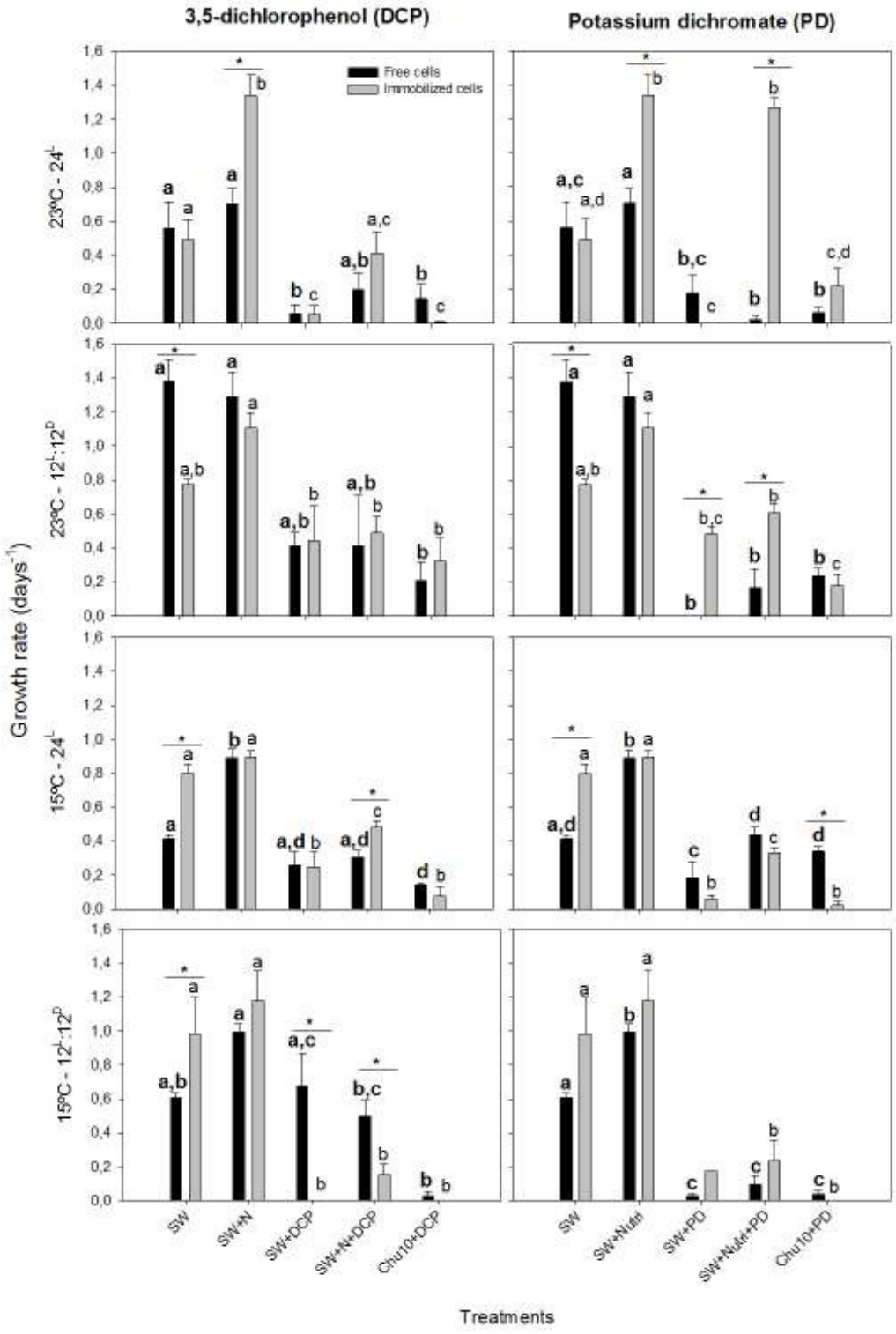

Fig. 2. Relative growth rates (simple ratio between the growth rates found in blank Chu10 and each test condition) of free and encapsulated cells of *N. libonensis* exposed to different treatments of stream water (SW) and Chu10 non-spiked and spiked with 3,5-dichlorophenol (DCP; left-hand panel) or potassium dichromate (PD; right-hand panel). Error bars represent standard errors. Different letters above error bars indicate significant

differences (P<0.05) between responses yield under different treatments when tested within free cells (light black letters) and within encapsulated cells (bold letters). The asterisks stand for significant differences between the responses of free and immobilized cells within each treatment.

Table 5. Physical and chemical characterization of the natural stream water.

