Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Dr. Amadeu Soares, Professor Catedrático, e do Dr. António Nogueira, Professor Associado com Agregação, do Departamento de Biologia da Universidade de Aveiro

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To Ricardo

To my mother

For all support, love and care

To my brother

To Sandra and Irene

For the friendship and solidarity

o júri

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palavras-chave *Chironomus riparius*, bioensaios, qualidade da água, contaminação de rios.

resumo

Este trabalho debruça-se sobre a utilização de bioensaios *in situ* com larvas de *Chironomus riparius* na avaliação da qualidade da água e do risco de contaminantes nos ecossistemas lóticos.

Estes foram realizados sazonalmente em locais seleccionados em rios contaminados e rios de referência no norte e centro de Portugal, para determinar a sua eficácia na avaliação da qualidade da água nos ecossistemas lóticos. Várias respostas biológicas das larvas (desenvolvimento, crescimento, sobrevivência e taxas de ingestão) foram determinadas, bem como os parâmetros bióticos, físicos e químicos de cada local. Verificou-se que os bioensaios avaliaram com sucesso a qualidade da água, com excepção dos locais com contaminação orgânica.

O impacto dos pesticidas (usados em larga escala na agricultura) nos rios e macroinvertebrados, é difícil de avaliar devido à sua rápida degradação. Para determinar os efeitos dos pesticidas nos parâmetros biológicos das larvas de *C. riparius* (desenvolvimento, crescimento, sobrevivência, taxa de ingestão e massa corporal), foram realizados bioensaios em rios artificiais (contaminados com o insecticida lambda-cialotrina) e em campos de arroz (contaminados com o insecticida endosulfão e os herbicidas molinato e propanil). A exposição ao insecticida nos rios artificiais resultou numa diminuição do crescimento e a uma inibição do desenvolvimento larvares. Nos ensaios realizados nos arrozais o crescimento das larvas foi apenas inibido pelo insecticida.

As actividades mineiras e os efluentes provenientes de minas abandonadas provocam um grande impacto químico no ambiente. Bioensaios *in situ* foram realizados em habitats lóticos impactados por uma mina de tungstênio activa e utilizados para monitorizar a variação da contaminação durante um processo de reabilitação ambiental de uma mina de ouro abandonada. Verificou-se que o crescimento e desenvolvimento larvares permitiram distinguir os locais altamente contaminados dos de baixa contaminação e permitiram detectar diferenças na contaminação dos rios durante o processo de reabilitação ambiental da mina.

Foram também realizados bioensaios em laboratório (1) para verificar se os testes *in situ* apresentam resultados similares em condições laboratoriais, (2) para comparar a sensibilidade dos parametros biológicos (desenvolvimento, crescimento e massa corporal) à contaminação por metais na água e no sedimento e (3) para determinar a importância da toxicidade causada pelos metais pesados que entram no organismo através do material ingerido. Verificou-se a) que a toxicidade causada pelos metais pesados e pelos pesticidas nos bioensaios *in situ* foi também observada em condições laboratoriais, b) que as larvas foram mais afectadas pela contaminação por metais pesados no sedimento do que na água, e c) uma diminuição da toxicidade quando a ingestão de sedimento contaminado foi evitada.

Em conclusão, esta tese permitiu avaliar a eficácia dos bioensaios com larvas de *C. riparius*, bem como as suas limitações, na avaliação da qualidade da água na presença de vários tipos de contaminantes.

keywords

Chironomus riparius, bioassays, water quality, river contamination.

abstract

This thesis deals with the use of *in situ* bioassays with *Chironomus riparius* larvae to assess water quality and the risk of contaminants on freshwater ecosystems.

These bioassays were seasonally deployed in selected sites on contaminated and reference rivers of North and Central Portugal, to evaluate their performance in assessing water quality in lotic ecosystems. Several biological responses (development, growth, survival and post-exposure feeding rate) were determined and the biotic, physical and chemical parameters were collected for each site. It was found that *in situ* bioassays successfully evaluated the water quality of rivers, except when nutrient pollution was present.

The impact of pesticides (used worldwide in agriculture) on river and macroinvertebrates is difficult to assess due to its quick degradation. To determine pesticide water contamination effects on biological parameters of the *C. riparius* larvae (development, growth, survival, post-exposure feeding rate and biomass), bioassays were carried out in indoor artificial streams (contaminated with the insecticide lambda-cyhalothrin), and in rice fields (contaminated with the insecticide endosulfan and the herbicides molinate and propanil). Exposure to the insecticide in artificial streams resulted in significant impairment in growth and inhibition in development of larvae. In rice fields, only larval growth was inhibited by the insecticide.

The mining industry and mine drainage from abandoned mines are often the cause of environmental damage due to its chemical impact. *In situ* bioassays were carried out in streams impacted by a tungsten mine and also used to monitor metal contamination variation in rivers during an environmental rehabilitation process of an abandoned goldmine. It was observed that growth and development of larvae could discriminate between high and low metal contaminated sites and could detect differences in river contamination throughout the environmental rehabilitation process.

Laboratory bioassays were also performed (1) to verify if *in situ* bioassays responded in the same way as in laboratory conditions, (2) to compare sensitivity of biological endpoints (development, growth and biomass) to metal contamination in water and sediment and (3) to determine toxicity of heavy metals that enter the organism through ingested material. It was found a) that the toxicity caused by heavy metals and pesticides during *in situ* bioassays, was also detected in laboratory conditions, b) that the larvae were affected mostly by metal contamination on sediment, and c) a decrease in toxicity when the sediment ingestion by larvae was avoided.

In conclusion, this thesis allowed us to evaluate the efficacy of the bioassays with *C. riparius* larvae, and their limitations, in the assessment of water quality in the presence of several contaminants.

indice

Introduction

This thesis is a collection of scientific papers focused on the validation of a methodology for the ecological assessment of water quality. For this purpose, throughout this thesis, *in situ* bioassays with *Chironomus riparius* larvae were used with key sources of contamination on Portuguese rivers. But first, I will address the main questions and observations that were the basis of this thesis.

Water quality

Ecological assessment of water quality is essential to the management of surface waters and the protection of aquatic ecosystems. In view of the complexity of factors that determine water quality and the large choice of variables used to describe the status of water bodies in quantitative terms, it is difficult to provide a simple definition of water quality (Meybeck and Helmer 1989). In theory, the physical and chemical quality of pristine waters would be as it was in pre-human times, i.e. with no signs of anthropogenic impacts (Meybeck and Helmer 1989). Also, the individual patterns of physical and chemical characteristics of each freshwater body, which are determined largely by the climatic, geomorphological and geochemical conditions prevailing in the drainage basin and the underlying aquifer, could, nevertheless, vary between different drainage basins. In practice, pristine waters are very difficult to find as a result of atmospheric transport of contaminants and their subsequent deposition in locations far distant from their origin (Meybeck and Helmer 1989). Significant concentrations of certain contaminants are even being observed in Arctic and Antarctic snow and ice (Gregor and Gummer 1989).

