INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Mineral and Organic Pollution in River Sabor (Northeastern Portugal): Ecotoxicological Effects on Freshwater Fauna

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Dissertation presented to the School of Agriculture of Bragança in partial fulfillment of the requirements for the degree of Master of Science in Biotechnological Engineering

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BRAGANÇA

JULY 2016

*Dissertation made under the agreement of **Double Diploma** between the Escola Superior Agrária de Bragança|IPB and the High Institut of Biotechnology of Monastir|ISBM, Tunisia to obtain the Degree of Master in Biotechnological Engineering*

AKNOWLEDGEMENTS

For the realization of the dissertation, I thank my advisors, Professor Amilcar Antonio Teiga Teixeira and Professor Conceição Fernandes, from School of Agriculture of Bragança, IPB, *PORTUGAL* and Professor Imed Massaoudi from Higher Institute of Biotechnology of Monastir in *TUNISIA.* Thank you for the patience, all the knowledge imparted by me have suggested this project and the support and wisdom that were an essential pillar so that this work was possible by the enormous enthusiasm for nature that infected me, for the friendship and support at all times;

To Professors Anabela Martins, Paula Rodrigues and António Peres, from the Scientific Comission of the Master of Biotechnological Engineering, School of Agriculture, for all collaboration during my Masters studies in IPB;

To all the teachers I had throughout the Master of Biotechnological Engineering, School of Agriculture, Polytechninc Institute of Bragança, Portugal, to contribute to my education my most heartfelt thanks;

To Mr Fernando Miranda and Mr Daniel Saraiva, superior technicians of School of Agriculture, IPB, for the essential help in fieldwork;

And a special thanks to Erasmus ICM+, namely to Professor Luis Pais, Vice-President of IPB, and to the International Relations Office (IRO), namely Dra Joana Aguiar for their competence and kindness, for this opportunity to do my Master Degree in Portugal and for open a lot of chances in life.

To my close friends for their support and help throughout this time to the completion of my work;

To My family, Mom Samira, Dad Mohamed , my sister Sana and my brother Wassim the deepest gratitude for the transmitted and essential motivation for the completion of another stage of my life.

ABSTRACT

Aquatic ecosystems are final collectors of all kinds of pollution as an outcome of anthropogenic inputs, such us untreated industrial and municipal sewage and agricultural pollutants. There are several aquatic ecosystems that are threatened by mineral and organic pollution. In Northeastern Portugal, near Bragança, different watercourses are suffering negative impacts of human activities. It has been developed several studies in the monitoring of environmental impacts in these river basins, namely in Rio Fervença, affected by organic pollution, and in Portelo stream, affected, since 2009, by the collapse and continuous input of mining deposits.

In this sense, the present study aimed to continue the monitoring study of ecological status of freshwater ecosystems of Northeastern Portugal, namely the following objectives: a) mineral pollution effects of mining deposits sudden incorporated into Portelo stream; b) organic pollution due to domestic and industrial inputs in River Fervença. Also, since fish are useful experimental models to evaluate toxicological mechanisms of contaminants, c) acute toxicity tests with Cu were conducted in laboratory conditions.

During 2015/2016, it was made abiotic and biotic characterization of 16 sampling sites distributed by both Portelo and Fervença rivers, tributaries of main River Sabor (Douro Basin). Several physicochemical parameters were determined and Riparian Quality (QBR Index) and Channel Quality (GQC) Indexes were determined for habitat evaluation. Fish and invertebrate communities were sampled, according to protocols of Water Framework Directive (WFD). Several metrics were determined, with particular emphasis on the Biotic Index IBMWP and the Northern Portuguese Invertebrate Index ($IPtI_N$). Acute toxicity tests were conducted with an Iberian fish species, common barbel (*Luciobarbus bocagei*) and some plasmatic electrolytes levels were evaluated, to assess their contribution to mitigate osmoregulatory adverse effects of Cu. Also, same electrolytes were measured after changing to clean water, in attempt to assess fish capacity to reverse this situation.

Results obtained for both rivers showed a significant level of disturbance that affected decisively water, habitat and biological quality of aquatic ecosystems.

Due to this change of environmental conditions in Portelo stream (extreme pH values, high conductivity and presence of heavy metals), several biological metrics (e.g. taxonomic richness, abundance, diversity, evenness) confirmed, comparatively with reference sites, a substantial decrease on ecological integrity status. The same pattern was found for Fervença River; however other water parameters, namely the content of most limiting nutrients (e.g. N and P) seemed to have more influence in the composition and structure of macroinvertebrate and fish communities. In fact, despite the operation of the Sewage Treatment Plant of Bragança, Fervença River presented significant levels of disturbance that affected decisively the quality and ecological integrity of the aquatic ecosystem. The synergic effect of domestic and industrial pollution, intensive agriculture, regulation and degradation of aquatic and riparian habitats contributed to the decrease of ecological condition, namely in the downstream zones (after Bragança).

The results for acute toxicity, showed that fish can change Na^+ and K^+ levels face to Cu exposition and, depending of Cu concentration tested, can also return to normal levels, providing some insights to that are believed to occurred in fish population, near the Portelo mines.

The low ecological integrity status detected in the lotic ecosystems in NE Portugal as a result of mineral and organic pollution deserves the development of several measures for rehabilitation and improving of water quality. On the other hand, environmental education actions are needed to contribute to improvement of ecological integrity of the river and its conservation.

Key-words: *aquatic organisms, ecological integrity, pollution, toxicity*

RESUMO

Os ecossistemas aquáticos são os recetores finais de todos os tipos de poluição, caso dos esgotos industriais e urbanos não tratados e dos poluentes agrícolas. No Nordeste de Portugal, na proximidade de Bragança, diferentes cursos de água são alvo de impactos negativos resultantes de diferentes atividades humanas. Nos últimos anos têm sido desenvolvidos diversos estudos na monitorização dos impactos ambientais nessas bacias hidrográficas, seja no Rio Fervença, afetado pela poluição orgânica, seja na Ribeira do Portelo, afetado, a partir de 2009, pelo colapso das escombreiras e entrada contínua de depósitos de minério.

Neste sentido, o presente estudo teve como objetivo continuar o estudo de monitorização do estado ecológico dos ecossistemas aquáticos do Nordeste Portugal, nomeadamente: a) efeitos da poluição mineral resultante do colapso e entrada na rede hídrica de depósitos de minério, outrora explorados nas minas do Portelo; b) efeitos da poluição orgânica devido às entradas de efluentes domésticos e industriais no rio Fervença. Para além da abordagem ecológica foi ainda realizada uma abordagem fisiológica baseada em testes de toxicidade aguda. Uma vez que os peixes são modelos experimentais úteis para avaliar mecanismos toxicológicos de contaminantes foram realizados c) testes de toxicidade aguda com cobre (Cu) em condições de laboratório.

Durante 2015/2016, foi feita a caracterização abiótica e biótica em 16 locais de amostragem distribuídos por ambos os rios Portelo e Fervença, afluentes do rio Sabor (Bacia do Douro). Foram determinados vários parâmetros físico-químicos e avaliada a qualidade do habitat ribeirinho e aquático através de índices de Qualidade Ripária (Índice QBR) e do Canal (GQC). As comunidades de peixes e invertebrados foram amostrados de acordo com os protocolos da Directiva-Quadro da Água (DQA). Foram calculadas várias métricas, com particular destaque para o Índice IBMWP e o Índice de Invertebrados do Norte Português ($IPtI_N$). Realizaram-se testes de toxicidade aguda numa espécie piscícola presente na bacia hidrográfica, caso do barbo comum (*Luciobarbus bocagei*) tendo sido avaliados alguns eletrólitos plasmáticos níveis, para avaliar a sua contribuição na mitigação dos efeitos adversos de osmorregulação

associada ao Cu. Além disso, os mesmos eletrólitos plasmáticos foram medidos depois de mudar a água, na tentativa de avaliar a capacidade dos peixes para reverter esta situação.

Os resultados obtidos em ambos os rios mostraram um nível significativo de perturbação que afetou decisivamente a qualidade da água, habitat e biota. Devido a essa mudança nas condições ambientais da ribeira do Portelo (e.g. valores de pH extremos, elevada condutividade e presença de metais pesados), várias métricas biológicas (e.g. riqueza taxonómica, abundância, diversidade, equitabilidade) confirmaram, comparativamente com os locais de referência, uma diminuição substancial na integridade ecológica dos ecossistemas. O mesmo padrão foi encontrado para o rio Fervença. No entanto, outros parâmetros da água, como por exemplo, a concentração de nutrientes limitantes (e.g. N e P) parecem ter mais influência na composição e estrutura das comunidades de macroinvertebrados e peixes. Na verdade, apesar do funcionamento da ETAR de Bragança, o rio Fervença apresentou níveis significativos de perturbação que afetaram decisivamente a qualidade e integridade ecológica do ecossistema aquático. O efeito sinérgico da poluição doméstica e industrial, agricultura intensiva, regulação e degradação de habitats aquáticos e ribeirinhos contribuiu para a diminuição da condição ecológica, especialmente nas zonas a jusante (depois de Bragança).

Os resultados de toxicidade aguda, mostraram que os peixes podem mudar os níveis de Na^+ e K^+ para enfrentar a exposição ao Cu e, dependendo da concentração de Cu testado, podem também retornar aos níveis normais, fornecendo algumas informações acerca do que poderá ter ocorrido na população nativa de peixes, localizada na proximidade das minas do Portelo.

A baixa integridade ecológica detetada nos ecossistemas lóticos do NE Portugal, afetados pela poluição mineral e orgânica merece o desenvolvimento de várias medidas para a reabilitação de habitats e melhoria da qualidade da água. Por outro lado, ações de educação ambiental são necessários para contribuir para a melhoria da integridade ecológica do rio e sua conservação.

Palavras-chave: *organismos aquáticos, integridade ecológica, poluição, toxicidade*

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SYMBOLS

WFD- The Water Framework Directive

EPT- Number of Families of the orders Ephemeroptera, Plecoptera, Trichoptera

GQC- Channel Quality Index

ASPT: Average Score by taxon

IBMWP- Iberian Biological Monitoring Working Party

INAG- National Institute of Water

$IPtI_N$- Portuguese Northern Invertebrates Index

IUCN- International Union for Conservation of Nature

NMDS- Non-metric multi-dimensional scaling

QBR- Riparian Quality Index

RQE - Ecological Quality Ratio

TDS- Total dissolved solids

BMI - Benthic Macroinvertebrates

Cu- Copper

K- Potassium

Na- Sodium

ppm- One part per million

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CHAPTER 1

GENERAL INTRODUCTION

Nowadays, aquatic environments are exposed to extreme pressure, as final recipients of numerous contaminants (e.g. inorganic, organic, microbiological and pharmaceutical) from various sources (e.g. industry, mining, municipal waste waters, and agricultural runoff). According to environmental agencies, mining effluents and agricultural runoff can be considered as the one of the most serious threats to freshwater ecosystems (ENVIRONMENT AGENCY, 2006). The former due to high acidity and high metal concentrations, and the latter mainly as a source of different types of organic contaminants (BARIŠIĆ et al., 2015). Therefore, environmental pollution is considered the unfavorable alteration of our surrounding world, wholly or largely as a product of man’s actions, radiation levels, chemical and physical constitution and abundances in organisms. These changes may affect human kind directly, through the supply of water, agricultural and other biological products (KORMONDY, 1996).

From the many aquatic ecosystem (ocean, sea, lagoon, river) affected by pollution, fluvial ecosystems could be mentioned to support rich and diverse assemblages, with adaptations that allow them to prosper in these environments, and which make them very vulnerable to possible alterations in the habitat. In this sense, human activity often causes severe ecological damage to river systems. These disturbances produce alterations in the water quality (e.g. physical and chemical parameters), habitat conditions (riparian and channel hydromorphological features) and on the composition and structure of aquatic communities (e.g. plants, invertebrates and fish) (OLLER & GOITIA, 2005; BENETTI et al., 2012). For example, the freshwater biodiversity provides a broad variety of valuable goods and services for human societies like food, entertainment, and some of them are irreplaceable, but human activities have always affected aquatic ecosystems. Rivers are highly vulnerable to change caused by anthropogenic impacts, and their flow is often manipulated to provide water for human use (BREDENHAND & SAMWAYS, 2009).

Globally, the biodiversity of freshwater ecosystems is rapidly deteriorating as a result of human activities (DAHL et al., 2004; BENETTI et al., 2012). The combination of these threats resulted in biodiversity decline, leading many freshwater species towards extinction (DARWALL et al., 2008). To face this problem, international laws, such as the Water Framework Directive (WFD), have driven the need to assess the ecological status of the water bodies through an integrative ecosystem approach using elements of biotic quality. One of the main objectives of WFD is the protection, improvement and restoration of surface water bodies of all country members, in order to achieve good environmental status. The ecological state is characterized by biological (e.g. phytoplankton, macrophytes and phytobenthos, benthic invertebrates and fish communities), physico-chemical (e.g. temperature, oxygen dissolved, nutrients) and hydromorphological (e.g. riparian and channel habitats) elements. The ecological status is defined by the deviation (ecological deviation) between the characteristics of a watercourse in natural conditions (reference site) and its characteristics when subjected to one or more pressures (e.g. organic/industrial pollution, regulation). The WFD distinguishes five levels of ecological status: excellent, good, moderate, poor and bad (DIRECTIVE 2000/60/EC).

Bio-indicators:

McCARTY & MUNKITTRICK (1996) defined bioindicators as "anthropogenically-induced variations in biochemical or physiological components or functions (i.e., biomarkers) that have been either statistically correlated or causally-linked, in at least a semiquantitative manner, to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization" (Figure 1.1). For ADAMS & GREELEY (2000) the bioindicator concept is defined "to be multiple measures of organism, population, or community-level health which include several levels of biological organization and time scales of response".

Biological communities reflect the environmental status of the systems (i.e., its physicochemical conditions, biological and hydromorphological) and by integrating the effects of different stressors often complex and interdependent provide a measure of its cumulative impact (BARBOUR et al., 1999).

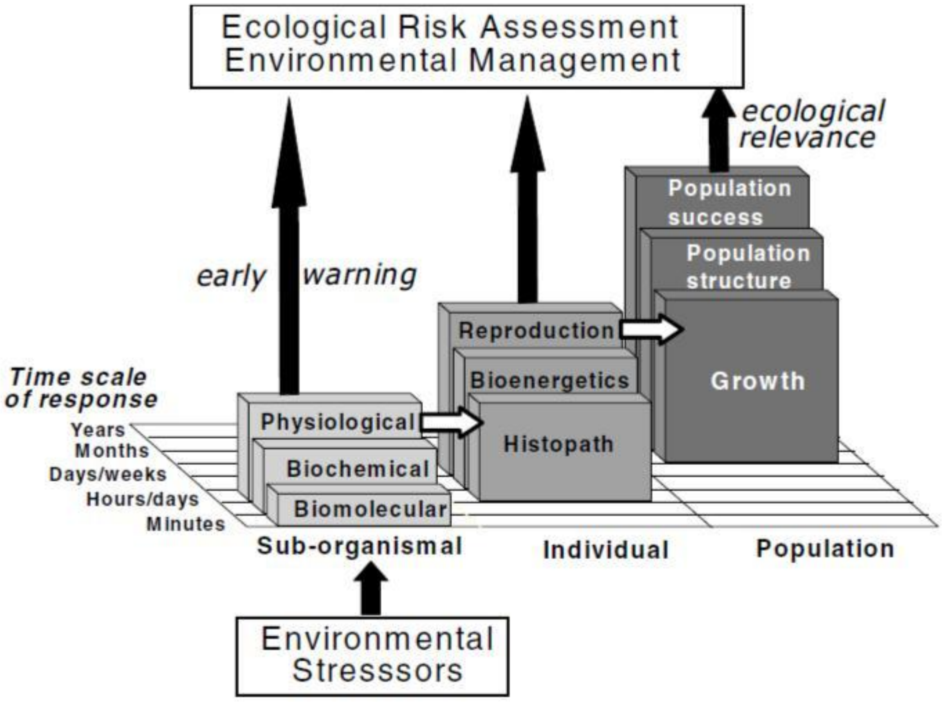

Figure 1.1. Hierarchical response of organisms to environmental stressors illustrating the more sensitive early warning indicators at the lower levels of biological organization and the slower-responding but more ecologically relevant indicators at the higher levels of organization (adapted from McCARTY & MUNKITTRICK, 1996)

Among the fauna of rivers that should be highlighted, macroinvertebrates occupy a key position because they maintain the balance of the food chain. This group of great diversity and ecological importance consists of invertebrates of macroscopic size, normally more than 0.5 mm, living permanently or during certain periods of their life cycle linked to the aquatic environment. They include different faunistic groups like insects, crustaceans, annelids, molluscs, leeches. Macroinvertebrates are excellent indicators of human impacts, especially contamination. Most of them have quite narrow ecological requirements and are very useful as bioindicators in determining the characteristics of aquatic environments (BENETTI & GARRIDO, 2010), to identify the segments of a polluted river where self-purification of organic inputs is under process (BENETTI et al., 2012). The quality of aquatic ecosystems is, nowadays, evaluated by several ecoindicators (BARBOUR et al., 1999; SIMON, 2003). For example, fish are increasingly considered as key organisms in biomonitoring programs, as they enable the assessment of ecological status, based on the following aspects (FAUSCH et al., 1990; HARRISON and WHITFIELD, 2004; OLIVEIRA et al., 2007): a) Are present in almost all types of aquatic ecosystems; b) Their communities are persistent and recover quickly from natural disturbances; c) Its taxonomy, ecological requirements and life cycles are aspects generally more known relative to other faunistic groups; d) Occupy different hydromorphological habitats and are indicators of the physical structure of the rivers in different spatial scales; e) The migratory behavior of some species indicate the connectivity conditions of the system; f) Represent a wide variety of trophic levels and thus reflect environmental effects at all levels of the food chain; g) Can display external anatomical pathology, easily identifiable in the field as a result of the pollutants; h) Because of its longevity are bioindicators for relatively long periods; i) Have economic value, and are considered how important environmental resources.

Mineral and organic pollution are two of the main negative impacts identified on the decreasing of aquatic ecosystems integrity in Portugal. As mentioned, the School of Agriculture has developed important work in the evaluation of environmental impact near the Portelo mines. In this sense, the present work aims to continue this study by monitoring the ecological *status* and the evolution of some freshwater ecosystems located in Northeastern Portugal.

OBJECTIVES AND ORGANIZATION OF THE DISSERTATION

The **specific objectives of the dissertation** will be the medium-long term monitoring analyses, take into consideration:

a) **Assessment of the environmental effects of mineral pollution**, resulted from the collapse of Portelo mine wastes, in December 2009, located in the Montesinho Natural Park (Northeastern Portugal);

b) **Assessment of the organic pollution**, resulting from an inadequate soil use and the input of domestic and industrial effluents in the Fervença River, near Bragança;

c) **Evaluation of cooper acute toxicity, under laboratory conditions and using fishes as experimental models;**

d) **Proposal of environmental awareness measures** to promote the rehabilitation of threatened habitats and ecosystems and the conservation of native species.

The **dissertation is organized into four chapters**, corresponding the first one, **CHAPTER 1**, to the **general introduction** where the definition of the main objectives of the dissertation is done. The following two chapters (Chapters 2 and 3) are presented in the form of scientific articles with the following titles:

CHAPTER 2: Evaluation of ecological status of lotic ecosystems affected by mineral and organic pollution: The study case of Portelo and Fervença rivers

CHAPTER 3: Effect of acute cooper exposure in aquatic vertebrates (*Luciobarbus bocagei*) on some biochemical indicators (Na+ and K+ plasma concentrations).

In the **CHAPTER 4 general conclusions and final considerations** of the study are made, based on a number of partial studies and presented separately in Chapters 2 and 3.

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CHAPTER 2

Evaluation of ecological status of lotic ecosystems affected by mineral and organic pollution: The study case of Portelo and Fervença rivers

ABSTRACT

From November 2015 to April 2016 the ecological integrity of Portelo and Fervença watercourses, both tributaries of Sabor River (River Douro basin, NE Portugal), were evaluated. The headstream of Portelo is located in the Montesinho Natural Park and is subjected, since December 2009, to the impact of mineral pollution, resulted from the collapse of mine wastes. Fervença River is negatively influenced by domestic and industrial effluents from rural and particularly from the urban area of Bragança city. Several abiotic (e.g. water quality and aquatic and riparian habitats) and biotic (e.g. macroinvertebrate and fish communities) parameters were measured in the 16 sampling sites selected along both river basins. The results showed, in general, good water quality in reference sites, with high levels of dissolved oxygen (DO> 8 mg/L) and low levels of dissolved salts (EC25 <100 μS/cm) and nutrients (NO_3^-, NO_2^- and P_{Total} <0.05 mg/L). Furthermore, all metrics determined for hydromorphological conditions and biotic evaluations (e.g. presence of stenobiont insects belonging to Ephemeroptera, Plecoptera and Trichoptera) confirm the good ecological integrity of these river zones. In highly disturbed sites it was found a clear pattern of decreased ecological integrity. In fact, it was detected for Portelo (mineral pollution), after 7 years, harsh environmental conditions, more visible during rainy periods, with low values of pH (pH < 5), high levels of dissolved salts (EC25 >100 μS/cm) and the input of heavy metals (e.g. Cu, Co, Al). The continuous input of fine sediments changed the aquatic habitats for invertebrate and fish communities and a low number of individuals and taxa were found. For Fervença River, the organic pollution affecting the downstream zone, also contributed to the diminishing of water quality (DO< 8 mg/L; EC25 >150 μS/cm; NO_3^- and P_{Total} > 0.1 mg/L) and the dominance of euribiont organisms (e.g. Diptera, Oligochaeta, Hirudinea) reflected the lower biological quality.

Key-words: *Mineral and organic pollution, Macroinvertebrates and fish community, Bioindicators, Water quality assessment*

2.1. INTRODUCTION

The protection and conservation of river resources has never been more important than it is today. Loss of habitat and the increase of anthropogenic pressures on freshwater are leading to water quality deterioration endangering many aquatic species (SANTOS et al., 2015; VALLE JUNIOR et al., 2015). Global freshwater systems are experiencing a general decline in biodiversity due to many factors: overexploitation, water pollution, river domestication and habitat degradation (DUDGEON et al., 2006). According to COLLEN et al. (2014), the global population of freshwater fish has declined by 76% since the 1970s. This trend is expected to worsen in the course of increased human water demand and reduced rainfall promoted or amplified by anthropogenic climate change (VÖRÖSMARTY et al., 2010; FONSECA et al., 2016)

In last few decades the increase in human population, heavy industrialization and agricultural activities have resulted in more and more wastes entering in freshwater resources. Aquatic organisms are affected by a number and variety of natural and man-induced stressors, such as variations in physicochemical factors (temperature, dissolved oxygen, nutrients and salinity regimes), changes in food and habitat availability, exposure to contaminants, and increases in nutrient (eutrophication) inputs. For example, bad agricultural practices can have adverse impacts on the health of aquatic systems through contaminant and nutrient increases (ADAM & GREELEY, 2000). Contamination of freshwater with a wide range of pollutants has become a matter of concern.

Furthermore, the rapid development of industry and agriculture has resulted in increasing pollution, especially by heavy metals (e.g. Cu, Zn, Co) which have significant environmental hazards for invertebrates, fish and humans (ULUTURHAN & KUCUKSEZGIN, 2007). Heavy metals released from domestic, industrial and other man made activities may contaminate the natural aquatic system extensively. These heavy metals have devastating effects on ecological balance of the recipient environment and a diversity of aquatic organisms (FAROMBI et al., 2007). Heavy metals and chemicals are toxic to animals and many cause death or sublethal pathology of liver, kidneys, reproductive, respiratory or nervous systems in both invertebrate and vertebrate aquatic animals (CHAVAN & MULEY, 2014).

Metal can seriously threaten the aquatic biota in certain regions where excessive industrial and anthropogenic activities exist. The Cu occurs naturally in unpolluted freshwaters, in concentrations ranging from 0.2 mg/L to 30 mg/L. However, Cu concentrations ranging from 50 mg/L to 4560 mg/L have been reported in polluted areas all over the world (USEPA, 2007; SAGLAM et al., 2013).