Parameters	Natural water sample
Dissolved oxygen (%)	44.5
Dissolved oxygen (mg L^{-1})	4.03
Conductivity (µS cm^{-1})	110
Total dissolved solids (mg L^{-1})	124
pH	7.7
NH_3 (mg L^{-1})	0.142
NH_4^+ (mg L^{-1})	0.134
PO_4^{3-} (mg L^{-1})	0
NO_2^- (mg L^{-1})	0
NO_3^- (mg L^{-1})	0
BOD_5 (mg L^{-1})	1.98
Total suspended solids (mg L^{-1})	7.64
Chl a (mg L^{-1})	0
Ca^{2+} (mg L^{-1})	3.3
Fe^{3+}(mg L^{-1})	0.07
K^+(mg L^{-1})	2.3
Mg^{2+}(mg L^{-1})	4.0
Si(mg L^{-1})	3.6

Table 6. Two-way ANOVA summary applied to the relative growth rate of *N. libonensis* exposed to different treatments (media spiked and non-spiked with 3,5-dichlorophenol and potassium dichromate) and cell encapsulation (i.e., free and immobilized cells), under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom.

	Test condition	**Source of variation**	**df**	**$MS_{residual}$**	**F ratio**	***P* value**
3,5-dichlorophenol (DCP)	23ºC, 24^L	Treatments	4	0.954	34.15	**<0.001**
		Free /immobilized	1	0.118	4.23	**0.054**
		Interaction	4	0.146	5.21	**0.005**
		Residual	19	0.028	-	-
	23ºC 12^L:12^D	Treatments	4	1.008	14.57	**<0.001**
		Free /immobilized	1	0.088	1.27	0.275
		Interaction	4	0.114	1.65	0.206
		Residual	18	0.069	-	-

	15ºC, 24^{L}	Treatments	4	0.533	61.00	**<0.001**
		Free /immobilized	1	0.068	7.78	**0.012**
		Interaction	4	0.049	5.61	**0.004**
		Residual	19	0.009	-	-
	15ºC, 12^{L}:12^{D}	Treatments	4	1.089	27.78	**<0.001**
		Free /immobilized	1	0.070	1.79	0.196
		Interaction	4	0.265	6.75	**0.001**
		Residual	20	0.039	-	-
Potassium Dichromate (PD)	23ºC, 24^{L}	Treatments	4	0.891	34.25	**<0.001**
		Free /immobilized	1	0.971	37.33	**<0.001**
		Interaction	4	0.513	19.74	**<0.001**
		Residual	20	0.026	-	-
	23ºC 12^{L}:12^{D}	Treatments	4	1.302	70.10	**<0.001**
		Free /immobilized	1	0.002	0.10	0.760
		Interaction	4	0.283	15.24	**<0.001**
		Residual	19	0.019	-	-
	15ºC, 24^{L}	Treatments	4	0.606	98.00	**<0.001**
		Free /immobilized	1	0.008	1.28	0.271
		Interaction	4	0.102	16.46	**<0.001**
		Residual	20	0.006	-	-
	15ºC, 12^{L}:12^{D}	Treatments	4	1.369	35.57	**<0.001**
		Free /immobilized	1	0.197	5.12	**0.035**
		Interaction	4	0.033	0.86	0.507
		Residual	20	0.039	-	-

Table 7. Summary of the one-way ANOVAs performed to assess the effect of cell encapsulation (i.e., free and immobilized cells) on the relative growth rate of *N. libonensis* within each treatment (media spiked and non-spiked with 3,5-dichlorophenol and potassium dichromate) tested, under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom.

Standard chemical	Test condition	Source of variation	Fixed factor	df	$MS_{residual}$	F ratio	*P* value
3,5-dichlorophenol (DCP)	23ºC, 24^{L}	Cell encapsulation	SW	1, 4	0.007	0.25	0.621
			SW+N	1, 3	0.578	20.72	**2.181E-4**
			Sw+DCP	1,4	7.310E-6	2.62E-4	0.987
			SW+N+DCP	1, 3	0.030	2.03	0.171
			Chu 10+DCP	1, 3	0.004	1.88	0.186
	23ºC 12^{L}:12^{D}	Cell encapsulation	SW	1, 3	0.013	35.59	**0.009**
			SW+N	1, 4	0.044	1.13	0.347
			Sw+DCP	1,3	0.090	0.01	0.916
			SW+N+DCP	1,4	0.146	0.06	0.814
			Chu 10+DCP	1,4	0.045	0.43	0.548
	15ºC, 24^{L}	Cell encapsulation	SW	1,4	0.005	25.40	**7.273E-5**
			SW+N	1,4	0.006	1.96E-3	0.965