Before pristine waters reach the polluted condition, two phases of water quality degradation occur (Meybeck and Helmer 1989). The first phase shows an alteration in water quality with some evidence of human impact but with no harm to the aquatic habitat and organisms. The next phase consists of some degradation of water quality. Once aquatic habitat and the biota have been markedly modified or water has become became unfit for certain uses, the water quality is usually defined as polluted. However, the concept of pollution is relative, since it reflects a change from

some reference value to a value that causes problems for human use. Therefore, a worldwide reference value is difficult to establish because insufficient monitoring occurred prior to changes in water quality due to human activities (Meybeck 1996). Furthermore, there is no universal reference of natural water quality because of the high variability in the chemical quality of natural waters (Meybeck 1996).

A fundamental aspect to be considered in the evaluation of water quality in freshwater ecosystems is sediment quality. Sediment is an essential, integral and dynamic part of river basins, with a great ecological value. It provides the habitat and food resources for many organisms and through a close interaction with the overlaying water it is the base of aquatic ecosystems (Brils 2002). Many pollutants that enter aquatic environments bind to the surface of suspended particles in the water column or settle into the sediment (Simkiss et al. 2001). As a result of this, the concentration of contaminants in the sediment may be much higher than in water (Simkiss et al. 2001). Eventually, they can be released into surrounding water and assimilated by organisms (Burton 1991; DeNicola and Stapleton 2002), representing a threat to organisms either when released back into the water or when intake occurs directly from the sediment (Simkiss et al. 2001). Therefore, it is clear that to achieve and maintain the "good status" of all surface and ground waters, the evaluation and proper management of sediment quality is indispensable (Brils 2002). Thus, the expression "water quality" is often used for simplicity but it refers to the overall quality of the aquatic environment and, thus the biological assessing of water quality it means assessing biological quality of the aquatic environment. For example, in aquatic ecosystems, the benthic macroinvertebrates communities often used to assess "water quality" can be more affected by chemical quality of sediment than by the chemical quality of water column. Several authors (e.g. Hoehn and Sizemore 1977; Letterman and Mitsch 1978; McKnight and Feder 1984; Gray 1996; Verb and Vis 2000; DeNicola and Stapleton 2002) found that precipitated forms of heavy metals in sediment can be more detrimental to benthic invertebrates communities than dissolved forms in the water column.

In contrast to the chemical quality of water, which can be measured by suitable analytical methods, the description of the biological quality of water is a combination of qualitative and semi-quantitative characterization (e.g. biotic indices, species inventories) and quantitative measurements (e.g. toxicity tests). The majority of methods for biomonitoring of fresh waters have been based on measures of community structure, focusing on benthic macroinvertebrates (Rosenberg and Resh 1993) although this does not imply that other communities do not have important roles to play. For example, plant monitoring is more suitable for detecting pollution by herbicides, since herbicides cause greater effects on plants than on macroinvertebrates (Hawkes 1979). Most of the methodologies for the analysis of collected data involve the calculation of

indices including measures of species richness, composition or diversity. In order to use these biological measures to determine water quality, observed values need to be compared to a reference condition, which is achieved by comparing indices to a reference value determined for non-impacted sites in a region (i.e. regional reference condition) or to a value expected if the site was not impacted (i.e. site-specific reference condition). The former approach is used in North America where up to 10 different indices of macroinvertebrate community structure are combined to produce a multimetric index (Barbour et al. 1997), which is then compared to an ecoregional reference condition (Gerritsen 1995).

Biological monitoring of European river systems is based almost entirely on measures of community structure. The Belgium Biotic Index (BBI) and the Indice Biotique (France) are based on Trent Biotic Index (TBI), proposed in the UK by Woodiwiss in 1964. The Saprobic index (Germany, The Netherlands) is based on a system originally proposed by Kolkwitz and Marsson in 1902 and revised by Friedrich (1990). The Biological Monitoring Working Party (BMWP) score used in the UK is an extension of the TBI and is often expressed as the Average Score per Taxon (ASPT), to correct for sampling effort (Armitage et al. 1983). There are only a few studies focused on the applicability of biological methods in Southern Europe. Those few studies (e.g. Coimbra et al. 1996; Coimbra and Graça 1998) suggested that changes in water quality during high discharge periods (winter-spring) are predictable from the BMWP' score, adapted from the BMWP system to the Iberian Peninsula by Alba-Tercedor and Sánchez-Ortega (1988) and ASPT', derived from BMWP' (Klemm et al. 1990). But during low discharge periods (summer-autumn) these biotic indices show a lower ability to predict water quality. None of these methods, however, consider the effect of low water quality or contaminants on the sublethal responses of the organisms (e.g. growth). According to Charvet (2000) sublethal responses of the organisms are more suitable for biomonitoring of streams than taxonomic/community structure (i.e. composition and the abundance of species or other taxa), because they are more effective in discriminating reference, low contaminated and high contaminated sites and, also, to discriminating between different human impacts on the benthic macroinvertebrates of running waters. In addition, community structure is inappropriate for biomonitoring over large geographic regions since altitude and geological differences generate wide differences in taxonomic structure at the reference sites (e.g. missassigning water quality in slow-flowing lowlands streams) while sublethal responses of the organisms tend to homogenize between spatially distant locations (Charvet et al. 2000).

Furthermore, most of the "ecosystem services" (benefits that society obtains from properly functioning ecosystems, such as drinking, fishing) depend on the functional integrity of the ecosystem. Recently, several techniques have been developed, which in combination with more established methods, offer the potential to assess the basic functions of running-water ecosystems.

These include the use of nutrient diffusing substrates (Matlock et al. 1998) to assess nutrient limitation of primary production, and *in situ* testing with the deployment of test organisms in the field to measure how the local environmental parameters (e.g. pH, pollution) modifies their functional performance (e.g. feeding rate) (e.g. Crane et al. 1996). Ecosystem functions depend on the availability and processing of energy and the main energy sources in running waters are detritus and aquatic plant biomass, while the major consumers of this energy are invertebrates. The incorporation of biomass from detritus material into animal biomass depends on its utilization by shredders and collectors (Maltby 1996).