Pristine rivers are recognized as ecosystems of great biological value with high diversity communities with a complex structure and high ecological integrity. However, their special typology makes them fragile and vulnerable to environmental changes (DAHL et al., 2004; BENETTI et al., 2012). These ecosystems could be affected as a whole, but without doubt, each of the changes described have specific effects on the biota even acting synergically. For this reason it is very difficult to discover which of these have a greater or lesser effect on the balance of the impacts of the different human activities. Although, aquatic communities are accustomed to natural disturbances (e.g. drought, floods), in several occasions, the tolerance thresholds of many species could be surpassed and the structure of the community begins to change irreversibly (MARQUÉS et al., 2003).

The spilling of mines deposit in the river can change a lot in water's parameters. The physicochemical effects on aquatic ecosystems are basically summarized in changes in the following variables: (i) pH; (ii) concentration of ions; (iii) transparency of the water and (iv), structure of the riverbed. The changes in the concentration of metals, both in the water and in the sediments are clear evidence, many times, of the mine impacts (MARQUÉS et al., 2001; MARQUÉS et al., 2003).

According to author mentioning above, observation of whether some of the specific consequences of mining have a specific effect on the chosen biotic community: the benthic macroinvertebrates (BMI). So the structure knowledge of a specific BMI community provides precise and local information on recent events. In this sense, there are several biotic parameters which can demonstrate these changes, such as density, taxonomic richness and dominance.

Comparison of the BMI communities from the different zones affected by the mining activity, in respect to a reference site, provides information on the intensity of the mining impact, and in this sense, many times is used a similarity index. As opposed to other indexes, the similarity index is very sensitive to disturbances of metallic origin.

The use of BMI to ascertain the changes that can take place in fluvial ecosystems is not new. Their effectiveness has been sufficiently demonstrated in many papers which are now classics. Although, especially over the last few years, they have been used as a cost-effective tool for measuring the recovery of the fluvial ecosystems subjected to very diverse disturbances (HANNAFORD & RESH, 1995; RESH et al., 1995; MARQUÉS et al., 2003).

The increased pollution in ecosystems reinforces the importance of both chemical monitoring and biological monitoring of streams and rivers, as an effective and complementary water quality-based approach to assess aquatic ecosystem health (PINTO et al., 2009). That’s why biomonitoring seems to be one of the best ways to assess the effect of pollution because of the use of biological variables to survey the environment (GERHARDT, 2000). The first step in this type of monitoring is to find the ideal bioindicator whose presence, abundance and behavior reflects the effect of a stressor on biota (BONADA et al., 2006).

There are several metrics to monitor the health state of an aquatic ecosystem like biotic (e.g. IBMWP) or diversity (e.g. Shannon-Wienner) indexes. Benthic macroinvertebrates are considered good indicators of local scale conditions. These invertebrates live on the bottom of aquatic ecosystems, at least part of their life cycle, and are easy to be collected, normally using nets (handnet, kicknet) with a mesh size of 500 μm or less. Aquatic macroinvertebrates are used to bioassess aquatic ecosystem quality due to their great diversity of shapes and habits and variety of responses to different environmental conditions. According to JOHNSON et al. (1993) and BENETTI et al. (2012) a biological indicator has to fulfill different characteristics:

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- To be taxonomically easy to identify
- To be widely distributed
- To be abundant and easy to capture
- To present low genetic and ecological variability
- To have low mobility and a long life cycle
- To present well-known ecological characteristics
- To have the possibility of being used in laboratory studies.

Although aquatic invertebrates do not fulfill all these requirements , they do offer distinct advantages for biomonitoring (HELLAWELL, 1986; ROSENBERG & RESH, 1993; BARBOUR et al., 1999; RESH et al., 2004; BONADA et al., 2006), including: *a*) Their ubiquitous occurrence; b) Their huge species richness, which offers a spectrum of environmental responses; *c*) Their basic sedentary nature, which facilitates spatial analysis of pollution effects; *d*) The propensity of certain species to enter in the water column (i.e., drift), which may indicate the presence of a pollutant; *e*) The long life cycles of some species, this can be used to trace pollution effects over longer periods; *f*) Their compatibility with inexpensive sampling equipment; *g*) The well described taxonomy for genera and families; *h*) The sensitivities of many common species, which have been established for different types of pollution; and *i*) The suitability of many species for experimental studies of pollution effects.

Aquatic invertebrates are undoubtedly the most widely used organisms in freshwater biomonitoring ecosystem status (BONADA et al., 2006). However, some variation in macroinvertebrates communities and biological indices are the result of the natural functioning of aquatic ecosystems. Rivers in particular, are mostly characterized by a high degree of spatiotemporal variability (ELOSEGUI & POZO, 1994).

As a result, the concurrently occurring spatial and temporal variability in the absence of disturbances must be first described and then understood before possible anthropogenic impacts and disturbance effects can be distinguished from natural variability (LEUNDA et al., 2009).

Also, biotic indices are the most used because they are highly robust, sensitive, cost-effective, easy to apply and easy to interpret. Biotic indices are tools for assessing quality based on the different response of organisms to environmental changes (MINISTRY OF ENVIRONMENT, 2005).

There are many biotic indices developed for different regions, for instance the BMWP for UK, and the IBMWP for Spain or diversity indexes, like Shannon-Wienner or Margalef, based on aquatic invertebrates (BENETTI et al., 2012).

One of the most used biotic indices in the Iberian Peninsula is the IBMWP (Iberian Biological Monitoring Working Party) formerly BMWP (British Biological Monitoring Working Party Score (ARMITAGE et al., 1983) and it is extensively used as the standard biological index in water quality biomonitoring programs (ALBA-TERCEDOR & PRAT, 1992; LEUNDA et al., 2009). The taxonomic resolution of this index is mostly at family level. Each benthic macroinvertebrate family (or higher *taxa*) has a score in relation to their tolerance to pollution. So, the sum of the scores of the different taxa found in one site gives a total score allowing this sampling site to be classified in one of the five water quality classes (ALBA-TERCEDOR et al., 2002; BENETTI et al., 2012). Recently, according to WFD, it was developed sampling protocols (INAG, 2008a) and a multimetric index (INAG, 2009) more adapted for Portugal rivers, named IPtIN or IPtIS, i.e. Northern or Southern Portuguese Macroinvertebrate Index. This index integrates different metrics (e.g. IBMWP, H', J') and they have been used for environmental impact assessment of the ecological conditions of Portuguese rivers.

This study aims to contribute to the knowledge of the integrity of lotic ecosystems of Sabor Basin (Douro River Basin), located in northeastern Portugal. In detail, the **specific objectives** were:

1) **Assess the water quality** through the measurement of several physical and chemical parameters, **considering also the heavy metals inputs in Portelo stream**;
2) **Evaluate the water quality of Fervença River, subjected to organic pollution** from rural and urban areas near Bragança city;
3) **Determine the hydromorphological quality of both river basins**, based on two aquatic and riparian habitat indexes;
4) **Analyse the macroinvertebrate and fish communities**, in particular the response to mineral and organic pollution.

2.2. METHODOLOGY

2.2.1. Study Area

River Sabor is one of the main tributaries of River Douro. In the upper part of Sabor Basin, there are several headwater streams, such as Ribeira Portelo and River Fervença. The Portelo mines are located in Natural Park of Montesinho (NPM), near the border with Spain (Figure 2.1).

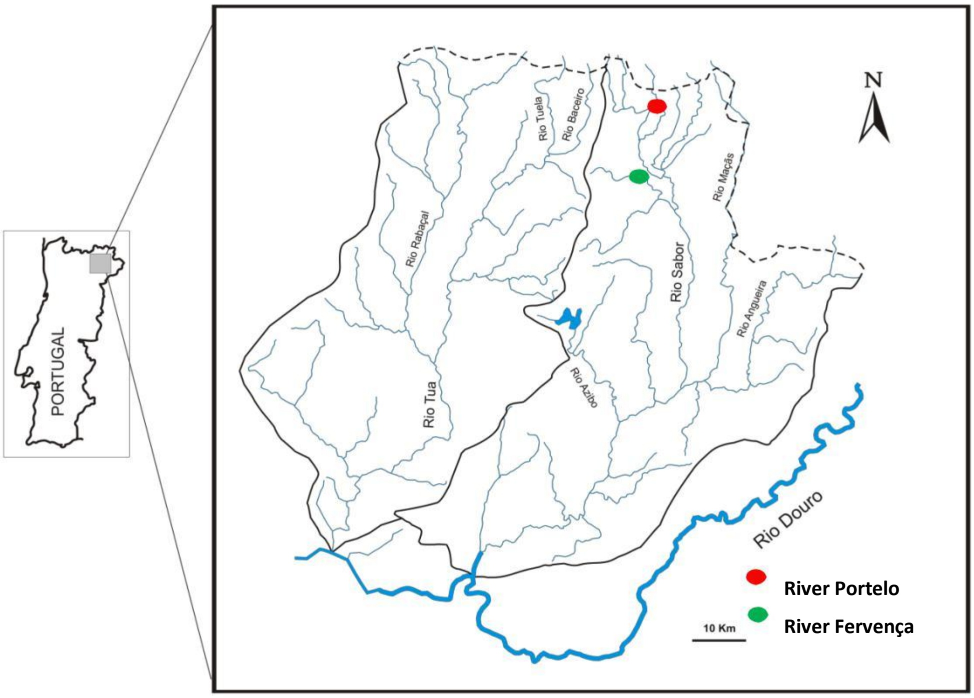

Figure 2.1. Map of the watercourses in Sabor Basin.

The landscape is dominated by natural vegetation composed by Pyrenean oak (*Quercus pyrenaica*) and holm oak (*Quercus ilex*). It can also be found plantations of chestnut (Castanea sativa) and different pine species (*Pinus pinaster*, *P. nigra* and *P. silvestris*). In the valleys, near rivers, often occur marshes and near rural areas the land use includes agriculture (e.g. cereals, horticulture) and livestock.

Portelo stream is a tributary of Aveleda stream (Sabor basin) and is a low order watercourse (Order 3), about 20 km long. In NPM the watercourses are heterotrophic and highly dependent on the input of allocthonous organic materials. The gradient is high and the water current contributes to the dominance of coarse substrates (eg large blocks, pebbles). The lithology is composed by schist and granite rocks, with low rate of mineralization, perceptivel on physical and chemical parameters, with low values of EC25 conductivity <50 µS.cm^{-1}, alkalinity <30 mg $HCO_3^-.L^{-1}$, total hardness <25 mg $CaCO_3.L^{-1}$, and nutrients such as nitrate NO_3^- <0.5 mg.L^{-1} and phosphates PO_4^{3-} <0.1 mg.L^{-1}. Riparian vegetation is well structured and developed and dominated by alder (*Alnus glutinosa*), but willow (*Salix* sp.), poplar (*Populus nigra*) and ash (*Fraxinus angustifolia*) trees are also present (NOGUEIRA, 2011). Generally a good or excellent ecological integrity can be found in streams of NPM. However, there are some negative impacts like river regulation (e.g. Serra Serrada and Alto Sabor dams) or mineral (e.g. Portelo mine) and organic pollution (e.g. Fervença River) and other human induced impacts are reported by several studies (NOGUEIRA, 2011; PARADA, 2012).

Sixteen sampling sites were selected and distributed along the River Portelo (P1 to P8) and River Fervença (F1 to F8) (Table 2.1).

Table 2.1. Location (geographical coordinates) of sampling stations in Sabor Basin.

Basin	River	Location (village)	Symbol	Latitude	Longitude
Sabor	**Portelo**	**Mine wastes**	**P1**	**41°56'0.33"N**	**6°44'14.93"W**
Sabor	**Portelo**	**Tributary**	**P2**	**41°55'57.58"N**	**6°44'16.25"W**
Sabor	**Portelo**	**After confluence**	**P3**	**41°55'57.74"N**	**6°44'13.73"W**
Sabor	**Portelo**	**Portelo village**	**P4**	**41°55'42.43"N**	**6°43'46.27"W**
Sabor	**Portelo**	**Near Calabor river**	**P5**	**41°55'59.29"N**	**6°43'3.34"W**
Sabor	**Calabor**	**Calabor**	**P6**	**41°56'4.54"N**	**6°42'53.12"W**
Sabor	**Aveleda**	**França**	**P7**	**41°51'48.47"N**	**6°44'4.80"W**
Sabor	**Sabor**	**P. Campismo**	**P8**	**41°51'22.17"N**	**6°44'39.96"W**
Sabor	**Fervença**	**Grandais**	**F1**	**41°48'59.21"N**	**6°48'58.91"W**
Sabor	**Fervença**	**Castro Avelãs**	**F2**	**41°47'40.50"N**	**6°48'2.95"W**
Sabor	**Fervença**	**Bragança (industrial)**	**F3**	**41°47'39.26"N**	**6°47'6.66"W**
Sabor	**Fervença**	**Bragança (ESA)**	**F4**	**41°47'54.38"N**	**6°45'53.90"W**
Sabor	**Fervença**	**Bragança (Polis)**	**F5**	**41°48'7.33"N**	**6°45'32.83"W**
Sabor	**Fervença**	**Qta dos Figueiredos**	**F6**	**41°46'33.84"N**	**6°42'53.33"W**
Sabor	**Fervença**	**Near Alfaião**	**F7**	**41°46'14.11"N**	**6°42'0.46"W**
Sabor	**Fervença**	**Confluence Penacal**	**F8**	**41°45'38.96"N**	**6°42'15.09"W**

The sampling sites were selected along the longitudinal gradient of each river, taking into consideration three type of sites: 1) **reference** (P2, P6, F1, F2), 2) **moderated disturbed** (P7,P8,F3,F4,F5**)**, and 3) **highly disturbed** sites (P1,P3,P4,P5,F6,F7,F8), affected by different impacts (e.g. mineral and organic pollution), in order to assess their ecological integrity (Figures 2.2 to 2.7).

Figure 2.2. Reference sampling sites (P2, P6) in Portelo Basin (near the village of Portelo).

Figure 2.3. Moderated disturbed sampling sites (P7, P8) in Sabor Basin (near the village of França).

Figure 2.4. Highly disturbed sampling sites (P3, P4) in Portelo Basin (near the village of Portelo).

Figure 2.5. Reference sampling sites (F1, F2) in Fervença Basin (near the village of Grandais).

Figure 2.6. Moderate disturbed sampling sites (F3, F5) in Fervença Basin (near Bragança city).

Figure 2.7. Highly disturbed sampling sites (F6, F8) in Fervença Basin (near the village of Alfaião).

2.2.2. Water quality: Physical and chemical parameters

A brief assessment of water quality was made, based on the physical and chemical measurements of the following parameters: A) ***in situ,*** using portable probes 1) Dissolved Oxygen (mg $O_2.L^{-1}$); 2), Temperature (°C); 3), Total Dissolved Solids (TDS mg/L), 4), Electrical Conductivity EC25 (µS.cm^{-1}), 5) pH and 6); Turvation (qualitative scale- from 1 minimum to 5 maximum) ; B) **in the laboratory** from water samples (1.5 L) transported in coolers (maintaining the temperature of 4°C) : 1) Nitrates (mg $NO_3^{-}.L^{-1}$), 2) Nitrites (mg $NO_2^{-}.L^{-1}$) and 3) Total Phosphorus (mg P_{total}/L) were only determined for Fervença river. All procedures were performed and determined according to the APHA (2005). The interpretation of the results was based on Portuguese legislation (Decreto-Lei 236/98 of 1 August). The physical and chemical parameters were measured in the spring season (April 2016), after the rainy period resulting in high discharge with flood events occurred in Sabor river basin (Figure 2.8).

Figure 2.8. In situ measurement of water quality in River Calabor (P6) (spring 2016).

2.2.3. Habitat evaluation: Channel and Riparian Indexes

The habitat evaluation in this study was made recurring to the indexes also adapted to the rivers of the Iberian Peninsula, from which it was possible to classify the hydromorphology and riparian quality of the sampling sites. Two following indexes, calculated in spring season of 2016, were used:

1) Habitat Index of Riparian Quality- QBR

The QBR index (MUNNÉ et al., 2003) was developed for the use of environmental managers and planners at national and regional levels to report on the riparian condition of streams. The QBR index is based on four compartments of riparian habitat: total riparian vegetation cover, cover structure, cover quality and channel alterations. This index also takes into account differences in the geomorphology of the river from its headwaters to the lower reaches. The differences are measured in a simple and quantitative way and the final index score varies between 0 and 100 points (see *Annex I*, for details). There are five quality classes of riparian habitat (Table 2.2) which broadly correspond to those suggested in the Water Framework Directive (DIRECTIVE 2000/60/EC).

Table 2.2. Quality classes according to the QBR index (MUNNÉ et al. 2003)

QBR range	Colour/Class	Riparian habitat quality class
≥ 95	I	Riparian habitat in natural condition
75 – 90	II	Some disturbance, good quality
55 – 70	III	Important disturbance, fair quality
30 – 50	IV	Strong alteration, poor quality
0 – 25	V	Extreme degradation, bad quality

2) Index of Channel Quality- GQC

The habitat assessment considering the scoring system of Channel Quality, GQC index (CORTES et al. 1999), is a broad measure of the physical structure, taking into account not only the channel conditions but also some river corridor features. The GQC index is based on eight components: Presence of artificial obstacles (e.g. weirs, dams), in-channel structure, sediments and stability of the channel, bank structure, and artificial alterations of the banks, channel heterogeneity, river bottom structure and embebedness. The final index score varies between 8 and 31 points (see *Annex II*, for details). There are five quality classes of channel habitat (Table 2.3).

Table 2.3. Quality classes according to the GQC index (CORTES et al. 1999)

GQC range	Colour/Class	Channel habitat quality class
≥ 31	I	Channel habitat in natural conditions, excellent quality
26 – 30	II	Some disturbance, good quality
20 – 25	III	Initial of important alteration of the channel, fair quality
14 – 19	IV	Strong alteration, poor quality
8 – 13	V	Extreme degradation (channelization, regulation) bad quality

2.2.4. Biota: Benthic Macroinvertebrate Communities

2.2.4.1. Sampling Procedures

The sampling procedures used for benthic macroinvertebrate communities followed the protocol established in the ambit of the Water Framework Directive (WFD) and commonly applied in Portugal (INAG 2008a).

Figure 2.9. Sampling procedures of benthic invertebrates collection (winter 2016).

From November 2015 until May 2016, with monthly periodicity, in each sampling site, a section of 50 m was selected and sampled. Different habitat (riffle, pool and run) and microhabitat (e.g. fine and coarse materials, leaves, and aquatic plants) were identified in erosion (turbulent flow) and adjacent sedimentation (laminar flow) units. Six subsamples were, then, collected according to their representativeness in the sampling river zone, using a handnet (25*25 cm dimensions, 500 μm of mesh size) and removing the substrata with the foot (1 meter extension) (Figure 2.9). Attached invertebrates to stable substrata (e.g. gastropods, caddisflies) were also collected using appropriate brushes. Invertebrates were captured and kept alive (in polyethylene bottles with river water coolers transported at low temperature) in order to facilitate the processing of the samples. In the laboratory, invertebrates were sorted and subsequently preserved in 70% alcohol. All invertebrates were counted and identified using a stereomicroscope SMZ10 with 10-132*x* zoom magnification and appropriate dichotomous keys (e.g. TACHET et al. 1981, 2010) (Figure 2.10). Except for Oligochaeta and Acari (Subclass level) all organisms were identified to the Family level.

Figure 2.10. Laboratorial procedures: macroinvertebrate sorting and identification.

2.2.4.2. Metrics used to environmental quality evaluation

The biotic response based on macroinvertebrate communities was evaluated through uni and multimetric variables, determined with the Software AMIIB@ (*http://dqa.inag.pt/implementacao_invertebrados_AMIIB.html*). Several metrics were calculated, such as:

1) Number of individuals (N) and number of *taxa* (S);

2) Diversity (*e.g.* H' index of Shannon-Wienner); (described in subparagraph 6)

3) Evenness (*e.g.* J' index of Pielou); (described in subparagraph 6)

4) Relative abundance of Ephemeroptera, Plecoptera and Trichoptera orders (% EPT);

5) Biotic Index IBMWP

The IBMWP index (ALBA-TERCEDOR. 1996) is a fast and simple method for assessing the biological quality of freshwater ecosystems, since only requires the identification of organisms to the taxonomic level of family. To each family is given a score ranging between 10 and 1 score, according to a gradient of pollution tolerance (see *Annex III*). The calculation of IBMWP final score is done by the sum of all the scores of the families present in each sample, classifying the biological quality of water on 5 defined classes (Table 2.4).

Table 2.4. Quality classes according to the IBMWP index

IBMWP range	Colour/Class	Biological quality class
> 100	I	Clean water, Excellent quality
61 – 100	II	Light polluted, Good quality
36 – 60	III	Moderately Polluted, Fair Quality
16 – 35	IV	Strongly polluted, Poor quality
<15	V	Extremely polluted, Bad quality

6) Portuguese Northern Invertebrate Index- $IPtI_N$

The **multimetric Index $IPtI_N$** (INAG 2009) integrates different metrics like nº of *taxa*, EPT, evenness of Pielou J’, diversity of Shannon-Wienner H’, IASPT and Sel. ETD, combined as presented in the following formula:

$$IPtI_N = N^o\ taxa * 0.25 + EPT * 0.15 + Evenness * 0.1 * (IASPT - 2) * 0.3 + Log(Sel.\ ETD + 1) * 0.2$$

where:

- **EPT**: Nº families belonging to Ephemeroptera, Plecoptera, Trichoptera orders;
- **Evenness**: Defined as Pielou index or evenness and calculated by the formula:

$$E = H'/Ln\ S$$ where:

H’ - diversity of Shannon-Wienner

S - number of present *taxa*

Ln - natural or neper logarithm

The **H’ Shannon-Wienner Index** is calculated by the formula:

$$H' = -\sum p_i\ Ln\ p_i$$ where:

$p_i = n_i/N$

n_i- nº of individulas of each *taxon i*

N- total nº of individuals present in sample

- **IASPT**: Iberian ASPT, corresponding to IBMWP (ALBA-TERCEDOR 2000) divided by the number of families present in the sample;
- **Log (Sel. ETD+1)** - Log_{10} of (1 + sum of individuals abundance of the families Heptageniidae, Ephemeridae, Brachycentridae, Odontoceridae, Limnephilidae, Goeridae, Polycentropodidae, Athericidae, Dixidae, Dolichopodidae, Empididae, Stratiomyidae);

The final score of **$IPtI_N$** depends on the sum of the weighted metrics. Two steps of normalization are performed and the index expressed in terms of Ecological Quality Ratio (RQE). For the normalization process is necessary to determine the ratio between the observed value and the reference value of each river type (median reference sites) (INAG 2009).

Table 2.5 shows the reference values and boundary values between the quality classes in RQE, according to the type of each sampling site selected in this study, taking into account the boundaries of the adjustment relating to classification criteria, revised in the Management Plan of River Basin in 2016 / 2021 (APA 2015).

Table 2.5. Median reference values and boundaries for the type of rivers under study (APA, 2015).

River Tipology	Reference Values	Excellent	Good	Fair	Poor	Bad
Northern Rivers Small Dimension (N1 < 100 km^2)	1.02	≥0.87	[0.68-0.87[	[0.44-0.68[	[0.22-0.44[	[0-0.22[
Northern Rivers Medium-Large Dimension (N1 > 100 km^2)	1.00	≥0.88	[0.68-0.88[	[0.44-0.68[	[0.22-0.44[	[0-0.22[

7) Functional Feeding Groups

As defined by MERRITT & CUMMINS (1996) “the macroinvertebrate functional feeding group (FFG) method is based on the association between a set of feeding adaptations found in freshwater invertebrates and their basic nutritional resources categories”. A general classification system for aquatic invertebrate trophic relations is presented in the following table (Table 2.6).

Table 2.6. Classification for invertebrate trophic relations (MERRITT & CUMMINS, 1996).