			Sw+DCP	1,4	0.021	0.01	0.912
			SW+N+DCP	1, 4	0.005	5.38	**0.032**
			Chu 10+DCP	1,3	0.006	0.65	0.431
	15ºC, 12^{L}:12^{D}	Cell encapsulation	SW	1,4	0.068	5.46	**0.030**
			SW+N	1,4	0.050	1.30	0.268
			Sw+DCP	1,4	0.057	1.75	0.201
			SW+N+DCP	1,4	0.020	4.52	**0.046**
			Chu 10+DCP	1,4	0.001	3.90	0.062
Potassium Dichromate (PD)	23ºC, 24^{L}	Cell encapsulation	SW	1,4	0.057	0.27	0.609
			SW+N	1,4	0.033	22.23	**1.327E-4**
			Sw+PD	1,4	0.016	1.71	0.206
			SW+N+PD	1,4	0.006	89.69	**7.833E-9**
			Chu 10+PD	1,4	0.017	1.46	0.241
	23ºC 12^{L}:12^{D}	Cell encapsulation	SW	1,3	0.013	23.82	**1.00E-4**
			SW+N	1,4	0.044	2.67	0.119
			Sw+PD	1,4	0.002	19.09	**3.304E-4**
			SW+N+PD	1,4	0.023	15.70	**8.353E-4**
			Chu 10+PD	1,4	0.010	0.27	0.606
	15ºC, 24^{L}	Cell encapsulation	SW	1,4	0.005	35.92	**7.347E-6**
			SW+N	1,4	0.006	2.77E-3	0.959
			Sw+PD	1,4	0.014	3.64	0.071
			SW+N+PD	1,4	0.004	2.91	0.103
			Chu 10+PD	1,4	0.002	24.76	**7.269E-5**
	15ºC,$12h^{L}$:$12h^{D}$	Cell encapsulation	SW	1,4	0.068	3.16	0.150
			SW+N	1,4	0.050	1.01	0.371
			Sw+PD	1,4	0.047	0.70	0.450
			SW+N+PD	1,4	0.027	1.07	0.359
			Chu 10+PD	1,4	7.570E-4	2.93	0.162

SW - stream water; SW+N - stream water enriched with nutrients; SW+DCP - stream water spiked with 3,5-dichlorophenol; SW+N+DCP - stream water enriched with nutrients and spiked with 3,5-dichlorophenol; SW+PD - stream water spiked with potassium dichromate; SW+N+PD - stream water enriched with nutrients and spiked with potassium dichromate.

Table 8. Summary of the one-way ANOVAs performed to assess the effect of the treatment (media spiked and non-spiked with 3,5-dichlorophenol and potassium dichromate) on the growth rate of *N.libonensis* within each cell immobilization factor level (i.e., free and immobilized cells), under different experimental conditions. Significant effects were highlighted bold. Df – degrees of freedom.

	Test condition	Source of variation	Fixed factor	df	$MS_{residual}$	F ratio	*P* value
3,5-dichlorophenol (DCP)	23ºC, $24h^L$	Treatments	Free cells	4,10	0.031	8.67	**3.693E-4**
			Beads	4,9	0.025	30.86	**4.508E-8**
	23ºC $12H^L$:$12H^D$	Treatments	Free cells	4,8	0.096	8.07	**0.007**
			Beads	4,10	0.047	6.31	**0.008**
	15ºC, $24h^L$	Treatments	Free cells	4,9	0.007	26.89	**1.363E-7**
			Beads	4,10	0.010	42.11	**3.434E-9**
	15ºC, $12h^L$:$12h^D$	Treatments	Free cells	4,10	0.031	9.46	**1.838E-4**
			Beads	4,10	0.048	25.08	**1.505E-7**
Potassium Dichromate (PD)	23ºC, $24h^L$	Treatments	Free cells	4,10	0.026	11.11	**6.528E-5**
			Beads	4,10	0.026	42.88	**1.534E-9**
	23ºC $12H^L$:$12H^D$	Treatments	Free cells	4,9	0.027	62.69	**1.113E-10**
			Beads	4,10	0.011	19.14	**1.865E-6**
	15ºC, $24h^L$	Treatments	Free cells	4,10	0.009	33.82	**1.221E-8**
			Beads	4,10	0.004	80.58	**4.825E-12**
	15ºC, $12h^L$:$12h^D$	Treatments	Free cells	4,10	0.004	141.48	**<0.001**
			Beads	4,10	0.073	11.52	**<0.001**

6.4. Conclusion and future perspectives

N. libonensis provided appropriate responses when immobilized in a calcium alginate matrix and exposed to different controlled conditions representing field and standard laboratorial contexts. Nevertheless, some methodological details of the bioassay need yet to be optimised in order to get more coherent trends and attain the validity criteria set in international standard guidelines. One possible amendment could be the use of fluorescence readings as a biomass surrogate, instead of performing time-consuming and labour-intensive cell counting under an optical microscope.