Aquatic toxicity and environmental risk assessment

With the industrialization and the increase of human populations, the range of requirements of water for "ecosystem services" has increased together with greater demands for higher quality water. Each use of water, including extraction of water and discharge of wastes, leads to specific impacts on the quality of the aquatic environment. Additionally, there are several human activities which have indirect and high negative effects on the aquatic environment, such as the uncontrolled land use for urbanization or deforestation and the release of chemical substances (pollutants).

In general, pollutants can be released into the environment as gases, dissolved substances or in the particulate form and can reach the aquatic environment through a variety of pathways, including the atmosphere and the soil (Meybeck and Helmer 1989). Pollution may result from point sources or diffuse sources (non-point sources). Point sources of pollution occur when harmful substances are emitted directly into a body of water and may be collected, treated or controlled, while non-point sources deliver pollutants indirectly through environmental changes. An example of this type of water pollution is when a fertilizer or a pesticide from a field is carried into a stream by rain, in the form of run-off which in turn affects aquatic life. The major point sources of pollution to freshwaters originate from the collection and discharge of domestic wastewaters, industrial wastes and acid mine drainages or from certain agricultural activities, such as animal husbandry (Meybeck and Helmer 1989). Most other agricultural activities, such as pesticide spraying or fertilizer application, are considered as diffuse sources. The atmospheric release of pollutants also leads to diffuse pollution of the aquatic environment. Therefore, determining potential ecological risks and investigating the nature of aquatic toxicity are critical elements in discharge monitoring and water quality management programs. Risk assessment of contaminated sites involves the use of bioassays which are performed under controlled conditions. However, laboratory toxicity testing does not always generate ecologically relevant information for the area in concern (Giesy and Hoke 1989), mainly because field situations may not be accurately simulated in the laboratory and sample collection (water or sediment), storage or handling can affect sample

toxicity (Chappie and Burton 1997). However, the impact of substances can also be assessed using more environmental realistic conditions through the use of mesocosms or field monitoring studies (Boxall et al. 2002).

No single type of bioassay or test organism is suitable for all situations, but the optimal assays also vary according to the study and its objectives (Burton 1991). Chironomids (i.e. *Chironomus tentans* and *Chironomus riparius*) are among the test species recognized as useful tools in studies of sediment toxicity (Ankley et al. 1994). Chironomid larvae are commonly used for the toxicity analysis of natural (Giesy et al. 1990; Hoke et al. 1993; Pellinen and Soimasuo 1993; Pery et al. 2003c) or spiked (Brown et al. 1996; Harrahy and Clements 1997; Pery et al. 2003b) sediments or bioaccumulation of sediment-associated contaminants (Ankley et al. 1994; Harrahy and Clements 1997). Although they have benthic larvae, chironomids are also appropriate for water-only tests (Bleeker et al. 1998; Stuijfzand et al. 2000). In addition to laboratory experiments, chironomids are also used for *in situ* bioassays (Chappie and Burton 1997; Tucker and Burton 1999; Castro et al. 2003). However, none of the test designs used so far have been widely accepted and different feeding levels, organism densities, organism health or test durations have been used. This lack of standardization means that different results are obtained for the same conditions of water quality and contamination exposure, preventing comparison of results obtained by different tests.

In this work we developed *in situ* bioassays with *Chironomus riparius* larvae to assess water quality and to monitor river metal contamination. Biological parameters, such as development, growth, survival and biomass, and the functional response post-exposure feeding rate, were used as endpoints. Laboratory bioassays were used to verify if *in situ* bioassays responded in the same way as in laboratory conditions and whenever site-specific ecotoxicological information was not needed. Bioassays were also conducted in artificial streams (mesocosms) and in rice fields to assess pesticide contamination in water because these compounds degrade very quickly, affecting the assessment of their ecological effects in running waters.

Chironomus riparius

This species from the dipteran family Chironomidae is widely distributed in the northern hemisphere, at temperate latitudes and it can be found in both lentic and lotic environments, usually in organic enriched waters (Armitage et al. 1995). The life cycle of these insects comprises aquatic stages (egg, four larva instars and a pupal stage) and an aerial adult stage. The larvae, which are collector-gatherers, feed on detritus deposited or mixed with sediment (Vos 2001). Chironomids are of great interest in ecology, since they represent a predominant part of benthic communities in virtually all freshwater habitats (Péry et al. 2002; Ristola 2000; Vos 2001). Berg and Hellenthal

(1992) reported an annual chironomidae secondary production in an American stream (northern Indiana, USA) that accounted for 80% of the total insect secondary production. Furthermore, they are important preys for other animals (e.g. fish and aquatic birds) in these ecosystems (van de Bund 1994; Prat and Rieradevall 1995; García-Berthou 1999). Chironomids are very useful for toxicity tests since they are easy to maintain in laboratory cultures, their life stages are easy to identify and they have a short life history under laboratory conditions (Péry et al. 2002). Their ecological diversity is corroborated by their physiological tolerance to environmental stressors, such as modification of salinity or temperature and they are able to survive and develop under hypoxic conditions (Armitage et al. 1995; Choi et al. 1999; Choi et al. 2001; Choi and Roche 2004). Due to these characteristics, they can play an important role as sentinel organisms in environmental monitoring (Choi et al. 1998).

Heavy metals

Mining industry causes serious environmental problems throughout the world due to chemical impact caused by production of exploitation residues and mineral processing, resulting in solubility and diffusion of heavy metals. The chemical impact of mine drainage from abandoned mines into the environment throughout the world is also large. These have eliminated several invertebrate species and significantly changed community composition in many metal contaminated areas (Clements 1994; Kiffney and Clements 1994). Metal contamination in rivers also causes a potential health risk for human populations living near contaminated rivers (Albering et al. 1999). It is now understood that to achieve a sustained development of mining industry it is essential to take into account environmental issues (Allan 1995). In Portugal, the potential environment impact from the 80 abandoned mine areas is great (Costa and Leite 2000). Studies on the impact caused by some of these mines showed negative effects on aquatic macroinvertebrates (Lopes et al. 1999; Castro et al. 2003), on leaf decomposing processes in rivers (Rocha 2004) and on human populations (Gomes 1999; Mayan et al. 2005). On the other hand, despite the immense potential risk to environment of the major Portuguese active mine (Panasqueira mine), which is the third major producer of tungsten in the world (Costa 1998; Romão 2001) its impact has not yet been evaluated.