Functional Group	Dominant Food	Feeding mechanism	Examples of taxa	Particle size of Food (µm)
Shredders	Living vascular hydrophyte plant tissue	Herbivores- chewers and miners of live macrophytes	Trichoptera	> 10^3
	Decomposing vascular plant tissue and wood- coarse particulate organic matter (CPOM)	Detritivores- chewers, wood borers, and gougers	Diptera Plecoptera	
Collectors	Decomposing fine particulate organic matter (FPOM)	Detritivores- filterers or suspension feeders	Hydropsychidae Simuliidae	<10^3
		Detritivores- gatherers or deposit (sediment) feeders	Ephemeridae Chironomidae	
Scrapers	Periphyton- attached algae and associated material	Herbivores- grazing scrapers of mineral and organic surfaces	Glossosomatidae Heptageniidae	< 10^3
Predators (engulfers)	Living animal tissue	Carnivores- attack prey, pierce tissues, cells and suck fluids	Hemiptera	
	Living animal tissue	Carnivores- ingest whole animals (or parts)	Perlidae	>10^3

2.2.5. Biota: Fish Communities

Monitoring of fish communities present in all sampling sites of both Portelo and Fervença rivers was performed by electrofishing (Figure 2.11) in April of 2016. The methodology used was according to the Water Framework Directive (INAG 2008b). It was used a portable electrofishing device with direct current output (ELT Hans Grassl, 300-600V), adapting the type of electric current to the conductivity values of water. All fish caught were identified, measured (precision 0.1 cm) and then returned to the river.

2.2.6. Data treatments

Data treatment was made through the univariate and multivariate analysis. The assessment of the significant differences was made taking into consideration the definition of three groups, i.e. 1) Reference, 2) Moderately disturbed and 3) Highly disturbed. Several analyses were done for physical, chemical parameters and different metrics by using non-parametric tests, such as Kruskall-Wallis test (H), since the data do not fit the normal distribution

(Bartlet was performed test). These analyzes, as well as BOX-WHISKERS graphics were performed with STATISTICA 7.0 package (STATSOFT 2004).

Figure 2.11. Fish sampling and biometric data obtained after electrofishing. April 2016.

A multivariate analysis technique was applied, including the multidimensional non-metric (NMDS) analysis, consisting of a sorting method, based on ranks established from the BRAY-CURTIS similarities of the abundance of macroinvertebrates data. It was also applied a non-parametric test (one-way ANOSIM tests) using Bray-Curtis similarity matrix to assess significant differences between sampling locations. These analyzes were performed with the package PRIMER 6 & PERMANOVA + β17 (CLARKE & GORLEY, 2006). Data from the invertebrate abundances were previously transfomed [Log (x + 1)].

2.3. RESULTS

The results allowed characterizing the physical and chemical parameters of water quality, the riparian and aquatic habitats and the impacts of mineral and organic pollution on macroinvertebrate and fish communities.

2.3.1. Physical and chemical analyses of water quality

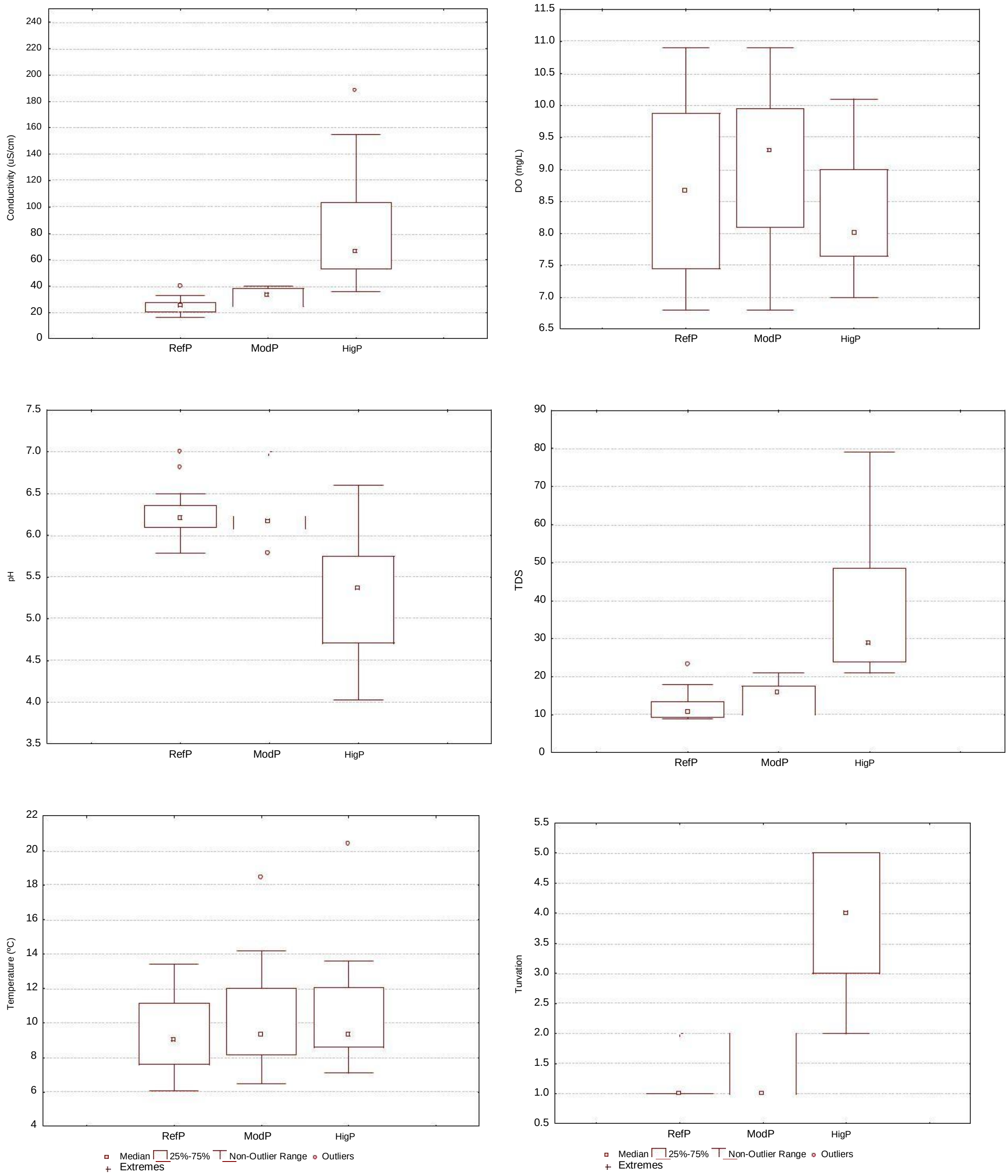

Figure 2.12. Dispersion of the physicochemical parameters for all sampling sites of Portelo basin, grouped according to the degree of disturbance: RefP- reference P2, P6; ModP- Moderated Disturbed P7, P8; and HigP- highly disturbed sites P1, P3, P4, P5. Parameters were obtained monthly, i.e. from November 2015 to April 2016.

The dispersion of the physical and chemical parameters for all sampling sites of Portelo (Figure 2.12) and Fervença (Figure 2.13) basins, are grouped according to the degree of disturbance, i.e. Reference (RefP, RefF), Moderated Disturbed (ModP, ModF) and Highly disturbed (HigP, HigF) sites.

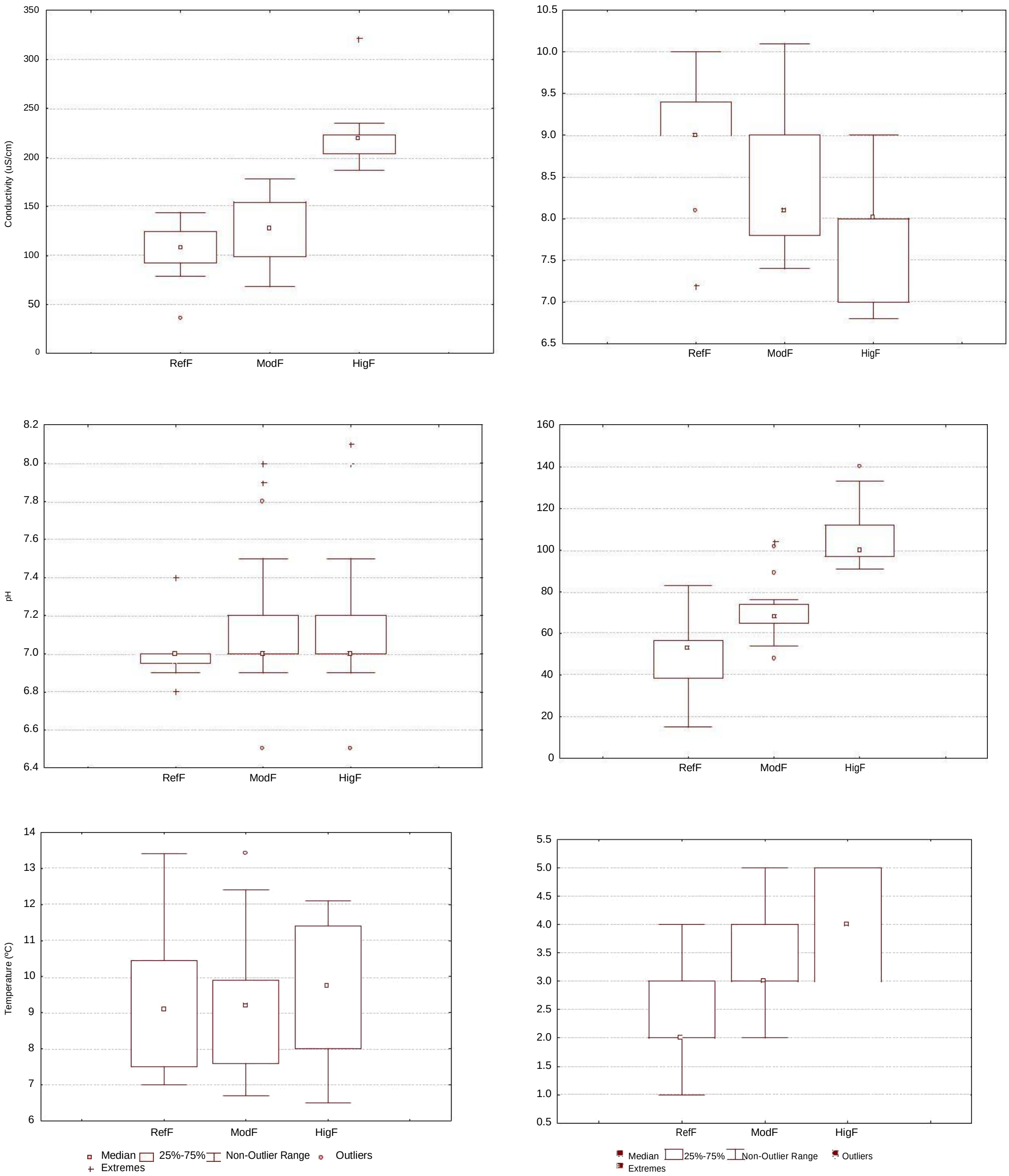

Figure 2.13. Dispersion of the physicochemical parameters for all sampling sites of Fervença basin, grouped according to the degree of disturbance: RefP- reference F1, F2; ModP- Moderated Disturbed F3, F4, F5; and HigP- highly disturbed sites F6, F7, F8. Parameters were obtained monthly, i.e. from November 2015 to April 2016.

The analysis of physical and chemical parameters of water, taking into consideration the Portuguese legislation (D.L. 236/98) showed some parameters outside of limit values, in particular:

A) **Portelo basin-** Analysing the dispersion charts it is possible verify the differences relatively to the parameters of pH, conductivity EC25, Total suspended Solids and Turvation between Reference (RefP) and Moderated disturbed (ModP) sites against highly disturbed (HigP). In fact, the pH of water, near the mine (P1, P3) reached minimum values of 4.0. This pattern was accompanied by the increase of conductivity (EC25 > 150 μS/cm), TDS (> 100 mg/L) and turvation (qualitative scale), as a result of the input of fine sediment from the waste deposits of the Portelo Mine. These impacts seemed to create more harsh environmental conditions after rainy periods, as verified during the autumn/winter season of 2015/2016.

B) **Fervença basin-** Relatively to the variation of physical and chemical parameters in Fervença basin, it was observed a similar tendency to the augmentation of dissolved and particulate substances in the aquatic ecosystem. In fact, higher values measured in Highly disturbed sites (HigF- F6, F7, F8) of conductivity (EC25 > 200 μS/cm), TDS (> 100 mg/L) and lower values of Dissolved Oxygen (DO < 8 mg/L) are typical of organic polluted rivers. The dilution effects related with higher discharge occurred during winter season contributed to the recovery of the aquatic ecosystem. However, this seasonal trend and the alternate periods of drought (summer season) and flood (winter season) events, tend to create also harsh environmental conditions and only resistant organisms can survive and colonize successfully these polluted systems.

The dispersion charts (Figure 2.12 and 2.13) showed for most variables a good power discriminant. In fact, highly significant differences were detected (P <0.001, Kruskal-Wallis H-test), namely between both Reference and moderated vs. highly disturbed groups, considering the physical and chemical variables of conductivity, pH, TDS and turvation for mineral pollution (Portelo river) and conductivity, DO and TDS for organic pollution (Fervença river).

In terms of Nitrogen and Phosphorus contents, determined for organic pollution (Fervença basin), the majority of sampling sites have lower values (<0.7 mg/L) but if there is a decrease in the scale (μg/L), it could have a meaning value. However, in downstream zones, after the influence of Bragança city, nitrate concentrations are notably higher than the other sites (e.g. maximum value of 4 mg/L in F7), explained by the influence of urban wastes from the city of Bragança (Figure 2.14).

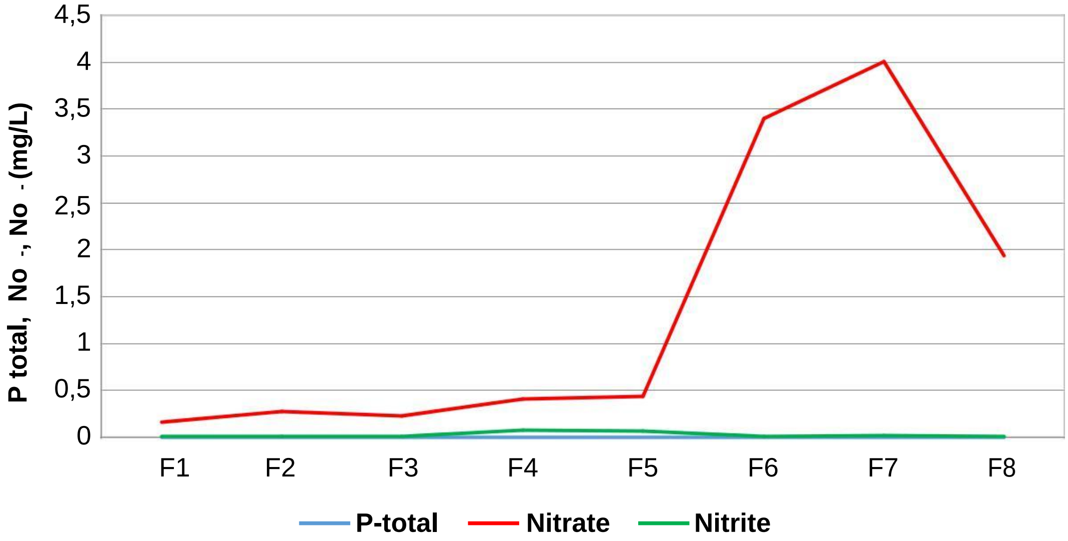

Figure 2.14. Variation of of Nitrite, Nitrate, Total Phosphorus (mg / L) in river Fervença (spring 2016)

2.3.2. Quality of aquatic and riparian habitats

The QBR and GQC indexes showed a good hydromorphological quality for reference sites of both rivers basins. Moderated disturbed sites present some signs of riparian and channel perturbation. The most disturbed sites corresponded to urban (F5, Bragança city- Polis Zone) and rural (P4, Portelo village) areas negative human impacts were more visible (Figure 2.15).

Figure 2.15. Excellent (left) and Bad (right) morphological conditions in Portelo river basin.

The classification of both QBR and GQC indexes for Portelo and Fervença river basins can be visualized in Table 2.7.

Table 2.7. Values of QBR and GQC indexes of Portelo and Fervença river basins.

River	Sampling site	Final Score		Classification	
		QBR	GQC	QBR	GQC
Portelo	P1	55	22	III	III
Portelo	P2	100	34	I	I
Portelo	P3	85	22	II	III
Portelo	P4	10	13	V	V
Portelo	P5	85	20	II	III
Calabor	P6	100	31	I	I
Aveleda	P7	60	26	III	II
Sabor	P8	70	28	III	II
Fervença	F1	85	30	II	II
Fervença	F2	75	32	II	I
Fervença	F3	80	27	II	II
Fervença	F4	80	29	II	II
Fervença	F5	10	15	V	IV
Fervença	F6	70	22	III	III
Fervença	F7	75	25	II	III
Fervença	F8	75	25	II	III

2.3.3. Macroinvertebrate Communities

During the study, considering the 16 sampling sites and 6 sampling periods, it were identified 38,083 individuals, distributed by 74 faunistic groups, mainly families, except for Oligochaeta and Acari (only subclass taxonomic level). The distribution of total number of individuals and taxa, for each type of disturbance (mineral and organic pollution), are presented in figures 2.16 to 2.19.

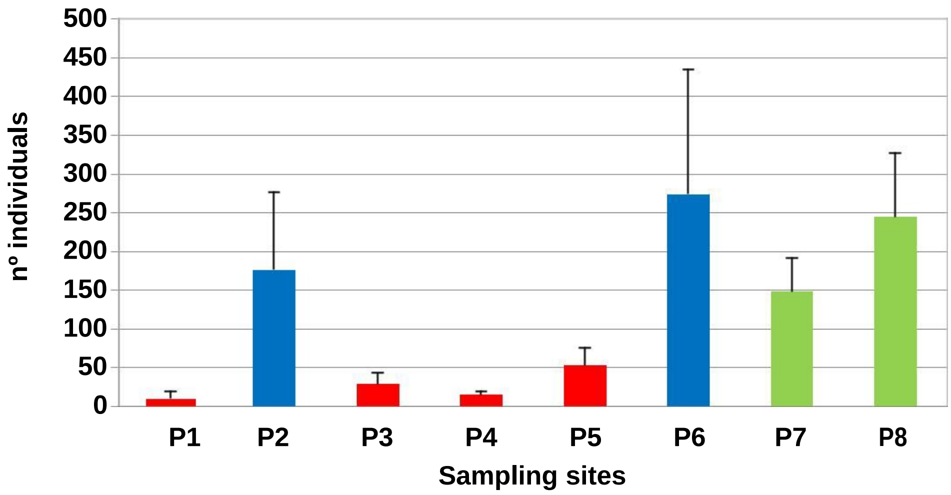

Figure 2.16. Number of individuals (mean+SD) captured in Portelo basin (blue bars- reference; green bars- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

Low number of invertebrates (< 50) was captured in highly disturbed sampling sites affected by mineral pollution (near mine collapse), comparatively with moderately disturbed (diluted effect) and reference (tributaries) sites.

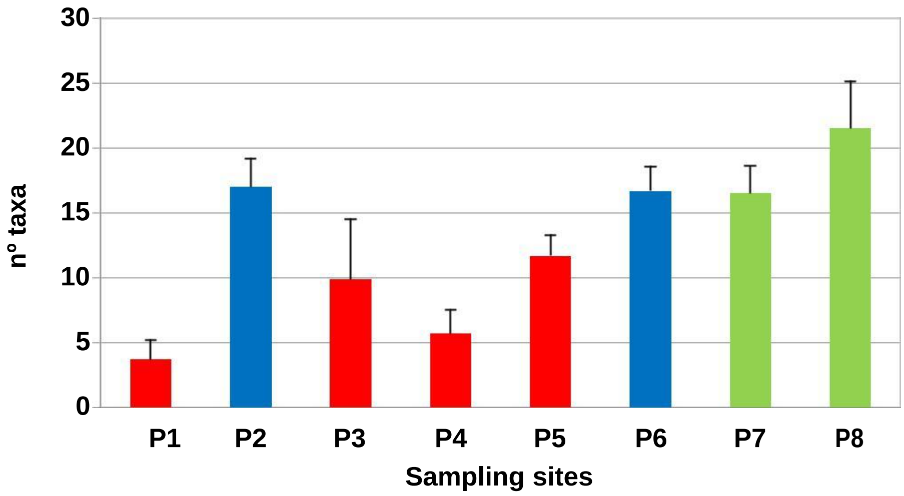

Figure 2.17. Number of taxa (mean+SD) captured in Portelo basin (blue bars- reference; green bars-moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

Furthermore, the same trend was observed for the number of taxa, i.e. lower in highly disturbed sites. Apparently, reference and moderated disturbed sites did not differ too much for these two metrics. However, differences in habitats must be considered (P2, P6- small tributaries *versus* P7, P8 main rivers).

A different tendency was observed in River Fervença. In fact, the higher abundance was obtained in the highly disturbed (> 350 individuals) sites.

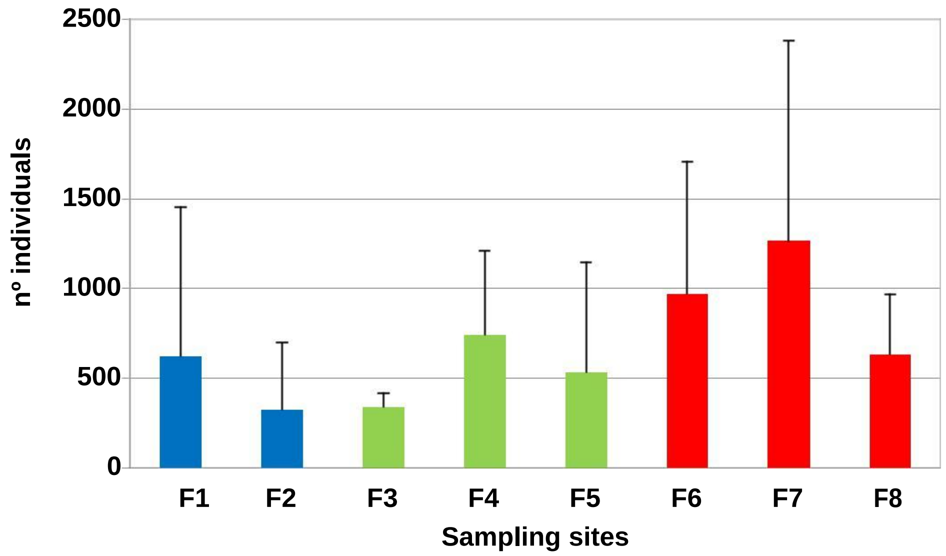

Figure 2.18. Number of individuals (mean+SD) captured in Fervença basin (blue bars- reference; green bars- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

However, the lower richness of taxa corresponded, precisely, to the highly disturbed (< 10 taxa) sites, revealing the euribiont character of most organisms found in these sampling sites (e.g. Gastropoda, Diptera and Annelida taxa).

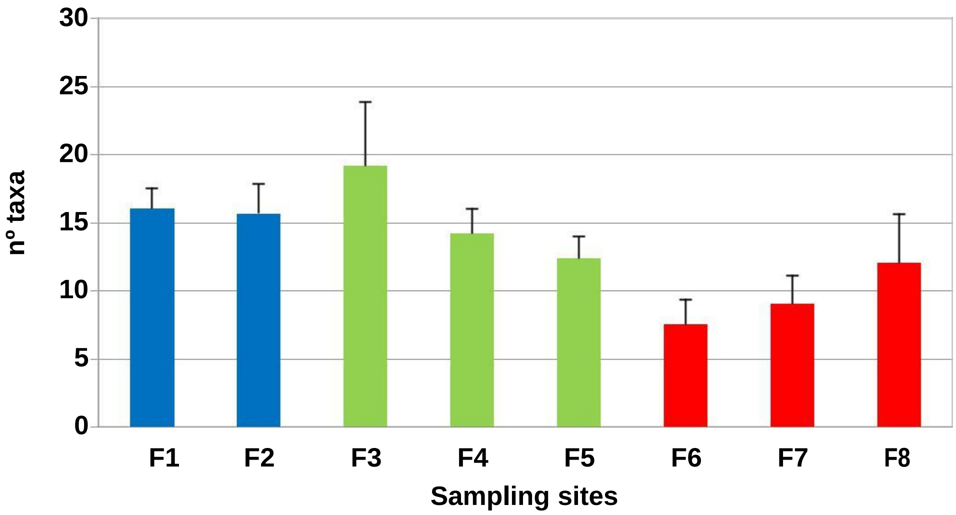

Figure 2.19. Number of taxa (mean+SD) captured in Fervença basin (blue bars- reference; green bars- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

2.3.3.1. Diversity (H') and Equitability (J') indexes

The variation of Shannon-Wienner diversity (H') and Pielou Equitability (J'), determined for all sampling sites, can be visualized in figures 2.20 to 2.23. The results obtained showed, as expected, an important reduction of aquatic macroinvertebrate diversity in both mineral and organic polluted sites.