In the first optimization trials, a slight increase of the diatom growth at higher temperatures, lower initial cell densities and shorter exposure under the artificial medium Chu10 was noticed. However, at temperatures close to the average found in Portuguese natural streams (15ºC), there was apparently a protective effect of the alginate beads over *N. libonensis* that promoted growth. Even though, there was not a consistent pattern of

the growth of free versus immobilized cells, through the conditions assessed. Free cells either evidenced higher, lower or similar rates comparatively to the encapsulated cells.

In the second set of experiments, the combined scenarios of temperatures and photoperiods did not apparently influence the sensitivity of this diatom (free and immobilized cells) to the reference stream water and chemicals tested. The growth of the diatom under a plain field water sample was usually slightly lower than that under nutrient enriched samples. In any case, the sensitivity of *N. libonensis* to the standard chemicals spiked into plain stream water was similar to that obtained when Chu10 was used as dilution medium, both for free and immobilized cells. Thus, there is a chance that under field scenarios immobilized *N. libonensis* retrieve a reliable ecotoxicological assessment. However this should be proven under more realistic scenarios involving the co-occurrence of different confounding factors, during direct exposures in *in situ* trials. Before that, a third trial is still to be performed, which will involve assessing the suitability of the optimised test procedure using microcosm systems where the contamination of both the sediment and the water column can be evaluated. Due to unexpected constraints, this part of the study was not included in this thesis and the final whole dataset will hence be published elsewhere.

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Chapter VII

Final Remarks

Final remarks

Much work has been done in order to apply and to develop the Water Framework Directive (WFD-2000/60/CE) (EU 2000) in lotic freshwater ecosystems, since its publication in 2000. However, more attention will be given in order to achieve the goals proposed by WFD until the end of 2015, not only establishing the classification of the water bodies, but also in the recovery of streams and rivers classified with less than good ecological status. European member states, from northern and central Europe, have devoted a lot of work in the optimization and harmonization of techniques and methodologies, due to their similar ecoregions; while the southern Europe, with different ecoregions, tries to follow and adapt the WFD methodology, regarding the ecosystems differences. Despite these efforts, for example, in Portugal and Greece, no river basin management plans have yet been adopted or reported to the European commission (Perni and Martinez-Paz 2013) regardless the work developed by national institutions (see, for Portuguese case APA 2013).

Presently, besides the evaluation of water bodies, streams and rivers, the researchers devoted their work to the development of cost-effective methodologies for river quality restoration. Guidance documents stated that cost- effective analysis (CEA) has been applied regarding the mitigation of eutrophication and diffuse pollution and also in designing measures for the reduction of groundwater and surface water withdrawal in areas of water stress. However, river restoration was not a straightforward process and depends on the complexity of the restoration target and natural complex interaction within and between species as well as interaction between species and habitats. Processes at the ecosystem level, naturally take time to develop important ecosystem functions, to result in a resilient and self-sustainable system (Pander and Geist 2013).

On the other hand, the assessment methods were very well defined in the WFD-transposed Portuguese law and in the nationally established criteria for the classification of ecological status of freshwater bodies (INAG 2009). Despite its comprehensiveness, WFD can be seen as an advantage in environmental monitoring actions. However, WFD is very complex in the sense that it requires very specialized work to sample, gather and integrate information from different sources including the biological communities inhabiting each assessed site. Furthermore, the WFD methodologies are time-consuming and costly, and hardly provide a clear view of the causes of the resulting effects. Thus, complementary methodologies are welcome in order to simplify the cost effective and technical complexity of WFD' methodologies.

Attending this challenge, the present work defined various stages of action in order to develop a first methodology that fulfills this need.

The methodology WFD application to Mau River leads to the conclusion that the river has a good water quality, despite the expected impacts from multiple stressors namely mine drainage, agricultural runoffs and sewage on specific locations of its extension. This achievement was supported both with the biotic indices and community structure approaches, which identified, in space and time, impacts on the macroinvertebrates assemblage. Both approaches revealed that impacts are negligible at the Mau River scale because the fate pollutants entering rivers depends not only on landscape filtering of diffuse and point sources but also on “instream” processes that may transform, immobilize or eliminate diffuse pollutants (Heathwaite 2010) and stream flow (Poff et al. 1997). However, the use of biotic indices was not as discriminating as the community structure analysis, in spite of the recommendation within the WFD scope. The latter approach explored the spatial and temporal trends, allowing a more detailed analysis of the species succession and also quantifies the environmental explanatory factors. In fact, more research is needed to solve remaining uncertainties, developing an integrated approach with surveys on other biological descriptors and *in situ* experimental design, namely the study of river functional ecological processes (*e.g.* leaf litter processing).