Pesticides

Pesticides are natural (e.g. pyrethrins produced from the flowers of *Tanacetum cinerariaefolium*) or synthetic (e.g. pyrethroids) chemicals that are mostly applied in agriculture to eliminate target organisms (e.g. insects, fungus, algae). Several pesticides are globally used in

agriculture and may enter aquatic environments through spray drift or runoff events, drainage or leaching, resulting in contamination of non-target environments such as surface and ground waters (Hamer et al. 1999; Leonard et al. 1999; Schulz et al. 2001a, 2001b; Cerejeira et al. 2003). Surface water contamination may have ecotoxicological effects for aquatic flora and fauna (Forney and Davis 1981; Mulla and Mian 1981), and for human health if used for public consumption (Funari 1995). However, the impact of these compounds on river organisms is usually difficult to assess because these compounds degrade very quickly (Barry and Logan 1998), because they can be absorbed onto the sediment (Hamer et al. 1999; Peterson and Batley 1993) and because peak concentrations of pesticides in rivers are rarely measured during storm runoff events, since sites are often inaccessible and these peaks may last only a few hours (Cooper 1996). In Portugal, Cerejeira et al. (2003) detected and quantified several insecticides (e.g. β-endosulfan, lindane, chlorfenvinphos) and herbicides (e.g. atrazine, molinate, propanil metabolite) during 1983–1999 in water samples collected in Tagus, Sado and Guadiana river basins. They found that high concentrations of pesticides in water were temporary, since they were only observed during the application season and decreased quickly throughout the summer, with non-detectable levels in September and October. However, the situation of pesticide pollution in other rivers is poorly known (Cerejeira et al. 2003). Furthermore, studies that evaluated the impact of these chemicals substances to aquatic organisms in Portugal are few (e.g. Cerejeira et al. 1998; Cerejeira et al. 1999; Pereira et al. 2000).

Outline

This thesis is structured in five chapters, which were laid out so they can be published in refereed scientific journals, in order to increase the dispersion of this knowledge. After these chapters, a general discussion and conclusions chapter deals with the relevance of the results and their implications for the use of *in situ* bioassays with *C. riparius* larvae for the ecological assessment of water quality and to monitor rivers impacted by heavy metals and insecticides. This structure is the result of a thesis rationale focused on very specific key questions:

1. Can *in situ* bioassays with *C. riparius* be used to assess river water quality as an alternative to biotic indices?
2. Which types of contamination can be assessed by *in situ* bioassays with *C. riparius*?
3. Can ecological responses of *in situ* bioassays be used to monitor contamination in rivers?
4. How reliable is the extrapolation of results from laboratory tests to *in situ* bioassays?
5. Does the sensitivity of *C. riparius* larvae to contamination differ between water and sediment contamination?

6. What is the relative importance of toxicity caused by contaminants that enter the organism through ingested material in comparison with other pathways?

In the **first chapter**, a bioassay with *C. riparius* larvae using ecological (survival, development and growth) and functional (feeding rate) responses as endpoints was developed to assess river water quality and contamination. The results of *in situ* bioassays conducted in reference rivers are compared with bioassays performed in organic/nutrient and metal contaminated rivers in Portugal. The relationships between biological endpoints, biotic indices and physical and chemical parameters were investigated.

In the **second chapter**, the bioassay developed in the previous chapter was used to study the impact of an insecticide (lambda-cyhalothrin) on *C. riparius* larvae. The bioassay was carried out in artificial streams in order to simulate the ecological conditions of natural lotic systems.

In the **third chapter**, bioassays were carried out in channels of rice fields, during the spraying period of several pesticides (molinate, endosulfan and propanil), to determine pesticide contamination effects on biological endpoints. Simultaneously, bioassays with contaminated water from rice fields and conditioned sediment were also run under laboratory conditions, in parallel to the *in situ* bioassays, to assess whether *in situ* and laboratory tests showed comparable results.

In the **fourth chapter**, bioassays were carried out in spring, in three consecutive years (2002-2004), throughout an environmental rehabilitation process of an abandoned goldmine. Laboratory bioassays, with field water and sediment, were also performed (a) to verify if *in situ* bioassays responded in the same way as in laboratory conditions, (b) to compare sensitivity of biological endpoints to metal contamination in water and sediment and (c) to determine toxicity of heavy metals that enter the organism through ingested material.

In the **fifth chapter**, the impact of metal contamination on the river ecosystems impacted by a tungsten mine is evaluated. For this purpose, an *in situ* bioassay was conducted in reference and metal contaminated specific sites in a stream that receives water directly from the mine galleries and water from the water treatment station.

All these chapters focus on specific issues that are important for the final goal of this work, that is, to increase our knowledge on the use of bioassays with *C. riparius* to assess river water quality and to biomonitor river contamination, so that these results can be useful for the long term monitoring of lotic ecosystems.

Chapter 1

Biological and functional responses of *in situ* bioassays with *Chironomus riparius* larvae to assess river water quality and contamination

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Abstract

Single species responses are more sensitive to river contamination and have the potential of measuring impacts at earlier stages than more traditional methods based in community structure. This study developed an *in situ* bioassay with biological (survival, development, growth) and functional (post-exposure feeding rate) responses of *Chironomus riparius* larvae to assess water quality and contamination in rivers. The bioassay with *C. riparius* third instar larvae was performed, seasonally, in reference rivers and in organic and metal contaminated rivers in Portugal. Biotic, physical and chemical parameters were collected for each site. Relationships of responses and biotic indices (BMWP' and ASPT') to the physical and chemical parameters were determined. Biotic indices were able to discriminate between contaminated rivers and not contaminated rivers in spring, but showed low ability to predict water quality in autumn. In spring, ASPT' was negatively related with ammonia concentrations. No significant differences in survival and post-exposure feeding rate were found between rivers. These responses were positively related with nitrite concentrations in autumn. Development was inhibited in the most metal contaminated river in autumn but pH accounted for 60% of development variation in this season. Growth was inhibited in metal contaminated rivers and an increase in growth was observed in spring. The pH and Mn concentrations accounted for 96% of growth variation in autumn. The results suggest that *in situ* bioassay with *C. riparius* larvae using growth as the endpoint is a responsive and suitable tool that can be used as bioindicator of metal pollution and to biomonitor water quality in metal contaminated rivers.