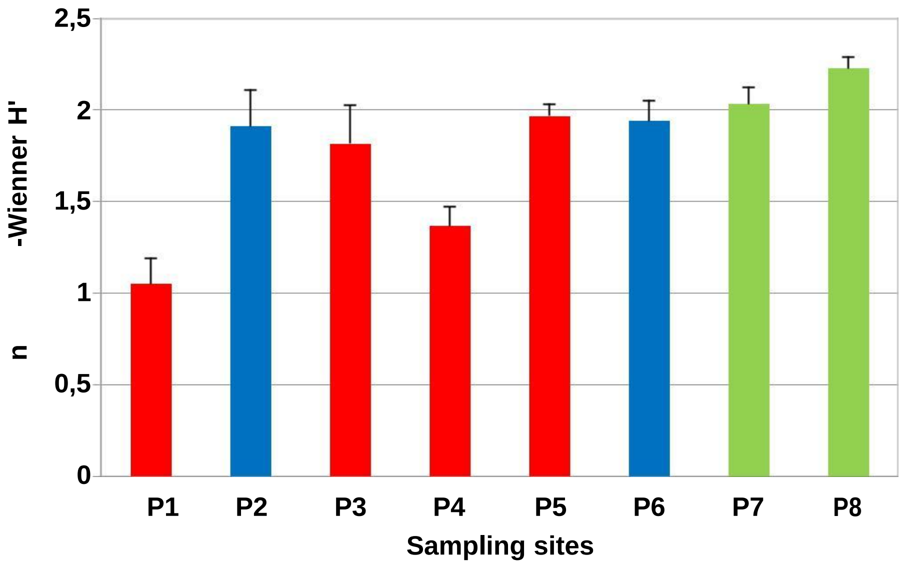

Figure 2.20. Shannon-Wienner diversity (H') (mean+SD) of Portelo basin (blue- reference; green- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

In Portelo river the decrease on H' diversity index was accompanied by the higher values of J' evenness index. Indeed, the harsh and inpredictable environmental conditions, suddenly created in this lotic system, can explain the lack of *taxa* dominance.

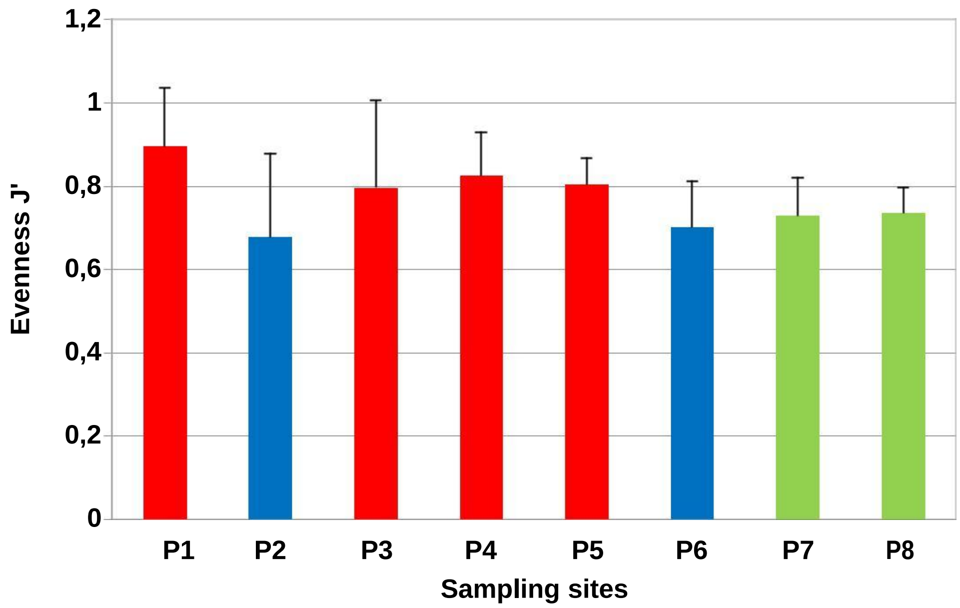

Figure 2.21. Pielou Evenness (J') (mean+SD) of Portelo basin (blue- reference; green- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

For Fervença river it was found lower values for both indexes, H' and J', as a result of environmental conditions more predictable for the organisms that usually live in organic polluted ecosystems, resulting from domestic wastes of urban and rural areas.

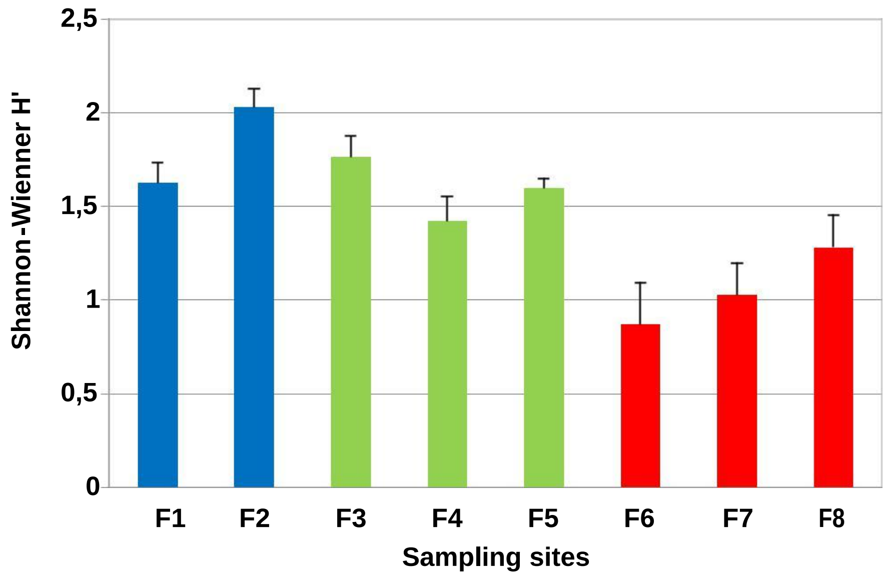

Figure 2.22. Shannon-Wienner diversity (H') (mean+SD) of Fervença basin (blue- reference; green- moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

In fact, the input of organic pollution implies the desapearence of stenobiont organisms, like several insects (e.g. Plecoptera, Ephemeroptera and Trichoptera), since their bioecological requirements don't allow the optimal conditions for the survival in these ecosystems. For that reason, the decrease in biodiversity normally means the increasing abundance and biomass, concentrated in lower number of taxa (e.g. Dipera, Annelida, Mollusca), more adapted to the available resources.

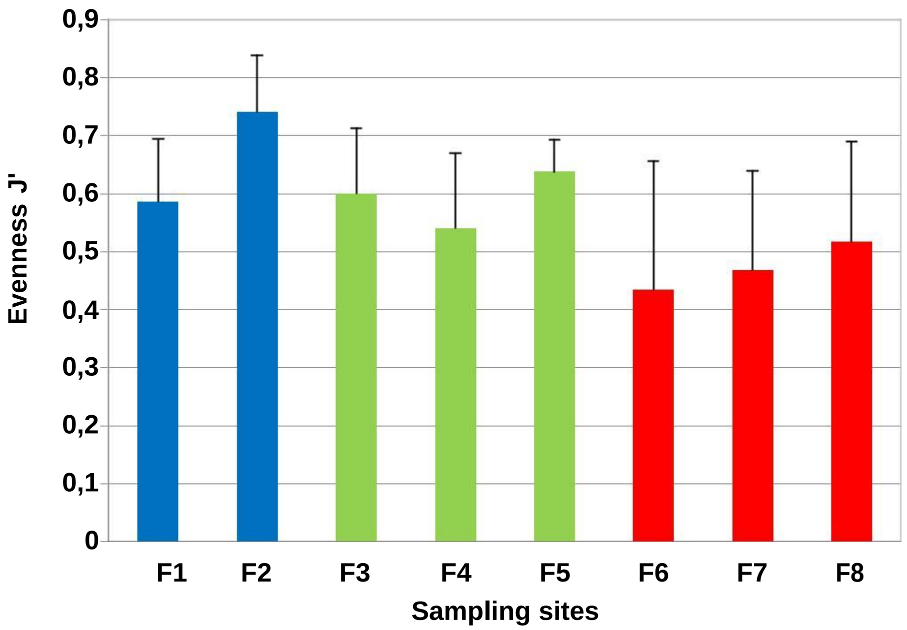

Figure 2.23. Pielou Evenness (J') (mean+SD) of Fervença basin (blue bars- reference; green bars-moderated disturbed; red bars- highly disturbed sites) (November 2015 to April 2016).

2.3.3.2. Faunal composition

The faunal composition of River Portelo basin is presented in Figure 2.24.

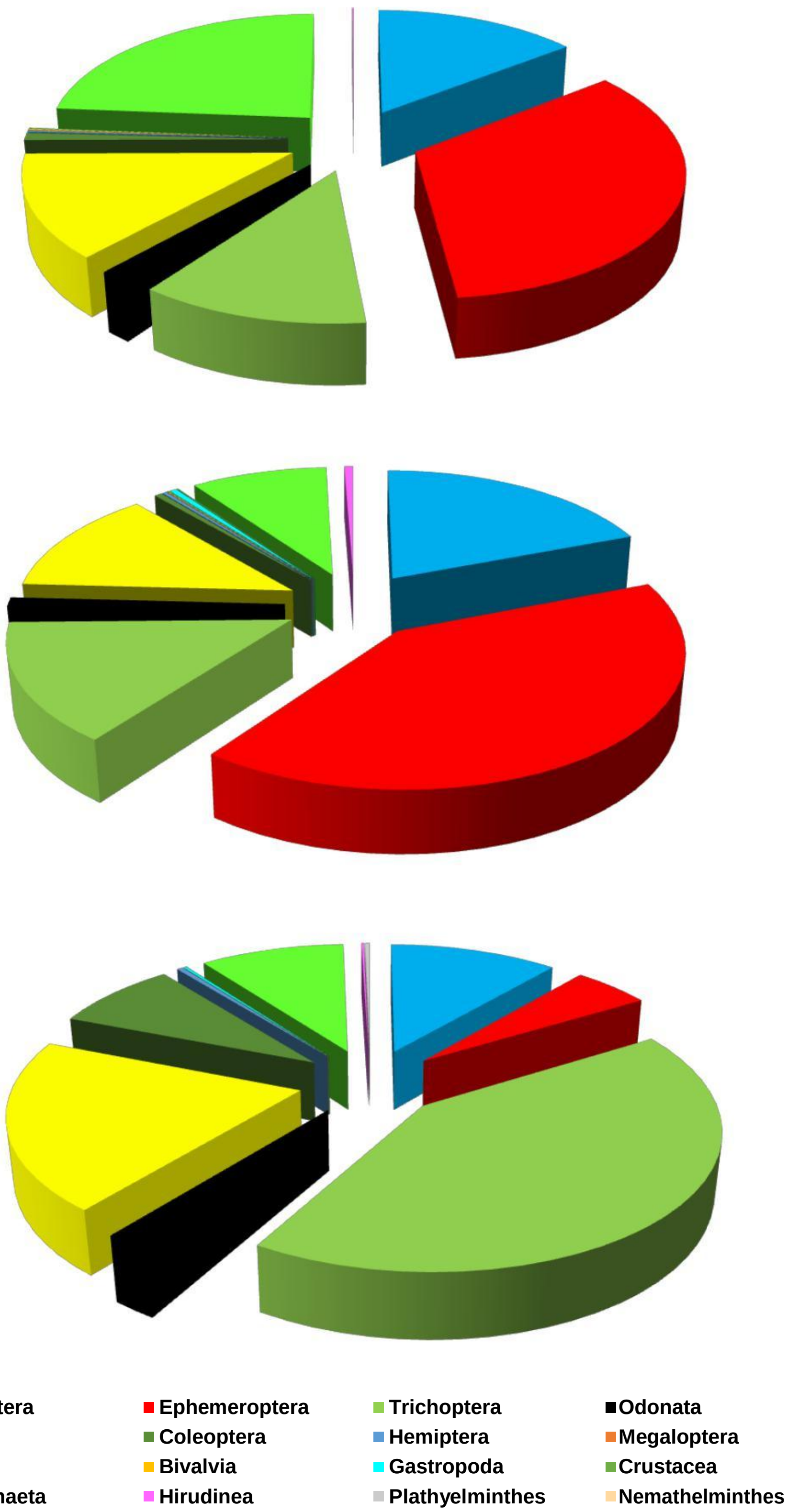

Figure 2.24. Faunal composition of invertebrates present in Portelo basin, distributed into 3 groups: reference (P2, P6); moderated disturbed (P7, P8) and highly disturbed (P3, P4, P5), considering the abundance (mean values) of all sampling periods (November 2015 to April 2016).

A more focused analysis, based on the monthly variability, showed different patterns from November to April (Figure 2.25).

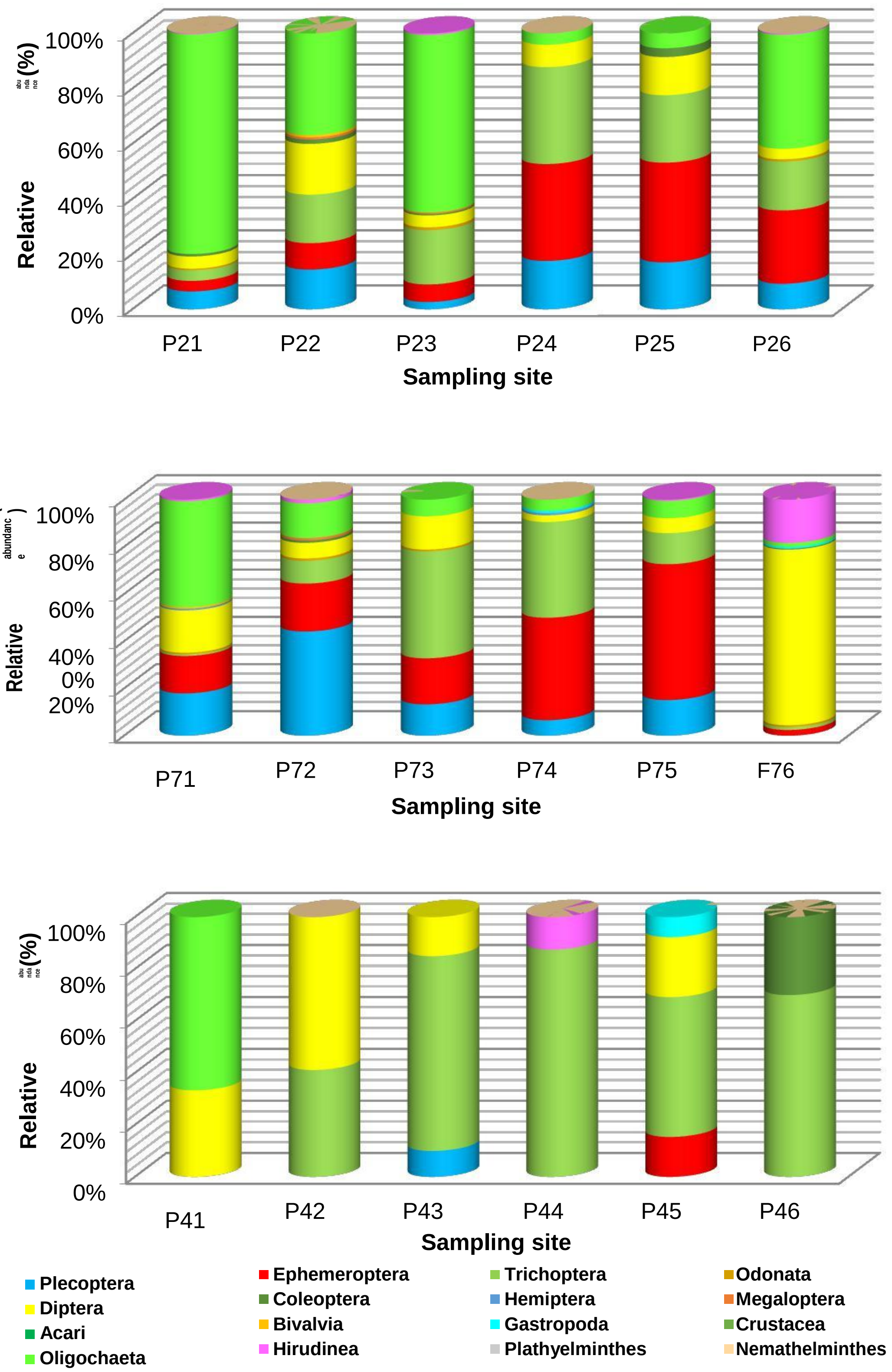

Figure 2.25. Monthly variation (relative abundance) on faunal composition in 3 sampling sites: 1) reference (P2); 2) moderated disturbed (P7); 3) highly disturbed (P4) (November 2015 to April 2016).

Relatively to the effects of mineral pollution (Portelo basin) on benthic macrofauna, the mais aspects that must be highlighted are:

- Dominance of Ephemeroptera, Plecoptera and Trichoptera (EPT taxa), normally bioindicators good ecological integrity of lotic ecosystems. Reference sites showed the higher relative abundance (EPT> 70%), comparatively to moderated (EPT = 60%) and highly (EPT= 58%) polluted sites;
- Low number of individuals were found in highly disturbed sites, namely in P1 and P4, however some of them belonging to stenobiont families, like Nemouridae (Plecoptera) Limnephilidae (Trichoptera) cohabiting with other families more resistent to environmental degradation, such as Chironomidae, Tabanidae, Tipulidae (Diptera) and Oligochaeta;
- A monthly/seasonal variability among macroinvertebrate assemblages, more visible and pronounced in reference (e.g. P2) and moderated disturbed sites (e.g. P7) than in highly disturbed sampling site (e.g. P4);

Relatively to the effects of organic pollution (Fervença basin) on macrofauna, several points deserve the following comments (Figures 2.26 and 2.27):

- Dominance of Ephemeroptera, Plecoptera and Trichoptera (EPT taxa), only on reference sites (P1, P2) (EPT> 55%), comparatively to moderated (EPT = 29%) and highly (EPT= 2%) polluted sites;
- The most representative families detected in reference sites and known by their high sensibility to pollution were Capniidae, Perlodidae, Nemouridae (Plecoptera) Baetidae, Siphlonuridae (Ephemeroptera) Polycentropodidae, Limnephilidae (Trichoptera);
- In the opposite, the representativeness of resistant organisms found in River Fervença, namely after the Sewage Treatment Powerpalnt Station of Bragança city, were Simuliidae, Chironomidae, Psychodidae (Diptera), Erpobdellidae, Glossiphoniidae Hirudidae (Hirudinea), Physidae (Gastropoda) and Oligochaeta;
- Similarly to the trend observed for mineral pollution a more detectable variation was found in reference and moderated disturbed sites. Conversely, highly disturbed sites mantain more stability in the abundance of euribiont taxa along the sampling period of this study.

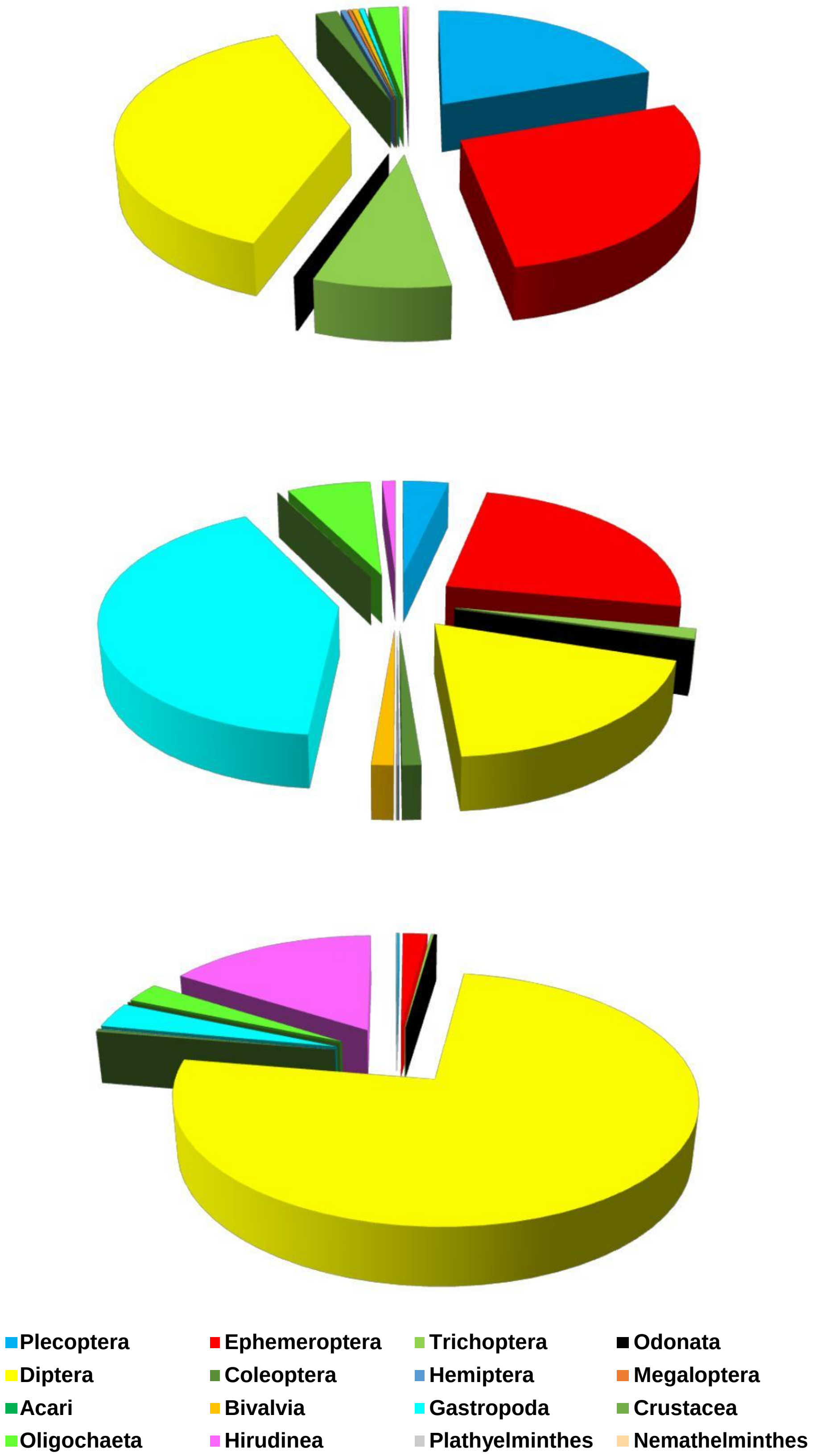

Figure 3.26. Faunal composition of invertebrates present in Fervença basin, distributed into 3 groups: reference (F1, F2); moderated disturbed (F3, F4, F5) and highly disturbed (F6, F7, F8), considering the abundance (mean values) of all sampling periods (November 2015 to April 2016).