As some authors stated (see Pinna et al. 2013), the WFD is constrained by the assessment tools, which are economically expensive, and long time lags. Therefore, complementary methodologies have been suggested in bibliography to overcome the expenses and time spent in evaluation of river ecosystem health. Namely, structural and functional measures, like organic matter breakdown (Young et al. 2008), leaf litter breakdown (Greenstein et al. 2004; Pascoal et al. 2003) and biological oxidative stress evaluated by biomarker response on caddisfly larvae belonging to the macroinvertebrates community (Prat et al. 2013). Still, Pinna et al. (2013), aimed at a reliable rapid assessment tool based on the selection of large size macroinvertebrates (> 2 mm), concluding that this information was adequate for rapid biomonitoring, in compliance with WFD.

Likewise the above authors, our work also aimed to contribute to the development of simplified methodology. Burton (2002) suggested that sediments constitute an additional source of contamination, since sediments can contain amounts of organic and inorganic material bounded to particles that when disturbed by stormwater runoff they can turn bioavailable as an important pollution source for both benthic and planktonic organism. Following this evidence, our work analysed the sediments of River Mau, trying to understand the episodic decrease of water quality

status as a consequence of the inflow of a mining effluent from Braçal mine and sediment resuspension. In order to discuss that possibility, a comprehensive ecotoxicological evaluation of sediments collected at two sites Braçal and Palhal mines, within river ecosystems potentially impacted by deactivated mines, were done through elutriate testing (USEPA 2001) with a battery of standard ecotoxicological organisms. The results obtained with the ecotoxicological test battery for Braçal mine corroborated our results already obtained using part of the WFD methodology. Thus, this ecotoxicological test battery may provide important information about the ecological status of each concerning site before investing in the application of time-consuming and costly methods defined by the WFD, constituting an additional methodology complementary to WFD (Vidal et al. 2012).

However, this methodology involves the collection of sediment which can promote the modification and/or loss of contaminants. To solve this potential problem, we developed a new methodology to obtain similar results, using the benthic diatom species *Navicula libonensis*, belonging to the Portuguese flora, sensitive to organic pollution and metals and good laboratorial handling size, as a test organism in ecotoxicological procedures. This methodology was optimized for application *in situ*, in order to obtain a very quick response on the degree of contamination of a site, providing also a complementary methodology to WFD's. It was the first attempt to create a new standard methodology for testing contaminated sediments in freshwater ecosystems considering both the water and sediments compartments, using freshwater benthic diatoms rather than planktonic microalgae. The latter have negligible prevalence in freshwater lotic systems, but was very popular as standard organisms in ecotoxicological tests and also known as very sensitive to several types of contaminants (Fairchild et al. 1997). For example, Moreno-Garrido et al. (2003) have already developed *in situ* test with marine benthic diatom. Our results showed that benthic diatoms suited well as bioindicators within the scope of WFD, regarding river quality assessment, showing to be very sensitive to the standard chemicals tested and one order of magnitude more sensitive than the standard freshwater planktonic microalgae used in procedures for ecotoxicological testing (ISO8692 1989; OECD 2011; USEPA 2002). The optimization of the technique, for application *in situ* of immobilized benthic diatoms, includes also field temperatures, photoperiods, natural stream water along with the standard chemicals tested. The species was successfully immobilized presenting approximately similar growth rate when comparing with free cells, in the same conditions. Also, the species was indeed shown to be very sensitive to the respective standard representatives potassium dichromate and 3,5-dichlorophenol, when tested both as free cells and as immobilized cells in alginate

beads. However, further studies are necessary to confirm the sensitiveness of *N. libonensis* to other metals and organic contaminants.

Based on the laboratorial data reported here, follow-up research is needed in order to develop *in-situ* testing protocols with the diatom *N. libonensis*, using immobilization matrices (see, e.g., Moreno-Garrido et al. 2005 for similar approaches focusing marine ecosystems). Field experiments are already planned to assess two well known deactivated mines (Braçal and Palhal) in order to obtain results that confirm if the species can be a reliable tool in field works to test the toxicity associated with river sediments. Finally, the two complementary methodologies developed in this study provided consistent results with the ones obtained using the WFD methodology in Braçal mine.

7.1. References

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