Keywords: *Chironomus riparius*, *in situ* bioassays, organic contamination, metal contamination

Introduction

Ecological assessment of water quality is essential for the management of surface waters and the protection of aquatic ecosystems. This assessment has been based on measures of community structure (e.g. species richness, composition, diversity and pollution tolerance), mainly of benthic macroinvertebrates (Rosenberg and Resh 1993). Such data have been used to calculate a number of different biotic indices. Biological monitoring of European river systems is based almost entirely on measures of community structure. For example, the Biological Monitoring Working Party (BMWP) score, used in the UK, separates invertebrate groups or taxa on the basis of their relative sensitivity to pollution with the more pollution sensitive taxa being allocated higher scores and the more pollution tolerant taxa lower scores. BMWP is often expressed as the Average Score per Taxon (ASPT), to correct for sampling effort (Armitage et al. 1983), which is obtained by dividing the BMWP score by the number of taxa present. Studies on the applicability of biological methods in Southern European and/or Mediterranean rivers are few. Those studies (e.g. Coimbra et al. 1996; Coimbra and Graça 1998) suggested that changes in water quality during high discharge periods (winter-spring) are predictable from the BMWP' score, adapted from the BMWP system to the Iberian Peninsula by Alba-Tercedor and Sánchez-Ortega (1988) and ASPT', derived from BMWP' (Klemm et al. 1990). But during low discharge periods (summer-autumn) these biotic indices show a lower ability to predict water quality.

Structure of benthic macroinvertebrates community is influenced by other factors besides pollution such as habitat characteristics and water hardness (Coimbra et al. 1996; Coimbra and Graça 1998; Ingersoll et al. 2001). Thus, methods based in the benthic macroinvertebrates community may fail in detecting contamination. Single-species responses are more sensitive to contaminants and can give a rapid indication of water quality because effects measured at the individual level will be manifested more rapidly (hours to days) than changes in community structure (months to years) (Maltby 1994; Maycock et al. 2003). Furthermore, community structure is inappropriate for biomonitoring over large geographic regions since altitude and geological differences generate wide differences in taxonomic structure at the reference sites (e.g. missassigning water quality in slow-flowing lowlands streams), while single species responses tend to homogenize between spatially distant locations and are more effective in discriminating different human impacts on the benthic macroinvertebrates of running waters (Charvet et al. 2000). Thus, methods based on responses of single species can be more suitable for biomonitoring of streams than community structure. Although the ultimate level of concern may be ecosystems, observing an

effect on a particular organism can be of ecological significance (Maltby 1999). Since pollutants rarely affect only a single species and because of the complex interactions (e.g. predator/prey) among organisms (Liu et al. 2002), the impacts on any species or group of organisms is likely to be disseminated throughout the whole ecosystem. If nothing else, an observed effect for a particular bioindicator indicates that further investigation is necessary.

Many *in situ* bioassays use single species, such as fish (e.g. Hatch and Burton 1999), amphipods (e.g. Chappie and Burton 1997), cladocera (e.g. Ireland et al. 1996) or chironomids (e.g. Chappie and Burton 1997; Castro et al. 2003) as tests organisms. Most of these studies use biological responses (survival, growth and development) as endpoints. These responses are ecologically relevant because they are important components of fitness and determinants of population health (Liber et al. 1996; Sibley et al. 1997a), structure and dynamics (Maltby 1994). However, biological responses are usually not suitable to be used as indicators of impact on functional integrity of ecosystem although most of the ecosystem services (such as drinking, fishing) depend on the functional integrity of the ecosystem. Recently, several techniques have been developed, which offer the potential to assess the basic functions of running-water ecosystems. These include *in situ* testing with the deployment of test organisms in the field to measure how the local environmental parameters (e.g. pH, pollution) modify their functional performance (e.g. feeding rate) (Crane et al. 1996; Maltby et al. 2002; McWilliams and Baird 2002b) Ecosystem functions depend on the availability and processing of energy. The main energy sources in running waters are detritus and aquatic plant biomass, while the major consumers of this energy are invertebrates. The incorporation of biomass from detritus material into animal biomass depends on its utilization by shredders and collectors (Maltby 1996).

This study focused on *C. riparius* larvae, a collector-gatherer (feeds primarily on fine particulate organic material deposited on sediment), which is a member of the widely distributed insect family Chironomidae. Chironomids are of great interest in ecology since they represent a predominant part of benthic communities in all freshwaters (Ristola 2000; Péry et al. 2002), play an important role in detritus processing, recycling organic matter and energy, and are an important prey species for fish and aquatic birds (Rieradevall et al. 1995). Their ecological diversity is corroborated by their physiological tolerance to environmental stressors, such as alterations of salinity or temperature and they are able to survive and develop under hypoxia conditions (Armitage et al. 1995; Choi et al. 1999; Choi et al. 2001). Due to these characteristics, they can play an important role as sentinel organisms in environmental monitoring (Choi et al. 1998).

The main goal was to develop a bioassay with an important collector species, using ecologically and functionally relevant responses as endpoints to assess and monitor water quality and contamination in rivers. An *in situ* bioassay with *C. riparius* larvae using survival, growth,

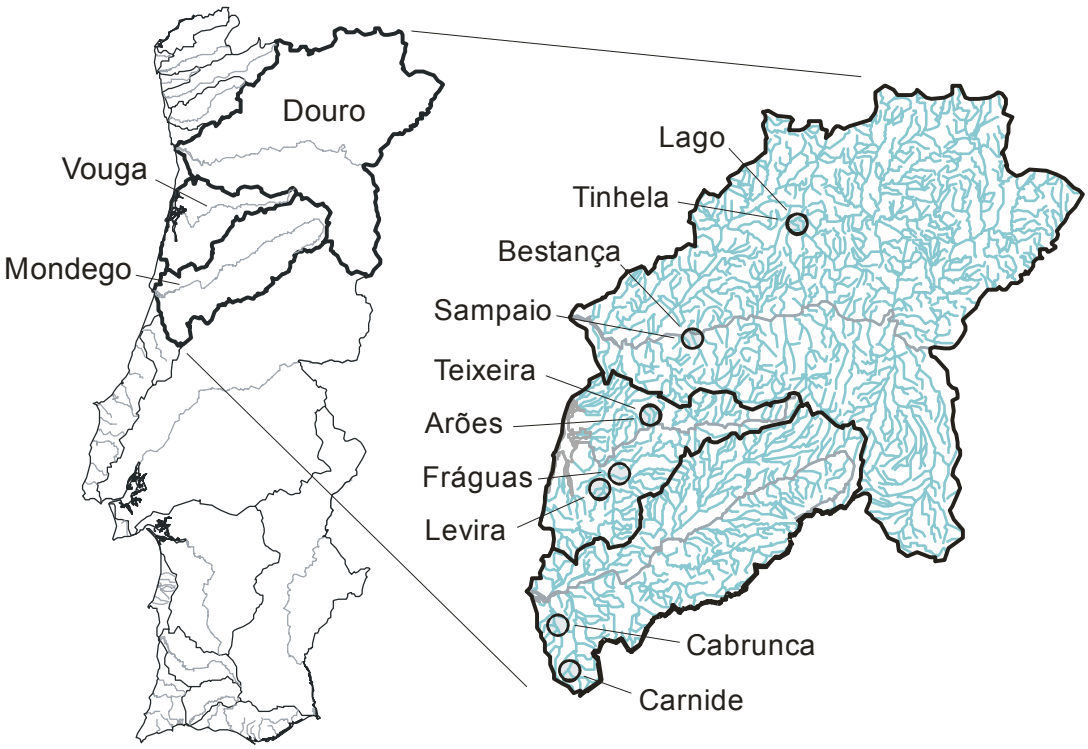

Fig. 1. The location of the sites where the bioassay was performed, selected in three Portuguese river basins (Douro, Vouga and Mondego).