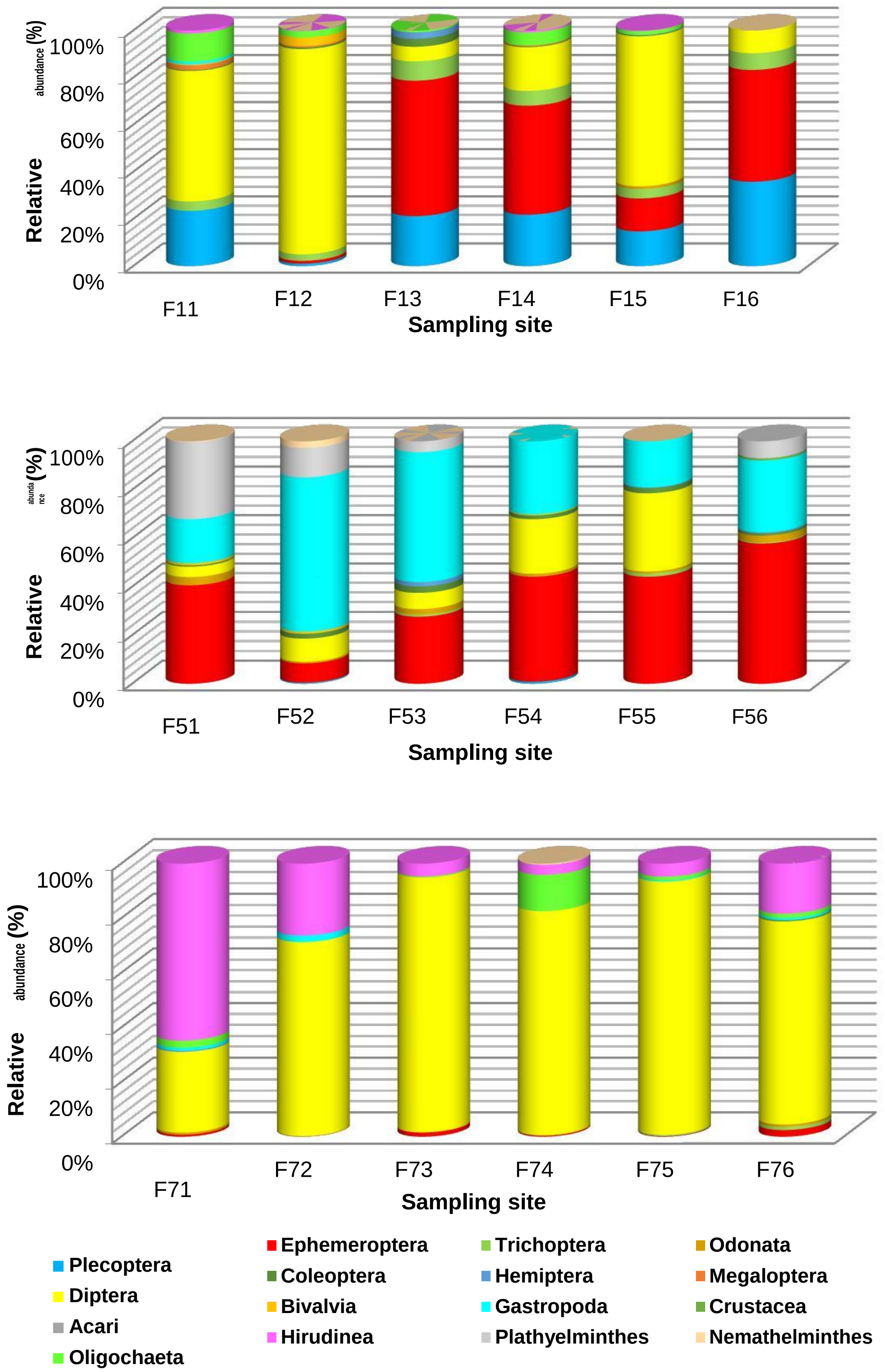

Figure 2.27. Monthly variation (relative abundance) on faunal composition in 3 sampling sites: 1) reference (F1); 2) moderated disturbed (F5); 3 highly disturbed (F7) (November 2015 to April 2016).

2.3.3.3. IBMWP and IPtI$_N$ indexes

Both IBMWP and IPtI$_N$ indexes were sensible to detect the most disturbed sampling sites (Table 2.8). From the results obtained was possible:

- Identify the most disturbed sampling sites (Portelo P1, P4, and (Fervença F6, F7, F8), usually colonized by euribiont families. In fact, the classification of ecological state was mainly **Fair**, **Poor** and sometimes **Bad**, reinforcing the tendency assigned by several metrics calculated by the AMIIB software;
- Highlight the excellent and good biotic condition for most undisturbed sampling sites (i.e. reference sites: F1, F2; P2; P6) for both basins;

Table 2.8. Biological classification using IPtIN and IBMWP indexes for all sampling sites and periods (November 2015 to April 2016). Symbols for ecological state: E- Excellent (blue); G- Good (Green); F- Fair (yellow); P- Poor (orange) and B- Bad (red).

Site	IPtI$_N$						IBMWP					
	Nov	Dez	Jan	Feb	Mar	Apr	Nov	Dez	Jan	Feb	Mar	Apr
P1	P	F	B	G	P	P	B	F	B	P	B	B
P2	F	G	G	G	G	G	E	E	E	E	E	E
P3	F	G	G	F	F	F	F	G	G	F	F	G
P4	B	F	F	P	F	F	B	F	F	B	F	P
P5	F	G	F	F	F	F	G	G	G	F	G	F
P6	G	E	G	G	G	G	E	E	E	G	G	G
P7	F	G	G	G	G	G	E	E	E	G	E	G
P8	G	E	E	E	E	E	G	E	E	E	E	E
F1	P	G	G	F	F	G	G	G	G	G	G	G
F2	F	G	G	G	G	G	G	E	G	G	G	G
F3	F	F	G	G	G	F	G	E	E	E	E	G
F4	P	P	F	F	F	P	F	F	G	G	G	G
F5	P	P	P	F	F	F	F	G	F	F	G	F
F6	P	P	B	P	P	P	P	F	P	P	P	F
F7	P	P	P	P	P	F	F	P	P	P	F	G
F8	P	P	F	P	P	F	F	P	F	G	F	G

- Observe, as expected, some discrepancy in the classification of each sampling site evaluated between both indexes, although IPtI$_N$ (multimetric index) be more rigorous than IBMWP (unimetric index, more adapted to organic pollution).

2.3.3.4. Functional Feeding Groups (FFG)

A complementary approach was made using the functional feeding groups. The following figures (Figure 2.28 and 2.29) show the relative abundance of each functional feeding group in the different type of impact groups defined.

For Portelo river, in Reference and Moderated disturbed sites the dominance belonged to shredders and gathering collectors. This pattern is usual for upstream zones where the importance of alochthonous sources of energy from riparian gallery is high and typical in tewrms of their functioning. On the other hand a more balanced trophic structure was found for highly disturbed sampling sites. However, these results were calculated based in a low number of individuals, justifying the low availability to colonize, adapt and use of energetic resources of this degraded zone.

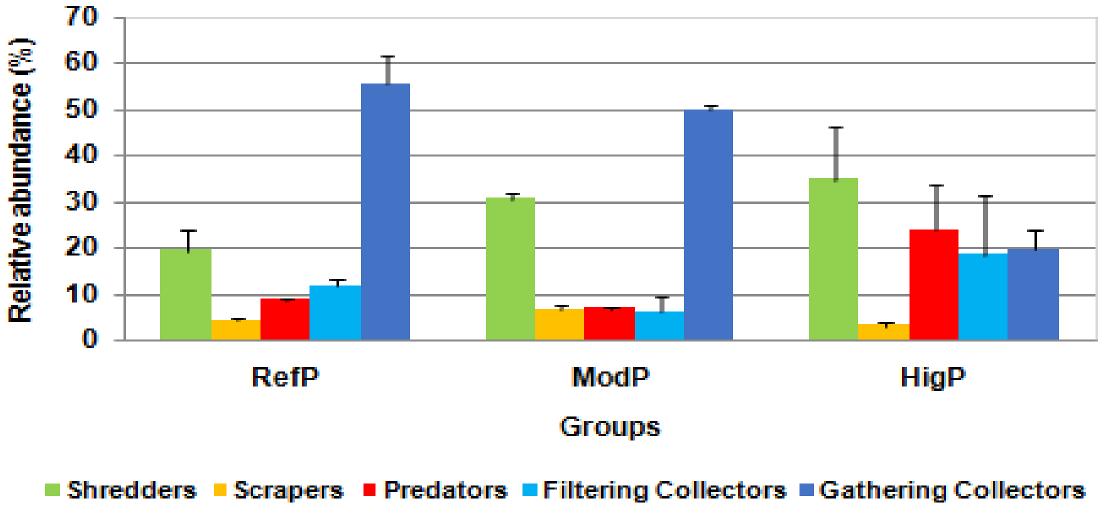

Figure 2.28. Relative abundance (mean+SD) of FFG for Portelo River basin, considering 3 groups: reference (P2, P6); Moderated (P7, P8) and Highly (P1, P3, P4, P5) sites.

For Fervença river basin it was foud the decreasing tendency of shredders, as expected since most of them are stenobiont organisms, in presence of organic pollution. The dominance is composed by filtering and especially by gathering collectors. In moderated polluted sites, scrapers obtain also relevant values as an answer of the nutrient input and the periphyton development in stable substrata.

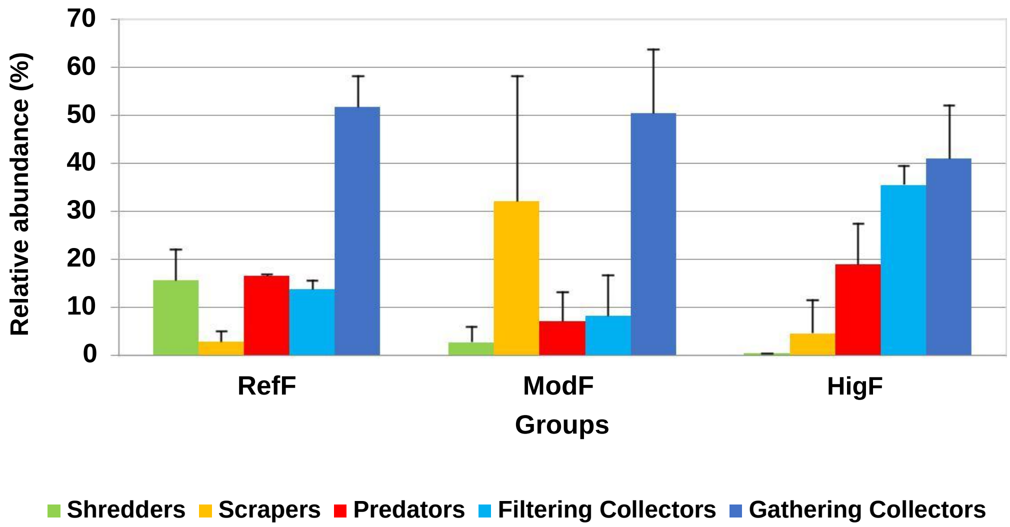

Figure 2.29. Relative abundance (mean+SD) of FFG for Fervença River basin, considering 3 defined groups: reference (F1, F2); Moderated (F3, F4, F5) and Highly (F6, F7, F8) sites.

2.3.3.5. Biotypology of macroinvertebrate communities

The NMDS analysis of all sampling sites revealed a clear separation for the type of impact: P- mineral pollution detected in Portelo Basin and 2) F- organic pollution observed in Fervença Basin, based on the good two-dimensional representation of the NMDS analysis, based on the 2D stress value of 0.19 (Figure 2.30). The ANOSIM analysis confirmed (P<0,001) the significant difference between impacts, since the typical invertebrate communities diverge almost completely.

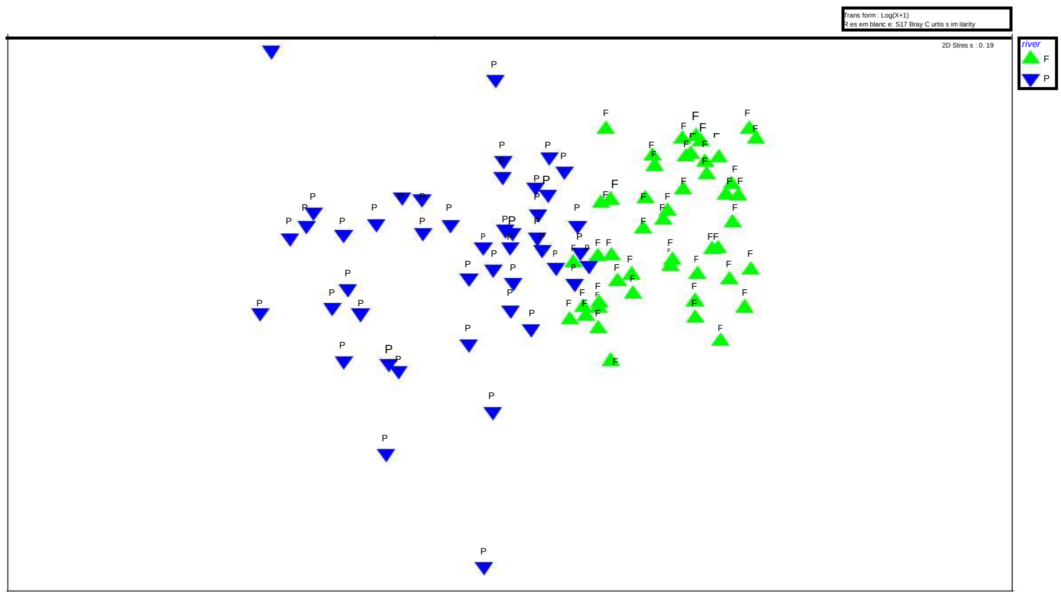

Figure 2.30. NMDS ordination of the sampling sites based on community invertebrates, considering the two types of studied impacts: P- Mineral pollution (Portelo Basin) and F- Organic pollution (Fervença Basin).

When the analysis was made considering the three defined groups with distinct impacts, it was more visible the differences between highly disturbed (HigF and HigP) groups and the remaining groups, i.e. reference (RefF and RefP) and moderated disturbed (ModF and ModP) groups was identified (Figure 2.31). However, the pairwise tests performed by ANOSIM showed very significant differences (P<0.001) between the majority of pair comparisons, except for moderated disturbed (ModP) and reference sites (RefP) of Portelo basin, that were only significant (P <0.05).

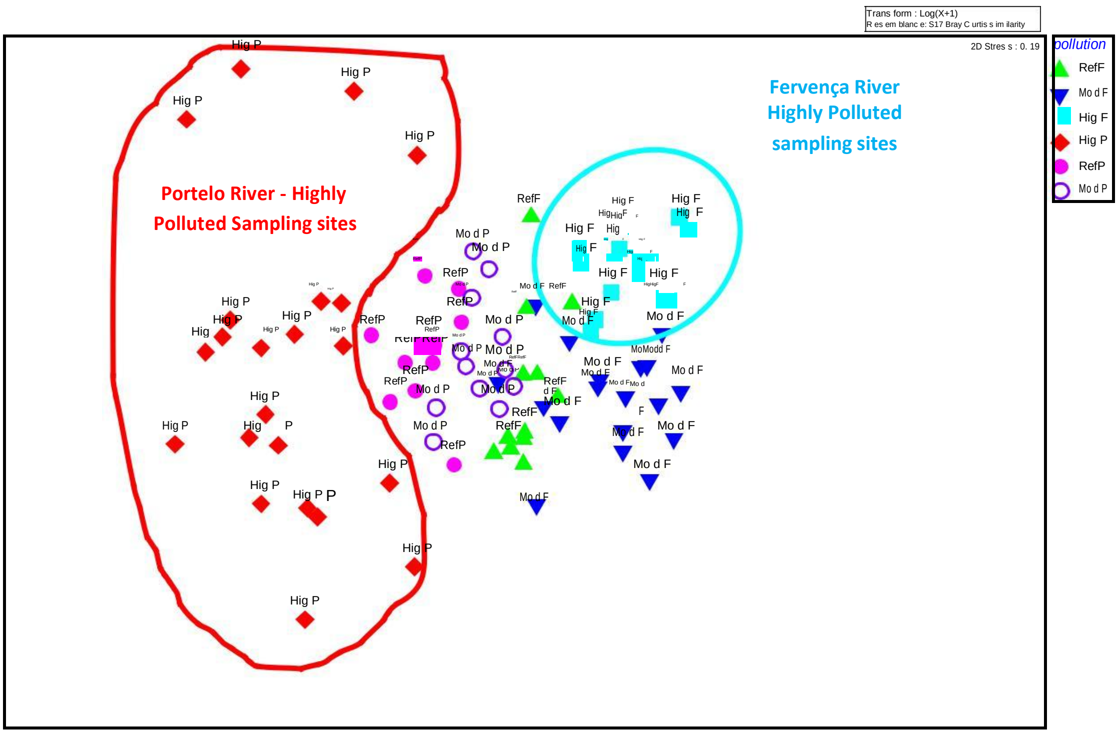

Figure 2.31. NMDS ordination of the sampling sites based on community invertebrates, considering the 3 groups defined for the two types of impacts: Reference (RefP, RefF); Moderated Disturbed (ModP, ModF) and Highly Disturbed (HigP, HigF).

Finally it was not found a clear pattern for month variation, namely for moderated and highly disturbed sampling sites. Apparently, the impact of pollution can be primordial in the structuring of macroinvertebrate communities of both river basins.

In Figure 2.32, the NMDS ordination of the macroinvertebrate communities of both river basins reflects the distribution of families, regarding to the environmental conditions. Thus, stenobiont taxa belonging to the insect orders of Trichoptera (Polycentropodidae, Calamoceratidae), Plecoptera (Perlidae, Perlodidae, Nemouridae, Leuctridae) and Ephemeroptera (Siphlonuridae, Ephemerellidae) are more linked to reference sites, while euribiont taxa like Diptera (Chironomidae, Psychodidae, Simuliidae) Hirudinea (Erpobdellidae, Glossiphoniidae) and Coleoptera (Hydrophilidae, Dytiscidae) are present in higher densities in highly polluted sites, namely in eutrophic conditions.

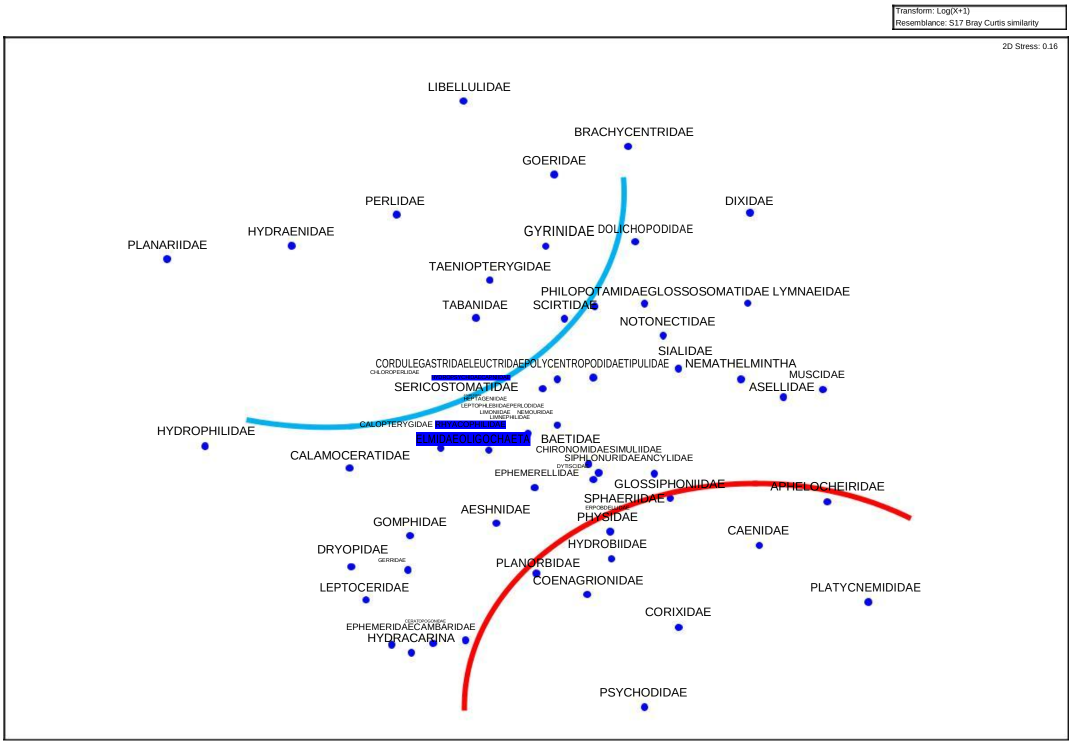

Figure 2.32. NMDS Ordenation of invertebrate communities of Portelo and Fervença Basins. Blue line present the stenobiant taxa and the Red line present the euribiont taxa.

2.3.4. Fish Communities

Fish fauna surveys allowed the detection of fish communities only in three sampling sites of Portelo Basin and two sampling sites of Fervença River. It were captured five (5) native species belonging to the families: Salmonidae (brown trout, *Salmo trutta*) and Cyprinidae (Iberian Northern chub, *Squalius carolitertii*; Calandino roach, *Iberocypris alburnoides*; common barbel, *Luciobarbus bocagei;* Douro nase, *Pseudochondrostoma duriense*) (Figure 25 and 26) and one (1) exotic species, belonging to the Cyprinidae family (carp, *Cyprinus carpio*) (Figure 2.33).

All native cyprinids are endemic species of Iberia. Some of them, belong to the IUCN (International Union for Conservation of Nature) RED List of Threatened Species (IUCN, 2015), such as *Pseudochondrostoma duriense*- Vulnerable (VU) and *Iberocypris alburnoides*- Vulnerable (VU).

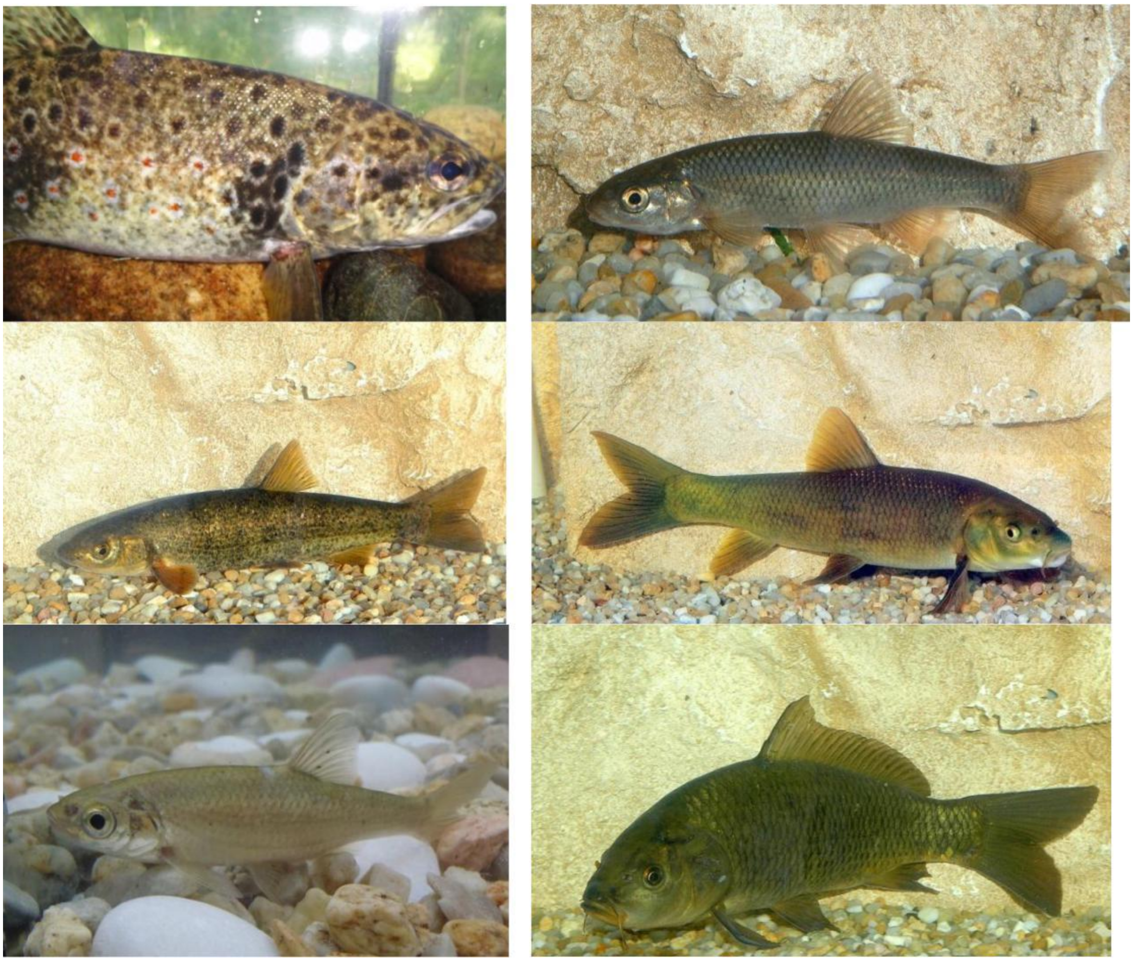

Figure 2.33. Fish species detected in the study (A- bown trout, B- Iberian Northern chub, C- Douro nase; D- Common barbel; E- Calandino roach and F- Common Carp) (spring 2016).

The distribution of different species by the different sampling sites is illustrated in Figure 2.34 and 2.35.

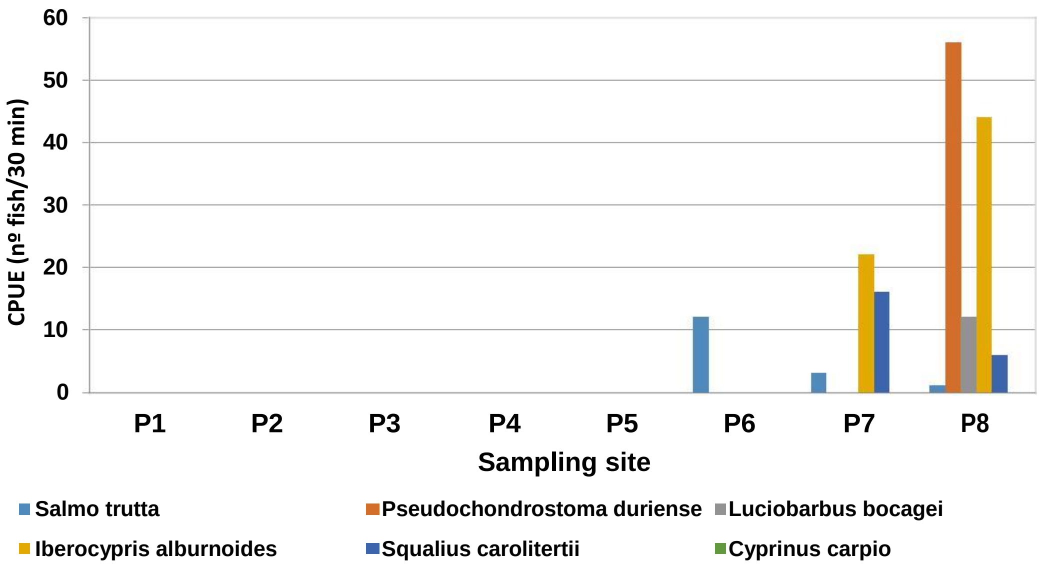

Figure 2.34. Composition and distribution of fish communities in the sampling sites of Portelo Basin (Spring 2016)

In spite of the good ecological condition in several reference sites (e.g. P2 and F1) it was not found fish communities because these watercourses have no stable conditions to mantain fish populations, namely running water during summer season. In the headstreams of Montesinho Natural Park only autochthnous fish populations can be detected, being an indicator of good ecological integrity of these watercourses, in contrast to the middle and final sections of the main stream (e.g. River Sabor), where already occur in high densities distinct exotic fish populations. The only exotic fish species detected, was introduced, unauthorized by Governmental Services, by fishermen in the Polis lentic weirs of the urban strecht of Fervença River.

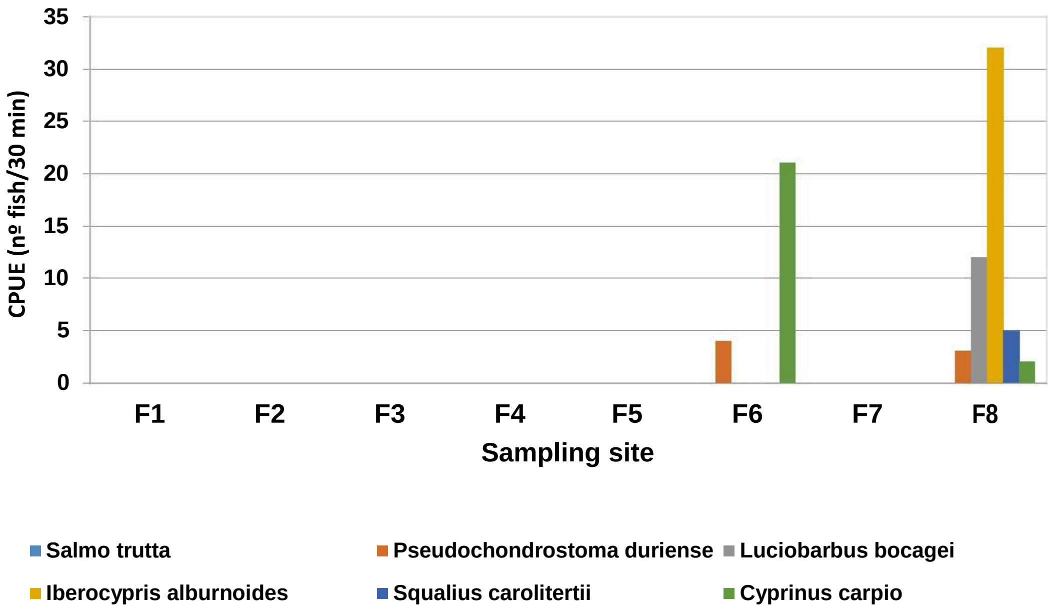

Figure 2.35. Composition and distribution of fish communities in the sampling sites of Fervença Basin (Spring 2016)

2.4. DISCUSSION

Based on this monitoring study it was possible continue to characterize the different pollution impacts in both Portelo and Fervença river basins. From winter 2015 to spring 2016 were identified the river zones where there are good ecological integrity status (e.g. reference sites) in contrast to others (e.g. moderated and highly disturbed sites) where obvious signs of disturbance appeared clearly reflected in the water and habitat quality and on composition and diversity of invertebrate and fish communities.

The evaluation of the environmental impacts resulting from the wastes collapse of the Portelo mines allowed to identify the effects of medium to long term (November 2015 to April 2016, after the wastes collapse of December 2009) occurred on the abiotic (e.g. water and habitats quality) and on biota (e.g. fish and macroinvertebrate communities). As detected by other studies (NOGUEIRA, 2011; PARADA, 2012) it was observed, after 7 years, the continuous input of significant amounts of sediment in water column and river bottom. This tendency has been verified with the gradual and complet embebedeness of substrata, leading to substantial changes and the lack of habitat for aquatic organisms. In the Figure 2.36 is visible the river bottom beetween the sampling sites of P5 and P7, showing the huge sedimentation in several small reservoirs of weirs (depht < 2m).

Figure 2.36. River bottom sedimentation in Portelo basin, near Aveleda village (Spring 2016)

This sudden change in environmental conditions is directly related with the drastic reduction in the abundance and diversity of invertebrate communities. Habitat loss (obliteration of interstices), asphyxia phenomena (clogging of the gills) and changes in the water quality (eg acidity and high levels of heavy metals in sediments and water column) can be cited as the main factors that explain the disappearance of stenobient taxa and inclusively the decrease of euribiont taxa. Comparatively with short term studies (NOGUEIRA, 2011; PARADA, 2012) developed in the same river, no clear signs of recovery in the macroinvertebrate community were detected. The low resilience registered can persist, since no rehabilitation/recovery measures / were developed, namely to avoid the continuous inflow of sediments in the river network, especially during rainy season.

The water acidification impacts are well documented (WINNER et al., 1980; FELDMAN & CONNOR, 1992; GRAY 1998; SOLÀ et al., 2004; BATTY et al., 2005) as one of the main causes of the decrease in diversity of aquatic ecosystems. A synergistic effect can also be linked to the decrease in pH, resulting in an increasing solubility of heavy metals in water (CAMPBELL & STOKES, 1985). For this reason, persistent phenomena of bioaccumulation can occur at the organism level and potential biomagnification in the food chain, contributing to a health problem for the ecosystem and also for human health. In fact, the results of this and previous studies showed high concentrations of some heavy metals, like aluminum (Al), copper (Cu) and cobalt (Co).

Relatively to Fervença River, the physical and chemical characteristics of the water showed clear signs of organic pollution, in particular from downstream of Braganca (F6) expressed, for example, by high values of conductivity (EC25), dissolved salts (TDS). However this tendency is more visible during summer season since eutrophication phenomena can occur as a consequent of the decrease of dissolved oxygen and water current and the higher content of nutients (N and P) (RODRIGUES, 2013). This decrease in dissolved oxygen content negatively affects the ecosystem and biodiversity of the aquatic environment. WINNER et al. (1980) and MASON (1996) reported, in similar studies, the dominance of tolerant taxa, such as Diptera (e.g. chironomids); Hirudinea (e.g. Erpobdella sp.) and Oligochaeta, in polluted zones. In the oposition, taxa very sensitive and previously dominant belonging to the orders of Ephemeroptera, Trichoptera and Plecoptera tend to disapear with this kind of pollution. Other metrics used in the study, i.e. the number of individuals and families, the diversity of Shannon-Wienner, H', the evenness of Pielou, J', the % EPT, proved to be enough sensitive to differentiate the three groups considered in the study (e.g. reference; moderated disturbed, highly disturbed). Also the analysis of the level dominant trophic regime has been used in other studies, such as traits in evaluation tests of water toxicity (GERHARDT et al., 2004; BATTY et al., 2005; DUCROT et al., 2005).

Finally, rehabilitation measures must be implemented for degraded river zones, like the elimination of waste inputs from Portelo mine (Portelo stream) and the improvement the efficiency of the Sewage Treatment Plant of Bragança (river Fervença). Furthermore, the development of monitoring actions, in order to detect and minimize any negative impacts that contribute to the degradation of the physical-chemical water quality, aquatic and riparian habitats and especially biota, and the establishment of awareness and environmental education programs must be urgently implemented to manage correctly the aquatic ecosystems and promote the conservation of native threatened species.

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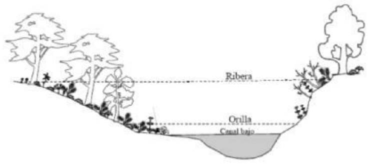
ANNEXES

Annex I: QBR INDEX

Riparian habitat quality



Station	
Classification	

The score of each of the four blocks can not be negative or exceed 25 points

1 - Total riparian cover - Score between 0 and 25

Score		
25	>80% of riparian cover (excluding annual plants)	
10	50–80% of riparian cover	
5	10–50% of riparian cover	
0	<10% of riparian cover	
+10 +5	If connectivity between the riparian forest and the woodland is total If the connectivity is higher than 50%	
-5 -10	Connectivity between 25% and 50% Connectivity lower than 25%	

2 - Cover structure -Score between 0 and 25

Score		
25	>75% of tree cover	
10	50–75% of tree cover or 25–50% tree cover but 25% covered by shrubs	
5	Tree cover lower than 50% but shrub cover at least between 10% and 25%	
0	<10% of either tree or shrub cover	
+10 +5 +5	At least 50% of the channel has helophytes or shrubs If 25–50% of the channel has helophytes or shrubs If trees and shrubs are in the same patches	
-5 -5 -10	If trees are regularly distributed and shrubland is >50% If trees and shrubs are distributed in separate patches, without continuity Trees distributed regularly, and shrubland <50%	

3 - Cover quality - Score between 0 and 25 *(the geomorphological type should be first determined*[a]*)*

Score		Type 1	Type 2	Type 3	
25	Number of native tree species	> 1	> 2	> 3	
10	Number of native tree species	1	2	3	
5	Number of native tree species	-	1	1 - 2	
0	Absence of native trees				
+10 +5 +5 +5	If the tree community is continuous along the river and covers at least 75% of the edge riparian area The tree community is nearly continuous and covers at least 50% of the riparian area When the number of shrub species is	> 2	> 3	> 4	
-5 -5 -10 -10	If there are some man-made buildings in the riparian area If there are some isolated species of non-native[b] trees Presence of communities of non-native[b] trees Presence of garbage				

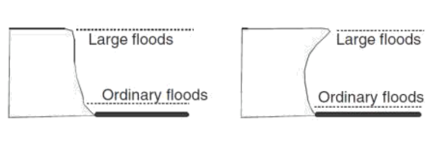
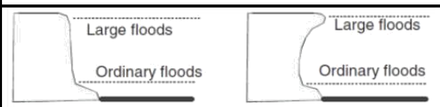
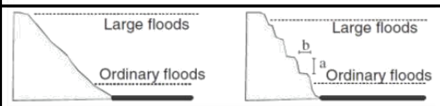
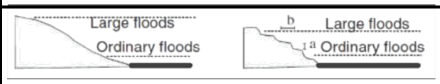
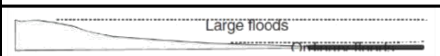
4 - Channel alteration - Score between 0 and 25

Pontuação		
25	Unmodified river channel	
10	Fluvial terraces modified and constraining the river channel	
5	Channel modified by rigid structures along the margins	
0	Channelized river	
-10 -10	River bed with rigid structures (e.g., wells) Transverse structures into the channel (e.g., weirs)	

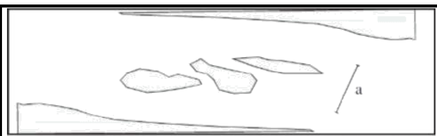
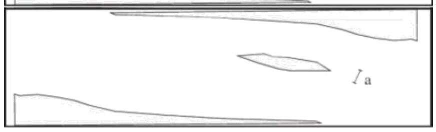
Final score (sum of four section scores)	

[a] **Type of the riparian habitat (to be applied at level 3, cover quality)**

The score is obtained by addition of the scores assigned to left and right river margins according to their slope. This value can be modified if islands or hard substrata are present.

		Score	
Slope and form of the riparian zone		Left	Right
Very steep, vertical or even concave (slope >75°), very high, margins are not expected to be exceeded by floods. *Slope is the angle subtended by the line between the top of the riparian area and the edge of the ordinary flooding of the river.*		6	6
Similar to previous category but with a bankfull which differentiates the ordinary flooding zone from the main channel.		5	5
Slope of the margins between 45° and 75°, with or without steps. (*a*>*b*)		3	3
Slope between 20° and 45°, with or , with or without steps. (a < b)		2	2
Slope <20°, large riparian zone.		1	1

Presence of one or several islands in the river

Width of all the islands "a" > 5 m.		-2	
Width of all islands 'a' < 5 m.		-1	

Percentage of hard substrata that can make impossible the presence of plants with roots

> 80%	Not applicable
60 – 80%	+6
30 – 60%	+4
20 – 30%	+2

Total score	

Geomorphological type following the score

> 8	**Type 1**	Closed riparian habitats. Riparian trees, if present, reduced to a small strip. Headwaters.
5 – 8	**Type 2**	Headwaters or midland riparian habitats. Forest may be large and originally in gallery.
< 5	**Type 3**	Large riparian habitats, and potentially extensive forests. Lower courses.

[b] **Non-native tree species in the study area**

(This should be listed for each study area)

e. g. in the studied area of Catalonia the following species are considered non-native: *Populus deltoides, Populus* x *canadensis, Populus nigra* ssp. *italica, Salix babilonica, Ailanthus altissima, Celtis australis, Robinia pseudo-acacia,Platanus* x *hispanica.*

Annex II: GQC INDEX - CLASSIFICATION OF CHANNEL QUALITY

Index of channel quality	Code:
(Conducted in at least three transects distance apart of 20 meters)	

1. Presence of retaining structures

Absence of structures	4
Semi-disaggregated rustic weir	3
Well established rustic weir	2
Dam or concrete dam	1

2. Channel Structure

W/D<7, It does not occur flood of the banks	4
W/D = 8-15, rare flooding of banks	3
W/D = 15-25, frequent flooding of banks	2
W/D> 25, very frequent flooding of banks	1

W - Average width of the wet bed obtained in transects

D – Average of maximum depth obtained in transects.

3. Sediments and stability of the channel

Absence of enlargement channel or accumulations of transported materials; single channel;	4
Some accumulation of transported material; single channel;	3
Lignes of gravel, sand and silt; the bed has full independent channels;	2
Channel divided into multiple lignes of sand and silt (or channelized river).	1

4. Structure of banks

Stable Banks with continuous and structurally complex riparian vegetation (trees and shrubs); without signs of erosion;	4
Stable Banks but with fragmented riparian vegetation; some eroded zones without vegetation;	3
Consolidated little Banks maintained by a sparse vegetation of grasses and shrubs;	2
Banks with very little and uniform vegetation, lowered by erosion along the stretch.	1

5. Artificial alteration of the banks

Almost complete absence of artificial change of banks;	4
One of the banks present moderate changes (e.g. rip-rap> 30% of the length section);	3
Both banks present moderate changes (e.g. rip-rap > 30%), or one of them is significantly altered (e.g. bank linearization)	2
As in the previous case, but the edge of the structure is of reinforced concrete or cyclopic.	1

6. Channel heterogeneity

Sinuosity of the channel and very marked lotic / lentic sequence;	4
Rectilinear channel with reduced lotic / lentic sequence;	3
Substantially constant velocity over the whole section;	2
Artificial lentic zone or channelized river	1

7. Bottom structure

Type 1	Headwater streams, low capability of supporting an extensive riverine forest;
Type 2	Middle zones of rivers, intermediate potential to support a riverine forest;
Type 3	Downstream zones with high potential to support a riverine forest;

Type 1 (Section on which predominates erosion)

> 50% of material comprises a particle size> 25 cm (boulders);	8
> 50% of material comprises particle sizes> 6.5 cm (pebble);	6
> 50% of material comprises particle sizes> 2.0 cm (gravel);	3
Predominates sand and silt (> 50%).	1

Type 2 (section in which predominantes transport)

> 50% of the material comprises boulders and pebbles (> 6.5 cm);	8
50% of material comprises pebble or higher (> 6.5 cm);	6
<25% of the material is larger than gravel (> 1.5 cm);	3
The bed is exclusively silt and sand (> 1.5 cm) is less than 10%.	1

Type 3 (Section on which dominates sedimentation)

> 50% of the material consists of larger than coarse sand (0.5 cm);	8
30-50% of the material consists of larger than coarse sand (0.5 cm) and the rest is formed by silt and sand;	6
<30% of the material consists of larger than coarse sand (0.5 cm) and the rest is formed by silt and sand;	3
The bed is only of silt and fine sand (<0.125 cm).	1

8. Deposition of fine interstitial sediments

% fines and <5%;	4
% fines is 5-25%;	3
% fines is 25-50%;	2
% fines is> 50%.	1

- *For Type 1 rivers, fines are considered <0.5 cm*
- *For Type 2 and 3 rivers, fines are considered <0.125 cm.*

Annex III: Scores assigned to different families of aquatic macroinvertebrates to calculate the IBMWP (adapted from ALBA-TERCEDOR 2000).

Families	Score
E: Siphlonuridae, Heptageniidae, Leptophlebiidae, Potamanthidae, Ephemeridae P: Taeniopterygidae,Leuctridae, Capniidae, Perlodidae, Perlidae,Chloroperlidae T: Phryganeidae, Molannidae, Beraeidae, Odontoceridae, Leptoceridae, Goeridae, Lepidostomatidae, Brachycentridae, Sericostomatidae D: Athericidae, Blephariceridae H: Aphelocheiridae	10
T: Psychomyiidae, Philopotamidae, Glossosomatidae O: Lestidae, Calopterygidae, Gomphidae, Cordulegasteridae, Aeschnidae, Corduliidae, Libellulidae C: Astacidae	8
E: Ephemerellidae, Prosopistomatidae P: Nemouridae T: Rhyacophilidae, Polycentropodidae, Limnephilidae, Ecnomidae	7
M: Neritidae, Viviparidae, Ancylidae, Thiaridae, Unionidae T: Hydroptilidae C: Gammaridae, Atyidae, Corophiidae O: Platycnemididae, Coenagrionidae	6
E: Oligoneuriidae, Polymitarcidae C: Dryopidae, Elmidae, Helophoridae, Hydrochidae, Hydraenidae, Clambidae T: Hydropsychidae, Helicopsychidae D: Tipulidae, Simuliidae Pl: Planariidae, Dendrocoelidae, Dugesiidae	5
E: Baetidae, Caenidae C: Haliplidae, Curculionidae, Chrysomelidae D: Tabanidae, Stratiomyidae, Empididae, Dolichopodidae, Dixidae, Sciomyzidae Ceratopogonidae, Anthomyidae, Limoniidae, Psychodidae, Rhagionidae Mg: Sialidae Pl: Piscicolidae A: Hidracarina	4
H: Mesovellidae, Hydrometridae, Gerridae, Nepidae, Naucoridae, Pleidae, Veliidae, Notonectidae, Corixidae C: Helodidae, Hydrophilidae, Higrobiidae, Dytiscidae, Gyrinidae M: Valvatidae, Hydrobiidae, Lymnaeidae, Physidae, Planorbidae, Bithyniidae, Bythinellidae, Sphaeriidae Hr: Glossiphoniidae, Hirudidae, Erpobdellidae C: Asellidae, Ostracoda	3
D: Chironomidae, Culicidae, Muscidae, Thaumaleidae, Ephydridae	2
O: Oligochaeta (All Families) D: Syrphidae	1

CHAPTER 3

Effect of acute cooper exposure in aquatic vertebrates on some biochemical indicators (Na^+ and K^+ plasma concentrations): the case of Iberian barbus (Luciobarbus bocagei).

ABSTRACT

In December 2009, heaps of Portelo mine witness a collapse and incorporation of a high quantity of heavy metals in the hydric system, namely copper (Cu) was observed. To understand which may have occurred in autochthones fish populations facing this specific contaminant, acute toxicity tests with Cu were conducted in laboratory conditions. In this sense, some biochemical indicators were evaluated to assess osmoregulatory adverse effects of Cu. Also, same evaluation was conducted after fish changing to clean water, in attempt to assess fish capacity to reverse the stress situation.

Biochemical indicators (Na^+ and K^+ plasmatic concentrations), and Iberian fish specie (*Luciobarbus bocagei*) were chosen. Acute toxicity tests were conducted on static system, with two different copper concentrations (0.06 ppm, 0.11 ppm) and a control group with no copper exposure. Water parameters, fish behavior and Condition Factor (*K*) are also evaluated during the experiments.

According to our results, barbels under Cu exposition showed several changes at physiological and behavioral levels, such as ion imbalance, decreased aerobic scope and mortality and reduced Condition Factor (*K*). One of the conclusions that emerge from this study is that the plasma K^+ levels may increase and Na^+ levels may decrease in fish exposed to Cu, allowing fish to an osmotic adjust in an attempt to survive under environmental stress. Under the conditions of these experiments, fish also showed ability to reverse the levels of plasma electrolytes, when they cease to be exposed to Cu; however that is depending of exposure concentration.

These results providing some insights to that are believed to occurred in fish population, near the Portelo mines

Key-words: copper, toxicity, biomarkers, plasma electrolytes, *Luciobarbus bocagei*

3.1. INTRODUCTION

Heavy metals are generally released in small amounts into the environment by nature processes, like weathering of rocks, volcanic eruptions, among others and their intake/exposure is necessary in trace amounts for good health. But, presently, there is a steady increase in their concentration in all habitats owing to anthropogenic action, such as mining, electroplating, paints and dye, battery making industries etc.

Water pollution has become a worldwide problem. Some essential metals for animal life, such as copper, are continuously increasing in water which may result in toxic effects on aquatic organisms, such as fish (HEATH, 1995).These trace metals, emanating either from natural sources or from anthropogenic activities, interact with aquatic organisms as mixture of more than one metal or in combination with organic pollutants. Therefore, biochemical interactions would determine the outcome of the toxic response (PANDEY et al., 2008). All these sources of pollution affect the physiochemical characteristics of the water, sediment and biological components, and thus the quality and quantity of fish stocks (AL-RAWI, 2005; SINGH et al., 2006; EBRAHIMI &TAHERIANFARD, 2009).