development and post-exposure feeding rate as endpoints was developed and used to compare contaminated and reference sites in Central and North Portugal. Seasonal deployment of the *in situ* bioassay was performed in autumn and spring at the selected sites in order to gain insight about temporal variability in response. Environmental and physical parameters were collected for each site including: altitude, latitude/longitude, alkalinity, pH, temperature, substrate composition. The biotic indices BMWP' and ASPT' were also determined for each site.

Material and Methods

Study sites

In situ bioassays of *Chironomus riparius* were carried out in reference and contaminated rivers of three river basins of North and Central Portugal (Fig. 1 and Table 1) in autumn 2001 and spring 2002.

The selected reference sites in rivers Bestança, Sampaio, Teixeira, Arões, Fráguas and Cabrunca were located in river segments where no significant sources of pollution were observed and far from major human or industrial agglomerates or intensive agricultural fields. The Tinhela river was considered a low contaminated site due to its proximity to an abandoned goldmine.

Table 1. Localization, altitude and status (contaminated or reference) of the sites where bioassays were performed.

Rivers	Localization	Altitude	Status	Type of contamination	Source of contamination
Douro basin					
Bestança	41º 04' N, 08º 04' W	96 m	Reference		
Sampaio	45º 04' N, 08º 07' W	150 m	Reference		
Tinhela	41º 28' N, 7º 33' W	731 m	Low contaminated	Heavy metals	Abandoned goldmine
Peliteira	41º 28' N, 7º 34' W	735 m	Contaminated	Heavy metals	Abandoned goldmine
Vouga basin					
Teixeira	40º 46' N, 8º 15' W	170 m	Reference		
Arões	40º 47' N, 8º 17' W	417 m	Reference		
Fráguas	40º 44' N, 8º 26' W	85 m	Reference		
Levira	40º 30' N, 8º 30' W	42 m	Contaminated	Difuse	Agriculture, ceramics industry and small urban centre
Mondego basin					
Cabrunca	39º 51' N, 8º 40' W	126 m	Reference		
Carnide	40º 00' N, 8º 43' W	27 m	Contaminated	Organic and pesticides	Agriculture (rice fields)

Although the selected site is located upstream of any contaminated tributary stream, there can be underground water and atmospheric contamination. On the other hand, the Peliteira stream, a tributary of Tinhela river, was considered to be contaminated since its drainage basin included this abandoned goldmine. This mine comprises underground galleries and a 14.4 ha open air deposit of mine residues containing high concentrations of arsenic (As), cadmium (Cd), copper (Cu), and zinc (Zn) (Bleeker et al. 2003). Gold mining in Jales dates back to Roman times and ended in 1992. Over many years, wind and water erosion resulted in spreading of contaminated particles into adjacent ecosystems (Bleeker et al. 2003). The contamination caused by the mine residues is translated in the presence of high concentrations of heavy metals in water. The Levira river contaminated site is located in the vicinity of a ceramics industry and agricultural fields. The contamination from the ceramics industry is not expected to be significant because this industry has a treatment system for the residues, mainly heavy metals from colouring and mineral from the ceramics powder. The contamination from agriculture is probably not very high because in this area there are just a few small agriculture fields. But the two sources of pollution jointly could create an important diffused contamination. The contaminated site in Carnide river is located adjacent to rice fields so we expected high organic and pesticide contamination especially in spring. *In situ* bioassays were performed in autumn and spring in all sites except for Teixeira and Sampaio rivers. In these rivers the human pressure (leisure activities) in spring prevented us from deploying the bioassays.

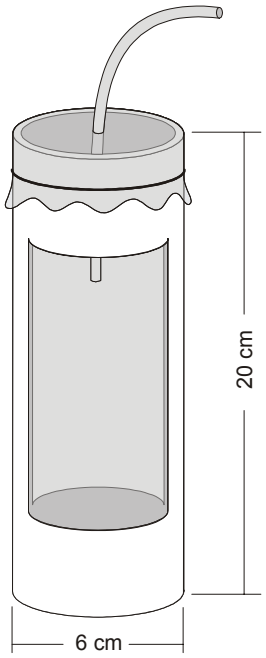

Fig. 2. Schematic diagram of the chamber used for *in situ* bioassays (see text for more details).

Test organisms

Larvae of *Chironomus riparius* used in the bioassays were obtained from laboratory cultures established at the Biology Department, University of Aveiro. The culture unit was an enclosed transparent acrylic box (120 cm x 60 cm x 40 cm), containing all the apparatus necessary to complete the whole life cycle of the chironomids and large enough to allow swarming and copulation of emerged adults (OECD 2000). Cultures were maintained at 20 ± 2ºC, with a 14 h light: 10 h dark photoperiod. At the start of a new culture approximately 120 newborn larvae were introduced into plastic beakers (27 cm x 13 cm x 11,5 cm) containing a 2 cm layer of sea sand (< 0.5 mm particle ranged size; supplied by Merck Co.) and ASTM reconstituted hard water (ASTM 200). A suspension of ground TetraMin® (Tetrawerke, Germany) was added as the food source and each beaker was gently aerated. Seven days later, larvae were transferred to new culture beakers with fresh media, sand and food (60 larvae per beaker) until emergence occurred. Adults were fed on a sucrose solution in paper, placed inside the culture unit. Freshly laid egg masses were transferred onto small plastic Petri dishes with culture medium until hatching occurred, after approximately 2-3 days. The new born larvae were then used to start a new culture.

Test chambers and experimental procedures

Test chambers (Fig. 2) consisted of PVC tube (20 cm high and 4.5 cm inner diameter) with four openings, two laterals (approximately 10 cm x 7 cm), one at the top and one at the bottom,

covered with 200-µm nylon mesh. They were initially developed and described by Castro et al. (2003) and modified for used in *in situ* tests that did not include emergence bioassays (Soares et al. 2005). The mesh that covered the opening on top of the chambers was bound to the chambers with a plastic string. Through this mesh a plastic tube was inserted and glued to the mesh, to introduce the larvae in chambers (Fig. 2). The size of the plastic tube depended on water depth.

Twenty-four hours before the larvae deployment, local sediment was collected and live visible organisms were sorted out. Five chambers with a layer of 5 cm depth of local sediment were placed in the river bed protected by a plastic cage.

On the initial day (day 0), five third instar larvae of *Chironomus riparius* were introduced through a plastic tube in each chamber and TetraMin® fish food (Tetrawerke, Germany) (30 mg in suspension) was added to each chamber to ensure that *C. riparius* larvae had an optimal growth and development. 1 mg larvae^{-1} day^{-1} was recommended by Naylor and Rodrigues (1995) as the optimal dose for *C. riparius* larvae and it is also the average between 1.4 and 0.6 mg larvae^{-1} day^{-1} Tetramin, determined by Péry et al. (2002) as the *ad libitum* and minimum food levels, respectively, for an optimal growth and development of *C. riparius* fourth instar larvae, without affecting survival. According to several authors (Ankley et al. 1994; Traunspurger and Drews 1996; Ristola 2000; Hooper et al. 2003; Péry et al. 2003a, 2003b) feeding is necessary in tests with chironomids because they may starve to death, especially when tests are initiated with young larvae and because of increased risk for false positives (reduced survival, growth, and development due to other reasons than toxicity) (Péry et al. 2003a). Body length and head capsule width of 30 additional larvae were measured to determine respectively initial body length and initial development stage.

At the end of experiment (day 6), the surviving larvae were counted and used to determine post-exposure feeding rate. The post-exposure feeding rate was adapted from the method developed by Soares et al. (2005). 15 of the larvae removed chambers were allowed to feed, individually, in a glass vial with 100 dead *Artemia* sp. *nauplii* (< 24 h old) for 2 hours in the dark. The number of remaining *Artemia* sp. was counted. Feeding rate was then calculated as the number of *Artemia* sp. eaten by larvae per hour. In a prior laboratory experiment, we allowed 30 *C. riparius* larvae to feed, individually, in flasks with ASTM water and 100 dead new born Artemia sp. for 1, 2 and 4 hours. We found no significant differences in feeding rate between the three time intervals (Anova, log feeding rate: $F_{2, 87} = 0.31$, $P > 0.05$) but the variability (standard error of the mean) found in feeding rate for 1 hour was higher ($SE_{1\,h}$: 1.07, $SE_{2\,h}$: 0.85, $SE_{4\,h}$: 0.86). Therefore we used the time interval with less variability for the post-exposure feeding rate experiments.

Finally, all larvae alive were killed and preserved in Von Törne conservant (1000 ml isopropylic alcohol: 60 ml acetic acid: 3ml formaldehyde at 30% (Gama 1964)) for later

measuring. Larval length and development were determined by measuring body length and head capsule width of larvae, respectively, using a stereomicroscope fitted with a calibrated eye-piece micrometer. Growth (body length increase) of larvae was calculated by subtracting the average initial length from each individual final length. Larvae from the chambers that were damage were not counted and measured. *C. riparius* larvae instar was determined according to Watts and Pascoe (2000): larval head capsule width between 0.29 to 45 mm corresponds to third instar, and between 0.49 to 0.63 mm corresponds to the fourth instar.

Physical and chemical parameters

Environmental and physical and chemical parameters were determined for each site including: altitude, latitude and longitude, maximum and minimum temperature, pH, dissolved oxygen, conductivity, nitrate, nitrite ammonia, phosphate, and hardness and organic matter content and particle size of sediment. On the initial day (day 0) and at the end (day 6) of the bioassays, pH, conductivity and dissolved oxygen (DO) were measured with hand-held meters. The minimum and maximum temperature throughout each bioassay was determined using a maximum-minimum thermometer. Water samples were collected on day 0 and at the end of the experiment (day 6) to determine nitrate, nitrite, ammonia and phosphate concentrations and to determine the hardness of the water. Two sediment samples were collected for sediment particle size analysis and organic content evaluation. The particle-size analysis was performed by dry sieving and organic matter was determined by weight loss on ignition of approximately 1 g of dried sediment, at 450ºC, for 5 h (Kristensen and Anderson 1987; Carvalho et al. 2001). The sediment was classified according to the Wentworth scale (Doeglas, 1968) and Larsonneur (1977).

Water samples were collected to plastic containers in all rivers to determine heavy metal concentrations, in the beginning (Day 0) of the bioassays. Water samples for heavy metal analysis were collected in all sites in autumn. In the Tinhela river and Peliteira stream water samples were also collected in spring, to verify if heavy metal concentrations in these two streams varied after winter rainfalls. Sediment samples were also collected in the Peliteira stream and Tinhela river for heavy metal analysis. Water samples were acidified (pH < 2.0) and sediment samples were frozen until analysis. Sediment was dried at 60ºC during 48 hours and digested in a mixture 1:3 of nitric acid (HNO_3 (70%)) and hydrochloric acid (HCL (37%)) on a hot (100-150ºC) plate (Clesceri et al. 1995). Heavy metal analyses were performed with inductively coupled plasma atomic emission spectrometry (ICP-AES) (Clesceri et al. 1995) at the Central Laboratory of Analysis in University of Aveiro.

Biotic indices

Macroinvertebrate collection to determine the biotic indices (BMWP' score system and ASPT') was performed by kick-sampling method as described by Fontoura (1985), in autumn and spring. Macroinvertebrates were identified according to Tachet et al. (1980).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey HSD multiple comparison tests were conducted to test for significant differences in responses of *C. riparius* between rivers and between seasons (Zar 1996). Responses were tested for normality using the Kolmogorov-Smirnov test. The percentage of larvae in fourth instar and survival data was arcsine transformed to stabilize the variance (Zar 1996).

Stepwise multiple regressions and linear regression analyses were used to investigate significant relationships between responses of *C. riparius* and physical and chemical parameters of water and OM content and particle size of sediment (Zar 1996). Stepwise multiple regressions and linear regression analyses were also conducted to identify significant relationships between biotic indices and physical and chemical parameters of rivers.

Pearson's correlation coefficient was used to determine relationships between responses of *C. riparius*, between biotic indices and, also, between responses and biotic indices (Zar 1996). Before the analysis, the absolute or relative data (except pH and DO) were log or arcsine transformed in order to stabilize the variance (Zar 1996). Since heavy metal concentrations in water were not determined for all rivers in spring, relationships between heavy metal concentrations and biotic indices and responses of *C. riparius* larvae were not determined in this season. Statistical analyses were performed using Minitab™ Release 14.