Metals are contaminants of great concern due to their persistence, susceptibility of accumulation in organisms and the possibility of reaching toxic levels. The presence of a given metal at high concentrations in water or sediments does not involve direct toxicological risk to fish, especially in the absence of significant bioaccumulation. It is known that bioaccumulation is to a large extent mediated by abiotic and biotic factors that influence metal uptake Bioavailability of metals can be influenced by organic and inorganic factors that control metal speciation and thereby bioaccumulation. Some heavy metals like lead have no function in biological systems, while others such as copper and zinc are essential for metabolism of living organisms, including fish. Nevertheless they may exert harmful effects, depending upon concentration (FERNANDES et al., 2008).

Environmental concentrations of heavy metals such as Zn, Cu, Fe, Co have been increasing in several European coastal areas due to industrial wastes, geochemical structure, agricultural and mining activities , that's why they became major environmental hazard which poses a level of threat to life by their capability to induce harmful effects on living organisms at ecological relevant concentrations and have been considered important environmental contaminants (CUNHA et al., 2007; SINGH et al., 2008). Metals are an important group of aquatic pollutants and can be taken up by aquatic vertebrate, like fish, from water, food, sediments and suspended particulate material. The presence of a given metal at high concentrations in water or sediments can disturb the integrity of biochemical and physiological mechanisms in aquatic life. However, it does not involve direct toxicological risk to fish, especially in the absence of significant bioaccumulation, which occurs when an organism absorbs toxic -substance at a rate faster than that lost by catabolism and excretion. It is known that bioaccumulation is to a large extent mediated by abiotic and biotic factors that influence metal uptake (FERNANDES et al., 2007; VIEIRA et al., 2009).

Copper (Cu) is widely distributed in nature (land or water). It is a trace element needed in very small quantities for the proper growth, development, and physiology of the organism and can plays a fundamental role in the biochemistry of organisms, such as cellular metabolism, but becomes toxic at elevated levels (PELGROM et al., 1995). It is essential for life however its accumulation can be toxic to cells. The mechanisms of toxicity are associated with oxidative stress. Indeed, Cu reacts with hydrogen peroxide (H_2O_2) to generate singlet oxygen and hydroxyl radical (NZENGUE et al., 2011).

Metals present in the water-column, sediment or food, are readily accumulated by aquatic organisms. So, exposure to toxic contaminants can be measured in terms of the biochemical responses of the organisms-so-called biomarkers (LIVINGSTON, 1993). The use of biomarker-based biomonitoring can be a promising approach that may provide early-warning signals of pollutants exposure. In many study concerning the pollution exposure, the blood parameters have been used as sensitive indicator of stress in fish exposed to different water pollutants and toxicants, such as metals (Cu, Fe, Co …), biocides, pesticides, chemical industrial effluents, etc. (SINGH et al ., 2008).

3.1.1. Model of Study*: Luciobarbus bocagei* (Steindachner, 1864)

According to Water Framework Directive (WFD), fish represent one of the most important key elements to evaluate the rivers ecological status. They are present virtually in all environments, and many species have been found to be susceptible to environmental pollutants (PEREIRA et al., 2013). Therefore, they can be a reliable biomarker of copper (Cu) pollution of aquatic bodies. Therefore, in this work we chose de Iberian barbel as a biomonitoring species.

Barbels are among the most widespread and diverse primary fishes in Europe, where only tetraploid species exist. Given its marked diversity, especially on the Iberian and Balkan peninsulas, this genus shows features that make it an ideal evolutive model for European freshwater fauna (DOADRIO et al., 2002).

Figure 3.1. Iberian Barbel (*Luciobarbus bocagei*) (WWW.fishbase.org).

The common barbel (*Luciobarbus bocagei*), used in the toxic assays , is an endemic cyprinid of the Iberian Peninsula (Figure 3.1), with a very wide distribution, occupied almost all of the watershed Portugal, with the exception of the basins of the rivers Guadiana, Mira and the Algarve Ribeiras. It is a very abundant t*axon*, occupying a wide range of habitats and presenting an omnivorous diet. The feeding pattern of the *L. bocagei* population is characterized by consumption of plant material and dipteran larvae associated with ephemeropteran nymphs, Trichoptera, Coleoptera and Mollusca (MAGALHAES, 1992). The breeding season runs from May to July, when carrying out migration to suitable for spawning, which usually are shallow areas with gravel or gravel substrate, and wherein water is present as well oxygenated.

3.1.2. Fish Gills

Fish, compared with invertebrates, are more sensitive to many toxicants and are the convenient test-objects for water quality assessment and toxicological effects. They are exposed to pollutants *via* two main routes, waterborne and dietary and, with time of exposure, even with low contamination levels, fish can develop early signs of toxic effects. Fish are relatively sensitive to changes in the environment and toxic effects of pollutants may start to occur in the cell and in metabolic pathways, before significant alterations in behavior or morphology can be identified. The knowledge of normal metabolic processes of major fish organs and alterations induced by pollutants exposure can be a tool for an early warning system in the evaluation and analysis of aquatic environment and fish population (FERNANDES et al., 2011).

The fish gill is an important organ with multiple functions, including gas exchange, ionic and osmotic regulation, acid-base regulation and excretion of nitrogenous wastes. The so called chloride cells, specific ionocytes, are the major cells in fish gill that actively transport ions. These ionocytes can secrete and absorb ions in seawater and freshwater environments respectively, as well as to carrying out acid-base regulation and ammonia excretion functions (EVANS et al., 2005; MARSHALL & GROSELL, 2005; EVANS & CLAIBORNE, 2008; GILMOUR & PERRY, 2009). The gills of freshwater fish comprise over half the total body surface area, and the mean blood to water diffusion distance here is only a few microns (HUGHES, 1984). Gills are the first organs which come in contact with environmental pollutants and so they are highly vulnerable to toxic chemicals because firstly, their large surface area facilitates greater toxicant interaction and absorption and secondly, their detoxification system is not as robust as that of liver (PANDEY et al., 2008). Many environmental toxicants not only enter through the gills but also exert their primary toxic effects right on the branchial epithelium by interfering with one or more of these essential physiological processes (WOOD, 1992).

The gill is particularly sensitive to physical and chemical changes in the aquatic environment and it is the main target organ in fish for toxic waterborne heavy metals (McDONALD & WOOD 1993). ATPases (Na^+/K^+-ATPase, Ca^{2+}-ATPase) are membrane-bound enzymes responsible for the transport of ions through membranes and thus help in regulation of osmotic pressure and membrane permeability (EVANS, 2008; ATLI & CANLI, 2011). The key mechanism of metal toxicity has been reported to be an osmoregulatory impairment associated with ATPase inhibition in the osmoregulatory tissues such as gill and kidney. It has been suggested that the assessment of ATPase activity may therefore be used as an early warning signal of pollutant-induced damage to the osmoregulatory and acid-based regulatory system in gills. Data from the literature have showed that there were considerable variations (inhibition and stimulation) in ATPase activities in fish exposed to several metals including Cd, Cu, Zn and Pb in hard waters (ATLI & CANLI, 2007; SAGLAM et al., 2013). Also, effects of metals on ionoregulatory gill functions have been well demonstrated, including regulation of plasma electrolytes (MAZON et al., 2002; GROSELL et al., 2003; CAO et al., 2013). In fish, measurement of major electrolytes (Na^+, K^+) under stressful conditions can be used as sensitive biomarkers of chemical exposure and its effects on the ion regulating tissues (FERNANDES et al., 2007, 2009; SUVETHA et al., 2010).

Therefore, since fish are useful experimental models to evaluate toxicological mechanisms of contaminants, acute toxicity tests with Cu were conducted in laboratory conditions, in attempt to understand which may have occurred in autochthones fish populations facing this specific contaminant, as during collapsed mining deposits in Portelo mines.

- ✓ The common barbel, *Luciobarbus bocagei***,** was chosen as an autochthones fish species and plasmatic electrolytes levels (Na^+ and K^+) were chosen as bioindicators to assess osmoregulatory adverse effects of Cu.

✓ Also these electrolytes levels were measured after fish changing to clean water, in attempt to assess the fish capacity to reverse the stress situation.

3.2. MATERIAL AND METHODS

3.2.1. Fish Sampling

Fish were initially sampled from Ribeira da Vilariça one of the main stream of River Sabor, located in Northeast of Portugal (Figure 3.2). Sampling was carried out in 3 different sites along the River (Figure 3.3), between December 2015 and April 2016. Fish were caught by electrofishing (portable device Hans Grassl ELT, continuous current output of 300-600V), according to sampling methodology of INAG (2008).

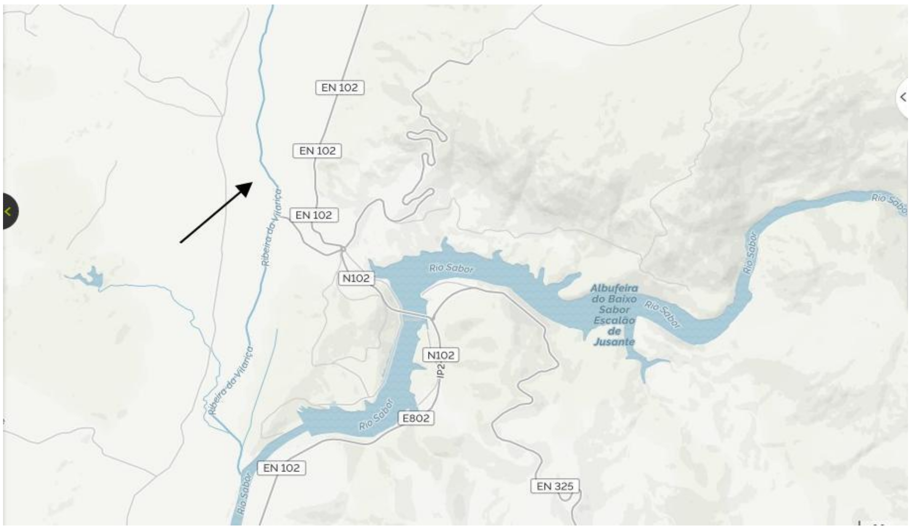


Figure 3.2. Location of Ribeira da Vilariça of River Sabor, showed by arrow. (www.mapquest.com/portugal)

After capturing, fish were transported alive in thermal bags with water to the Laboratory of Aquaculture at School of Agriculture, Polytechnic Institute of Bragança.

Figure 3.3. Sampling sites in the Ribeira da Vilariça

3.2.2. Acclimation Conditions

The toxicological tests were developed in the Aquaculture Laboratory at Agrarian School, Bragança, Portugal. Initially, fish were acclimated for a period of 7 days in 5 tanks of 16 L, in a closed system (Aquaneering Systems ®), under 16h/8h photoperiod, constant temperature of 20ºC and fed once a day, with commercial pellets, until 24 h before bioassays. This is a system of aquaculture (artificial aquariums), composed of 40 tanks of 16L each, with water recirculation system with physical filters, activated carbon and UV for maintenance of a good water quality, temperature control system and photoperiod (Figure 3.4).

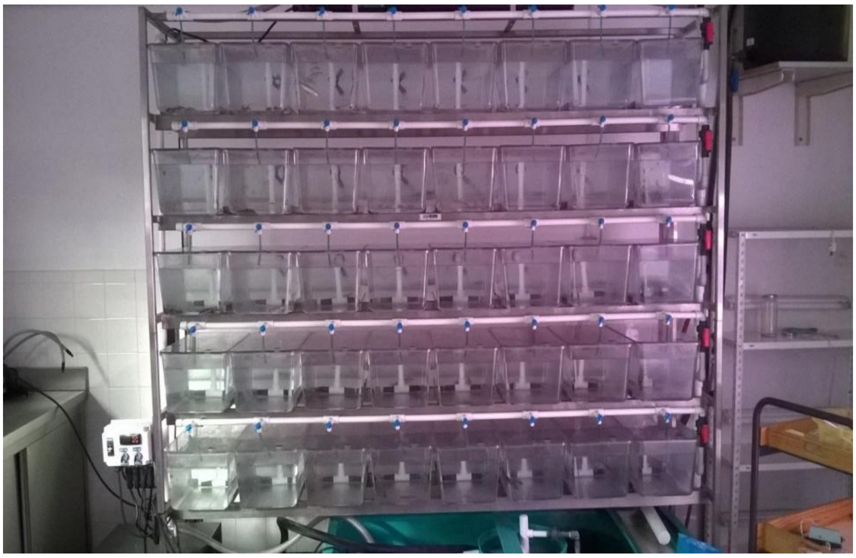


Figure 3.4. Tanks used in fish acclimatization (Aquaneering Systems ®).

3.2.3. Assays of Acute Cu Exposition

Toxicological tests were carried out with the *Luciobarbus bocagei* for 2 days and were carried out in a static system, with no fish feed. Plastic tanks, with 80 liter capacity were used, aeration was provided by means of pumps (aquarium pumps type), photoperiod was set on 16h:8h and under two different temperatures: 19°C±0.46 and 21°C±0.44.

Tanks were filled with river water and also with some tap water (volume of testing 70 L) and before the experiment water parameters were measure: pH, conductivity (Figure 3.5), temperature and dissolved oxygen (Figure 3.6). Analysis of the water hardness was performed using titration.

Figure 3.5. Instruments used for water parameters measurements: (a) pH (HANNA instruments HI8417); (b) conductometer (Consort K320)

Figure 3.6. Multi-parameter sensor (HANNA HI9828).

After acclimatization period, fish were divided into 3 groups (*n* = 10 each). One group is used as control and the other two as the exposed groups, with cooper exposition concentration of 0.06 ppm and 0.11 ppm. The Cu concentrations in tanks were obtained by dissolving certified standard solution (Merck) to desired tests concentrations.

For the first 24 hours we put 10 fish in each tank (Figure 3.7). In all tanks, the barbs were frequently observed during the period of the test to assess their behavior and their vital conditions, namely problems in the balance and swim. If is there any fish dead or with a total lack of movement, they were remove immediately for blood sampling. The observation of the fish were done at 0 hours (15 pm), after 6 hours (20 pm), after 12 hours (9 am) and after 24 hours (9 am) of Cu exposition.

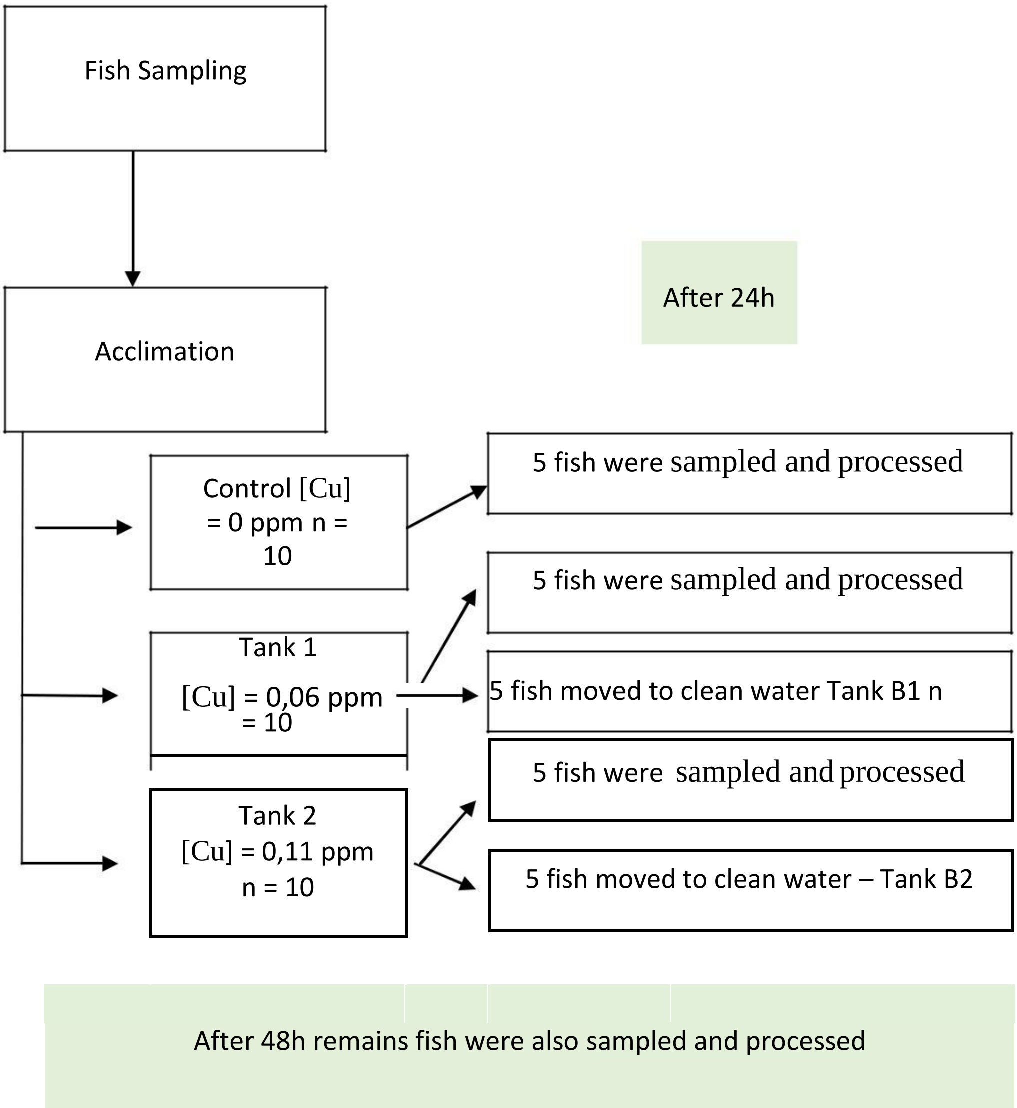


Figure 3.7. Scheme of the methodology used in the toxicity test

After 24h under cooper exposition, 5 fish were removed from Tank 1 and Tank 2 to blood sampling and the remains 5 fish moved to another tanks filled with clear water, respectively to Tank B1 and Tank B2 (Figure 3.7 and Figure 3.8). The objective was to assess the fish ability to revert the plasma electrolytes levels, arising after cooper exposition. After 48h the remaining fish, as well as the fish from control tank, were taken for blood sampling.

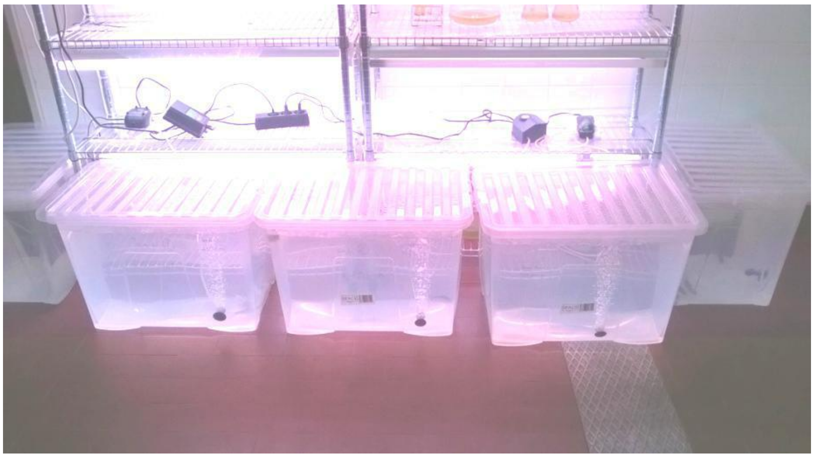


Figure 3.8. Tanks used in the Cu exposure tests, from left to right: Control tank, Tank 1, Tank B1, Tank 2, Tank B2.

3.2.4. Fish Processing and Blood Sampling

For the blood sampling the barbels were anesthetized (ether ethylene glycol monophenyl, MERCK) and then blood was drawn from the caudal vessels with heparinised syringes, to eppendorf tubes. Plasma was obtained by centrifugation (Centrifuge 5415 R) under refrigeration (15 min, 10.000 g, / 3 500 rpm, 4ºC) and frozen until analysis (Figure 3.9).

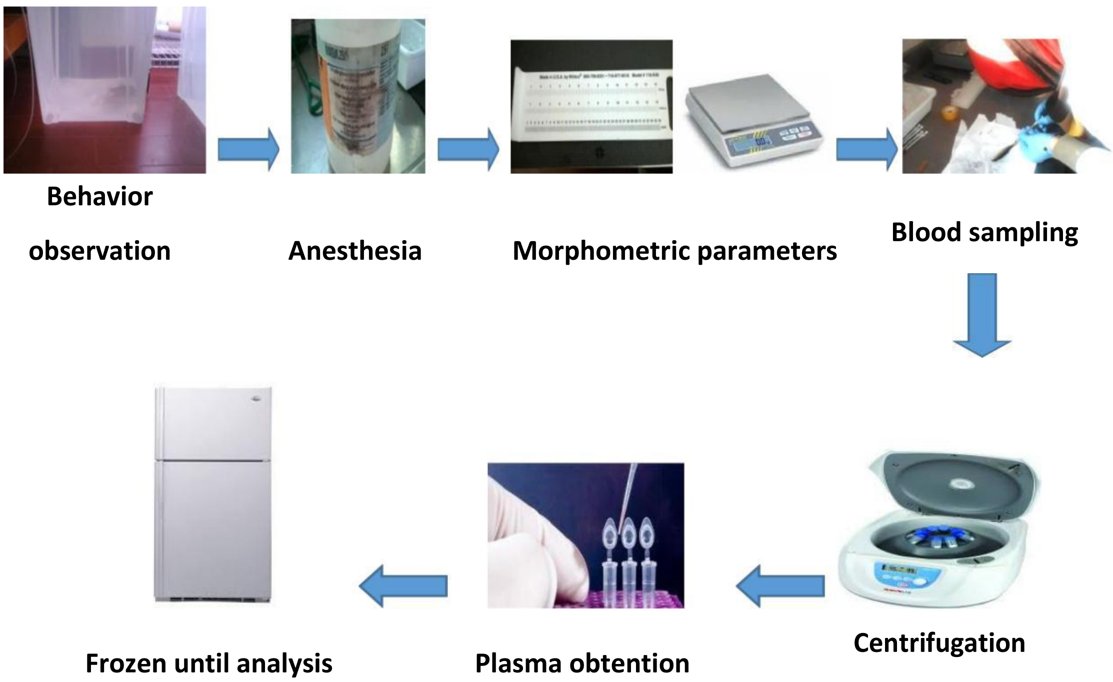


Figure 3.9. Scheme of the methodology used in the fish processing and blood sampling

Total body length and weight (Chyo MK-2000B) of each fish were recorded to calculate Condition factor (K):

$$K = \text{body weight (g)}/(\text{fish length (cm)})^3 \times 100$$

3.2.5. Water Hardness

Water hardness is not defined in a unified manner, but the simple definition is the sum of calcium and magnesium concentrations, the two most prevalent divalent metal ions. Here, water harness was measured by titration with an EDTA solution and expressed as mg/L, according :

$$\text{Hardness} = [Ca] + [Mg]$$

$[Ca] = V1 * 0.4008 * 1000/50$ (mg/L)

$[Mg] = V2 * 0.243 * 1000/50$ (mg/L)

Hardness = $(V1 + V2) * 1000/50$

Where: *V1* is the volum of EDTA (N/50) for titration of Ca; *V2* is the volum of EDTA (N/50) for titration of Mg.

3.2.6 Flame Atomic Absorption Spectroscopy (FAAS)

The Cu concentrations in Tank 1 and Tank 2 were measured by FAAS (Phillips PU9100X) and results were expressed as ppm.

Plasma samples were diluted with ultra-pure water (Reverse osmose – “Water Purification System”- Series WIN Topway Global, Inc) and then total concentrations of potassium (K^+) and sodium (Na^+) were measured by FAAS. Results were expressed as mM/L.

3.2.7. Data analysis

Results were expressed as mean values ± standard deviation (M±SD). Statistical analysis was carried out with SPSS 20.0 (SPSS Inc., Chicago, IL, USA) with a significance level of 5%. ANOVA was applied to identify differences in mean values between Cu concentrations, within each set of test (weight, Condition Factor (*K*), K^+ and Na^+. The Mann-Whitney test and Kolmogorov-

Smirnov test were used to identify differences in mean values, within each set of test, between different tanks.

3.3. RESULTS AND DISCUSSION

3.3.1. Water Parameters

Generally, water quality parameters during acclimation period (Table 3.1) were between desirable ranges, according OECD (1992).

Table 3.1. Water quality parameters in tanks used during acclimation of barbels (M±DP).

Parameters	M ± DP
T (°C)	18.37 ± 0.78
pH	8.02 ± 0.16
Conductivity (µS/cm)	352 ± 3.46
Hardness (mg/L)	58 ± **0.00**
O_2 (mg/L)	6.15 ± 0.87

During each assay of acute Cu exposition, water parameters between tanks (Control T1, TB1, T2, and TB2) were quite similar, except for Cu in water, and for this reason they were pool together. Although the differences observed in water parameters between the two assays of acute Cu exposition performed (Table 3.2), considering the species wider distribution across several river basins (ALEXANDRE et al., 2014), generally parameters were within the optimum conditions for the barbells in nature habitat. The water hardness shows a range close to the typical value of this region, with mean values of 58.9 mg/L (BRÁS, 2013).

Table 3.2. Water quality parameters in the tanks used in Cu exposure (M±DP).

Parameters	1st Assay	2st Assay
T (°C)	18.88 ±0.46	21.35±0.44
pH	7.74±0.16	8.17±0.04
Conductivity (µS/cm)	145.40±14.31	136.75±2.30
Hardness (mg/L)	74.80±5.59	54.25±1.71
O_2 (mg/L)	8.55±0.37	n.m.

n.m. - not measured

3.3.2 Fish Behavior

During acclimation period no fish mortalities were observed and behavior was considered normal. This is used as a basis for comparison to assess the health of fish during Cu exposition, since some fish mortalities and some behavior changes were observed. In fact, fish behavior changes were star to occur after 12h, in Tank 1 and in Tank 2, during 1st assay of Cu exposure experiments (19°C), as in table 3.3.

Table 3.3. Fish behavior during the first assay of Cu exposure

	Control	Tank 1	Tank 2
After 6h	Normal swim Normal equilibrium Good reflex	Normal swim Normal equilibrium	Normal swim Normal equilibrium
Between 12h-24h	Same as before	*Balance problems *Some fish swim in the bottom and other in the middle *There is some fish which can't swim easily	*Balance problems *Swim with circular rhythm *Every fish swim in different directions *Some fish stay quite *Some fish trying to get the surface *Some fish straggle to breath
	Control	**Tank B1**	**Tank B2**
Between 24h-48h	Same as before	Same behavior as the control tank	Same behavior as the control tank

For the Control Tanks (without Cu) the fish showed typical behavior during all experiment. However, in Tank 2 fish showed high stress behavior, with severe problems in balance and swim movements, including lethargic movement. On the other hand, behavior of fish in Tank 1 was less severe (Table 3.3), probably since fish were exposed to low Cu concentration. In this assay no fish mortalities were observed. After the remains fishes were transferred to clean water, behavior turn into normal condition and become similar to the Control Tanks.

During the second assay of Cu exposure (21°C), same behavior in Control Tank and in Tank 1 was observed; however all the fish in Tank 2 were dead. These fish mortality could be linked to exposition to higher Cu concentration associated to a higher temperature of this assay, compared to 1st one, enhancing in this way Cu toxicity. Also, after remain fish from Tank 1 were transferred to clean water, behavior turn into normal condition.

Fish as a bioindicator species plays an increasingly important role in water pollution monitoring because it responds with great sensitivity to changes in aquatic environment. The effects of exposure to sublethal levels of pollutants can be measured in terms of several responses, including behavioral responses (MISHRA & MOHANTY, 2008; KUMARI et al., 2011). Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment (LITTLE & BREWER, 2001). The results obtained here, namely swimming performance and activity of fishes significantly altered, are also previously observed in fish exposed to heavy metals (LITTLE & FINGER, 1990; ZHOU & WEIS, 1998; KUMARI et. al., 2014).

3.3.3. Morphometric Parameters

The assays were carried out with wild barbels randomly chosen, but as far as possible with same size class. For the 1st assay of Cu exposition, fish length range between 9.80 cm and 12.70 cm, whereas for the 2st one fish length range between 8.60cm and 13.50 cm. There were no differences in fish length between the two assays (Table 3.4). Considering the fish weight, the range for the 1 st assay was between 6.80g and 15.40g and between 4.30g and 15.60g for the 2st assay. Also, there were no differences in fish weight comparing the tanks from the 1st assay. However, fish weight from Tank B2 was lower than fish weight from the other tanks (Table 3.4).That could arise from the differences between fish that we pick up between 24 h and 48 h.

Table 3.4. Length and weight of barbells used in assays of Cu exposition (M ± DP).

	Length (cm)		Weight (g)	
	Assay 1	Assay 2	Assay 1	Assay 2
Control	11.10±0.84	10.92±1.70	10.42±2.66	10.27±4.14 ^{a}
Tank 1	11.22±0.88	10.26±0.78	9.78±2.31	7.28±1.04 ^{a}
Tank B1	11.42±0.73	9.98±0.52	10.46±2.13	6.90±1.16 ^{a,b}
Tank 2	11.18±0.85	11.01±0.51	10.26±2.70	10.51±1.45
Tank B2	11.62±0.50	10.05±0.07	10.46±1.86	5.35±0.78 ^{b}

Different letters in same row correspond to statistical differences between tanks.

Standard weight in fish is the typical, or expected, weight at a given total length for a specific species of fish. As fish grow in length, they increase in weight; however the relationship between weight and length is not linear. In fact, the weight of a fish could increase more than length, or could happen that length increases more than weight, depending, among others, from feeding conditions and changes in food reserves. Therefore, relationship between weight of a fish and its length, namely the condition factor (K), reflects the general condition of the fish. *K* is a commonly measured variable that can be used as a general index of fish health (EASTWOOD & COUTURE, 2002).

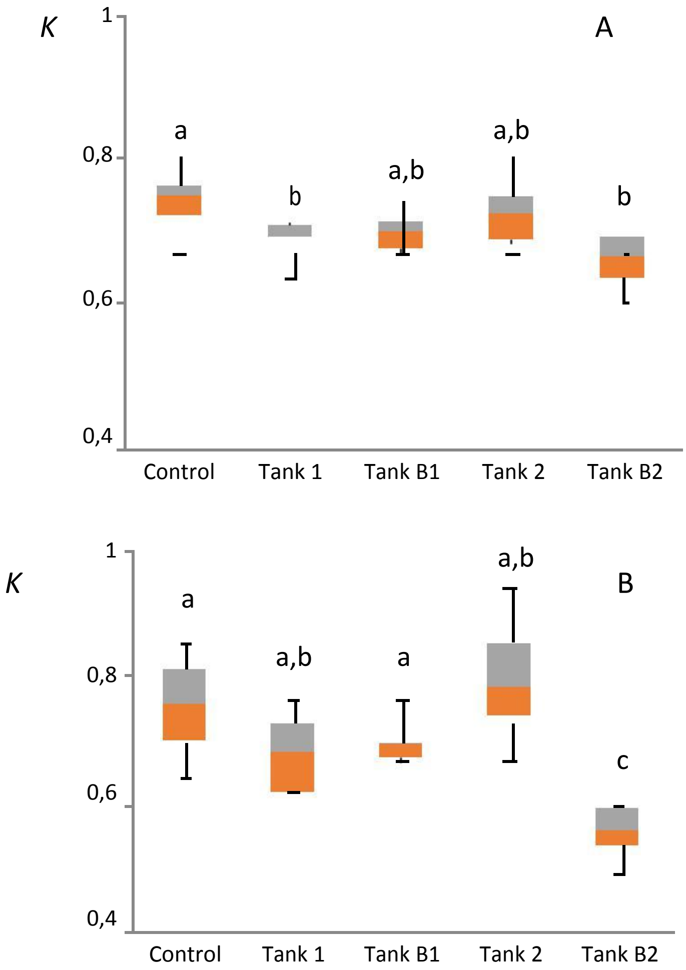


Figure 3.10. Condition Factor (K) of barbells used in Cu exposition assays: (A) 1st assay; (B) 2 st assay (different letters corresponded to significant differences).

As figure 3.10A shows, for 1st assay, although fish *K* from Control Tanks was higher than fish *K* from Tank 1, there was no differences between fish *K* from Tank 1 and Tank B1, neither between Tank 2 and Tank B2; i.e. all fish exposed to Cu, and the ones removed to clean water, had same *K*. These results suggested that Cu exposure, in these experimental conditions, do not affect *K*.

In turns, for 2 st assay (Figure 3.10B), although *K* was similar between fish from Tank 1 and Tank B1, there was a difference between fish *K* from Tank 2 and Tank B2. Once again, these results suggests that barbels exposed to higher Cu concentration in water under high temperature could be more stressed, reflecting It into a more severe behavioral response, including fish mortality, and in a lower *K*. The environmental factors affect the uptake and accumulation of metals in fish and under higher temperature, since metabolic rate is increased, higher uptake rates of metals can also occurs. Similar effects were shown by others studies on the toxicity of Cu and other heavy metals, with increase of temperature (MACINNES & CALABRESE, 1979; BAT et al., 2000; JEZIERSKA & WITESKA, 2006).

3.3.4. Quantification of Cu

The values of Cu quantified in the tanks (Tank 1 and Tank 2) were not different than expected. Indeed, a mean value of 0.067±0.001 ppm and 0.121±0.002 ppm were measured in Tank 1 and Tank 2, respectively (y=3.0193x + 0.0151; R^2=0.992; n=3), quite similar to expected values of 0.06 ppm and 0.11 ppm, respectively.

The choice of these Cu concentrations for the present toxicity tests, although slighter than the ones detected during mine spill, are based on previous results with same barbel species, who have been show, high fish mortality rates with higher Cu levels (MONTEIRO, 2012).

3.3.5. Plasma Electrolytes

The levels of electrolytes measured during the two assays of Cu acute exposition were presented in table 3.5. Also, results from previous work are presented for comparison purposes. Since there was no electrolytes differences between the two assays, comparing same type of tank, results were pool together.

Regarding Na^+, it is possible to observe that barbels from Tank 1 had lower plasmatic levels comparing to barbels from Control Tank and when they are moved to clean water they are able to increase sodium levels i.e. Tank B1 showed same mean values of Na^+ than fish from Control Tank.

Table 3.5. Plasma levels of Na^+ and K^+ obtained in present study and previous study (MONTEIRO, 2012) (M ± DP).

	Sodium (mM/L)		Potassium (mM/L)	
	Present study	*Monteiro, 2012*	Present study	*Monteiro, 2012*
Control	233.422±49.706[a]	*221.22 ± 37.06*	3.366±1.935[a]	*6.53± 2.01*
Tank 1	166.694±45.980[a,b]	*100.37 ± 34.57*	10.763±4.524[b]	*36.59± 16.99*
Tank B1	229.902±59.228[a]		6.107±4.811[a,b]	
Tank 2	33.239±24.554[c]	*85.85 ± 21.45*	88.313±12.346[c]	*37.41± 14.77*
Tank B2	11.810±4.437[c]		2.922±1.444[a]	

Different letters in same column correspond to significant differences

Comparing, both barbels from Tank 2 and Tank B2 showed lower values of Na^+ than Control suggesting a stress effect due to higher Cu exposition (Table 3.5 and Figure 3.11A). Surprisingly, was the levels reported in barbels from Tank B2, i. e. after changing to clean water these fish were not able to return to Na^+ normal (Control) levels. Probably with more time in clean water the fish can reverse the level of sodium in blood.

For the K^+ levels, results show a pattern, with an increase in plasma levels of barbels exposed to Cu, depending of Cu concentration, and a decrease after fish moving to clean water, reaching normal levels (Table 3.5 and Figure 3.11 B). These results suggest that K^+ levels increases with Cu concentration and also that fish have capacity to reverse this situation when they cease to be exposed.

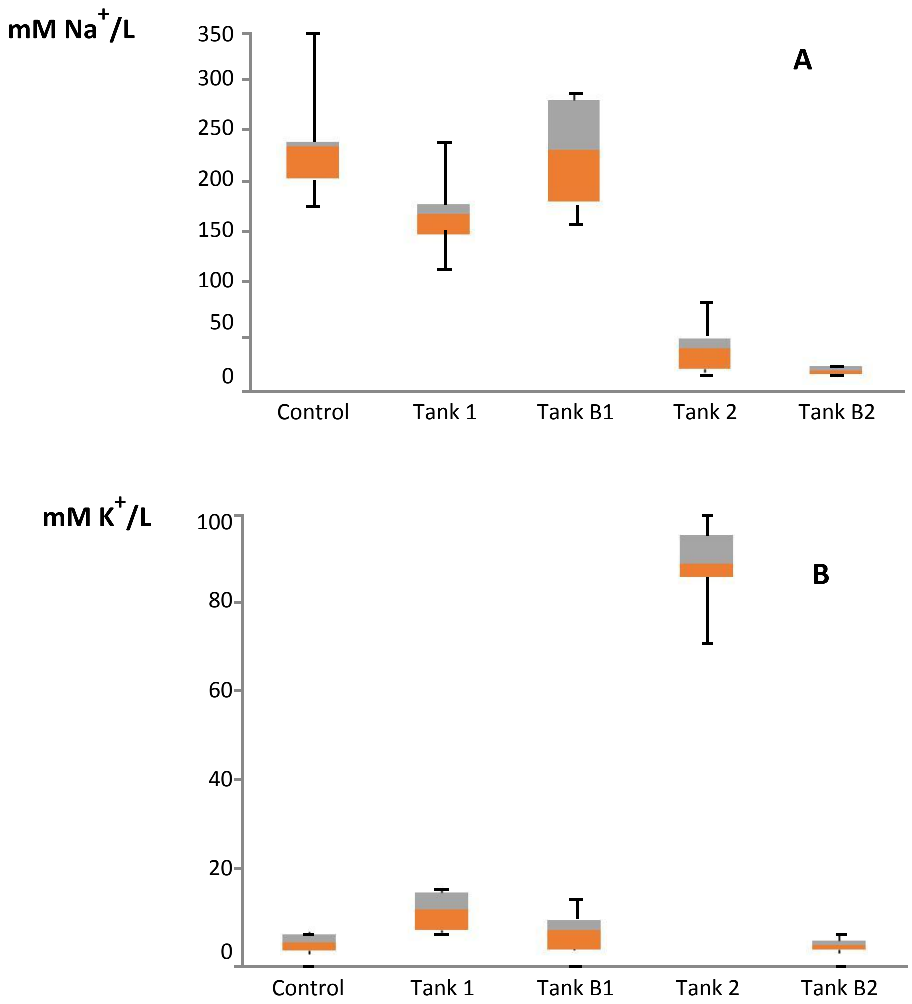


Figure 3.11. Levels of electrolytes measured in barbels after 24h of Cu exposure (Tank 1 and Tank 2) and 24 h in clean water (Tank B1 and Tank B2): (A) Na+; (B) K+

Metal ions such as Cu^{2+} affect the antioxidants of fish tissues including gills and also the active uptake of ions from the water is initially impaired, leading to disturbances of ionic homeostasis (PANDEY et al., 2008). The mechanisms of acute Cu toxicity include the osmoregulatory disturbances involving Na^{+}, $Cl^{–}$ and K^{+} uptake by the gill (MAZON et al., 2002; GROSELL et al., 2003). Plasma K^{+} levels may increase in fish exposed to Cu due to osmotic adjustment, when compensating for a decline of other plasma components, or resulting from disruption of K^{+} regulatory ability. Same trend of K^{+} levels increase and decrease values of Na^{+} have been also observed by MONTEIRO (2012); and FERNANDES et al., (2007; 2009), suggesting a compensatory measure to maintain ionic equilibrium. Also, exposure of freshwater fish to Cu in the water frequently leads to concentration related losses of plasma ions, in particular of sodium and chloride (LI et al., 1998).

Several studies have documented different species variation in degree of tolerance to metals, for example salmonids are very sensitive to contamination by metal (PELGROM et al., 1997; NIYOGI & WOOD, 2004), and some members of Percidae fish family are known as It is very tolerant to metal toxicity (NIYOGI & WOOD, 2004).

However, it is important to note that most of the studies on acute toxicity tests of metals are carried out under different experimental conditions, which may explain some variation when we compare this study with others. More studies address the effects of metals on gill ion regulation, including regulation of plasma electrolytes. GROSSEL et al (2003) examined the effects acute exposure to Cu (7 days) in two species of marine fish, *Raja erinacea* and *Myoxocephalus octodecemspinosus* at different concentrations and found that no concentrations of serum electrolytes were affected, in particular Na^+ plasma, and consequently suggested that the activity of the enzyme gill Na^+/K^+ - ATP-ase was not changed. They suggested that marine fish are less Cu sensitive to contamination, compared to freshwater fish, since high cation concentrations in seawater can perform some protection against the toxicity of Cu. These aspects were also mentioned by PAGENKOPF (1983) and GROSSEL & WOOD (2002), which shows that high sodium calcium concentrations can reduce absorption and Cu toxicity in water salt. Similar to our study WILSON & TAYLOR (1993) observe a decrease in plasma Na^+ and an increase in plasma K^+, in fresh water trout (*Oncorhynchus mykiss*) during the acute lethal exposure of copper.

The Cu promotes changes in ion regulation mechanism, leading to a rapid decrease in plasma electrolytes, and consequently the inhibition of activity of the enzyme Na^+/K^+-ATPase on the gills, leading to the influx of Na^+ (HEATH, 1995; MAZON et al 2002; GROSELL et al., 2003; TAYLOR et al., 2004).

Comparing electrolytes levels measured in Control Tanks, in this work, with controls from MONTEIRO (2012), we can conclude that values are very similar, however electrolytes levels measured after Cu exposure showed some differences. This situation could arise from duration of assays of acute Cu exposure, since levels quantified by MONTEIRO (2012) resulted of 48h of exposition.

During acute Cu exposure, barbels showed several changes such as the physiological and behavioral variation, like ion imbalance, decreased aerobic scope and mortality, reduced Condition Factor (*K*) and weight. One of the conclusions that emerge from this study is that the plasma K^+ levels may increase and Na^+ levels may decrease in fish exposed to Cu, allowing fish to an osmotic adjust in an attempt to survive under a stress environment. Under the conditions of these assays, fish also showed ability to reverse the levels of electrolytes in plasma, when they cease to be exposed to Cu; however that is depending of exposure concentration. Also, it is essential to consider the effects of temperature when assessing the toxic effects of heavy metals on the survival of aquatic organisms.

These results also providing some insights to that are believed to occur in fish population near Portelo mines. Although disappearance and/or reduction of fish species, and degradation of habitats, it is expectable that with gradual reduction of disturbance ecological status, a balance should be achieved.

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CHAPTER 4
CONCLUSIONS AND FINAL REMARKS

This work has provided an ecological characterization of river Portelo and river Fervença, with particular attention to physical and chemical parameters of water quality, to riparian and aquatic habitats and impact of mineral and organic pollution on macroinvertebrates and fish community. Also, acute toxicity tests with Cu providing some insights to that are believed to occur in fish population, near the Portelo mines.

- **Assessment of physical and chemical characteristics of the water**

The quality of the water, measured from references sites (P2 and P6) showed a relatively good parameters of water such as temperature values (T <20° C), electrical conductivity (EC25 <40 µS / cm) dissolved oxygen (OD> 8.5 mgO_2 /L), pH (5.5 < pH <7) and total dissolved solids (TDS <25 mg) typical for rivers in Natural Montesinho Park where the disturbance phenomena are tiny. In turn, the highly disturbance sites witness an increase for most physical and chemical parameters of water, for example, a high values of conductivity (EC25 > 150 µS/cm), dissolved salts TDS (> 100 mg/L) and a values of acid pH (pH=4.0) which are derived not only from human impacts, but also the very spatial variability face the contribution of the entire area of the watershed water quality.

For Fervença River, it was observed a similar tendency to the augmentation of dissolved and particulate substances in the aquatic ecosystem. In fact, higher values measured in highly disturbed sites of conductivity (EC25 > 200 µS/cm), TDS (> 100 mg/L), nutrients (nitrate concentration are much higher than the other sites) and lower values of Dissolved Oxygen (DO < 8 mg/L). This decrease in dissolved oxygen content negatively affects the ecosystem and biodiversity of the aquatic environment. These characteristics are typical of organic polluted rivers. This seasonal trend and the alternate periods of drought and flood events tend to create also harsh environmental conditions and only resistant organisms can survive and colonize successfully these polluted systems.

- **Assessement of the quality of aquatic and riverine habitats**

The Quality Index of Ecosystems Bordering (QBR) and Quality Grade Index Channel (CQC) used showed a good hydromorphological quality for reference sites of both rivers basins. Moderated disturbed sites present some signs of riparian and channel perturbation and for the high disturbed areas (F5 and P4) negative human impacts were more observable.

- **Evaluation of biological quality based on invertebrate communities**

Macroinvertebrates have proven to be excellent bio-indicators of environmental quality, and demonstrated sensitivity to identified disturbing phenomenon. For Portelo River, the number of individuals and taxa high disturbance sites was low compared to moderated disturbed reference sites. A different tendency was observed in River Fervença. In fact, the higher abundance (> 350 individuals) and the lower richness (< 10 taxa) were obtained in the highly disturbed site. For Shannon-Wienner Index (H') and evenness of Pielou (J') there was an important reduction of aquatic macroinvertebrates values in both rivers. In Portelo River the decrease on H' diversity index was accompanied by the higher values of J' evenness index whis can explain the lack of taxa dominance. For Fervença River it was found lower values for both indexes, H' and J', as a result of environmental conditions in organic polluted ecosystems. For others metrics, including IBMWP indices and especially IPtIN, these two indexes were sensible enough to detect the most disturbed sites: P1 and P4 for Portelo and F6, F7and F8 for Fervença River.

- **Evaluation of biological quality based on fish communities**

Fish communities were present in only in three sampling sites of Portelo Basin and two sampling sites of Fervença River. Despite the good ecological condition in several reference sites (P2 and F1), no fish communities were found because these watercourses have no stable conditions to maintain fish populations. Five native species were captured, belonging to the families: Salmonidae (brown trout, *Salmo trutta*) and Cyprinidae (Iberian Northern chub, *Squalius carolitertii*; Calandino roach, *Iberocypris alburnoides*; common barbel, *Luciobarbus bocagei;* Douro nase, *Pseudochondrostoma duriense*) and one exotic species, belonging to the Cyprinidae family (carp, *Cyprinus carpio*). In the headstreams of Montesinho Natural Park only autochthonous fish populations can be detected, being an indicator of good ecological integrity of these watercourses, in contrast to the middle and final sections of the main stream (e.g. River Sabor), where already occur in high densities distinct exotic fish populations.

- **Evaluation of Acute Cu exposure, using a native fish species**

Several metrics, i.e. water parameters, fish behavior, Condition Factors (*K*) and plasma electrolytes concentration were used to evaluate the acute Cu exposure. Barbels showed several changes at physiological and behavioral level, such as ion imbalance, decreased aerobic scope and mortality, as well as a lower Condition Factor (*K*).

The main conclusion that emerges from this study is that the plasma K^+ levels may increase and Na^+ levels may decrease in fish exposed to Cu suggesting a compensatory measure to maintain ionic equilibrium. Under conditions of these assays and depending of exposure concentration, fish also showed ability to reverse the levels of electrolytes in plasma, when they cease to be exposed to Cu. Also, it is essential to consider the effects of temperature when assessing the toxic effects of heavy metals on the survival of aquatic organisms.

These results provide some insights to that are believed to occur in fish population near Portelo mines. It is expectable that with gradual reduction of disturbance ecological status, a balance should be achieved.

As our modern society is facing a serious need for health care, the challenges encountered in this research field are becoming more and more important to face all kind of disturbance that may occur. For the development of healthy and environmentally friendly environment, a new quantified approach is needed to evaluate the overall impact of mining waste, industrial and domestic input and others on the health of humans and ecosystems.

Therefore, a need to increase the knowledge about the effect of all kind of pollution (mineral, organic, radiation…) is necessary to face the consequences that may happen for generation to come.