Results

Physical and chemical parameters

Physical and chemical parameters determined during bioassays are shown in the Appendix 1. Mean values of DO were equal or greater than 6.8 mg L^{-1} in autumn and 7.3 mg L^{-1} in spring, both values were found in the Tinhela river. The lowest levels of measured pH were observed in the Peliteira stream (5.5 in autumn, 6.2 in spring) while the highest level was found in the Levira river (7.7 in autumn and spring). Mean values of conductivity were higher in southern sites and ranged from 33 to 526 μs cm^{-1} in autumn and between 27 - 451 μs cm^{-1} in spring, except for the Tinhela river, a northern river with high conductivity (210 μs cm^{-1} in autumn, 121 μs cm^{-1} in spring). Similar to conductivity, hardness was higher in southern sites and ranged from 4.5 to 91.9 mg

Table 2. Heavy metals concentration in rivers in autumn. Heavy metals concentrations in spring in parentheses. n.d. = not detected. n.a. = not analysed.

	As	Cd	Pb	Ni	Zn	Fe	Mn	Cr	Cu
Water (mg L^{-1})									
Bestança	n.a	n.d.	n.a	n.d.	n.d.	0.04	0.002	n.d.	n.d.
Sampaio	n.a	n.d.	n.a	n.d.	n.d.	0.02	0.002	n.d.	n.d.
Tinhela	n.a	n.d.	n.a	n.d.	0.12 [a]	0.17 (0.22)	0.02 (0.02)	n.d.	n.d.
Peliteira	n.a	0.03 (0.01)	0.58 [a]	0.02 (0.01)	2.33 (1.16)	0.09 (0.18)	2.24 (1.11)	n.d.	n.d.
Teixeira	n.a	n.d.	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arões	n.a	n.d.	n.a	n.d.	n.d.	0.02	0.002	n.d.	n.d.
Fráguas	n.a	n.d.	n.a	n.d.	0.04	0.09	0.01	n.d.	n.d.
Levira	n.a	n.d.	n.a	n.d.	0.02	0.43	0.05	n.d.	n.d.
Cabrunca	n.a	n.d.	n.a	n.d.	n.d.	0.06	0.01	n.d.	n.d.
Carnide	n.a	n.d.	n.a	n.d.	n.d.	2.31	0.10	n.d.	n.d.
Sediment (mg Kg^{-1})									
Tinhela	0.35	n.d.	0.17	0.21	0.49	256	2.07	0.20	0.10
Peliteira	29.1	0.07	4.12	0.38	3.64	492	4.13	0.36	0.20
Detection limit (mg L^{-1})	0.025	0.005	0.025	0.01	0.01	0.01	0.001	0.01	0.01

[a] Values obtained from Rocha (2004)

$CaCO_3$ L^{-1} in autumn and from 5.7 to 206.4 mg $CaCO_3$ L^{-1} in spring. The highest level of phosphate in autumn was observed in the Arões river (1.39 mg L^{-1}) while in spring the highest level was found in the Bestança and Carnide rivers (0.26 mg L^{-1}). Higher differences were found in nitrate levels between rivers. Higher levels of nitrates were found in the rivers Levira (2.9 mg L^{-1} in autumn, 7.07 mg L^{-1} in spring) and Carnide (3.09 mg L^{-1} in autumn, 3.62 mg L^{-1} in spring). The lowest values of nitrates were found in the Tinhela river in autumn (0.14 mg L^{-1}) and the Arões river in spring (0.18 mg L^{-1}). Ammonia was also higher in contaminated rivers, with the highest levels found in the Levira river (0.83 mg L^{-1}) in autumn and in the Carnide river (0.43 mg L^{-1}) in spring. Nitrite levels were lower than 0.03 mg L^{-1} in all rivers except in the Carnide river in spring where the concentration of nitrites in water was 0.1 mg L^{-1}.

Heavy metal concentrations

The Peliteira stream was the most metal contaminated river (Table 2). In the Peliteira stream water was detected the presence of arsenic (As), lead (Pb), cadmium (Cd), and nickel (Ni) besides iron (Fe) and manganese (Mn), found also in all other rivers. The highest water concentration of Mn was found in the Peliteira stream (2.24 mg L^{-1}) but the highest concentration of Fe (2.31 mg L^{-1}) was found in the Carnide River. Although the highest water concentration of Zn (0.58 mg L^{-1}) was found in the Peliteira stream, the presence of Zn was also observed in the Tinhela, Levira and Fráguas rivers. In spring there was a decrease in heavy metal concentrations in water of the Peliteira and Tinhela rivers. Heavy metal analysis showed high concentrations of Fe (492 mg Kg^{-1})

Table 3. Organic matter (OM) content and particle size of sediment.

Rivers	OM (mg mg_{sed}^{-1})	gravel (%)	coarse sand (%)	medium sand (%)	fine sand (%)	silt & clay (%)
Bestança	0.09	15.88	52.87	23.29	7.85	0.11
Sampaio	0.24	8.44	77.47	4.02	10.01	0.07
Tinhela	0.03	0.55	5.39	48.79	45.03	0.23
Peliteira	0.03	35.71	57.90	3.01	3.18	0.20
Teixeira	0.03	53.35	33.33	7.68	5.41	0.22
Arões	0.03	0.85	47.09	35.24	16.61	0.20
Fráguas	0.05	37.06	37.44	14.34	11.10	0.06
Levira	0.09	3.62	55.73	35.88	4.76	0.00
Cabrunca	0.21	6.72	23.44	29.31	39.99	0.54
Carnide	0.23	1.15	46.75	45.63	6.46	0.01

Sediment classified according to the Wentworth scale (Doeglas, 1968) and Larsonneur (1977): gravel > 2 000 μm; 500 μm ≤ coarse sand < 2000 μm; 250 μm ≤ medium sand < 500 μm; 63 μm ≤ fine sand < 250 μm, silt & clay < 63 μm.

and As (29.1 mg Kg^{-1}), Pb (4.12 mg Kg^{-1}), Zn (3.64 mg Kg^{-1}), and Mn (4.13 mg Kg^{-1}) and low concentrations of Ni, chromium (Cr) and copper (Cu) and Zn in the sediment of the Peliteira stream (Table 2). The analysis also showed high concentration of Fe (256 mg Kg^{-1}) and low concentrations of As, Pb, Zn, Ni, Cr, Cu, and Mn in the Tinhela river sediment (Table 2). Zn concentrations in the Tinhela river and Pb concentration in the Peliteira stream (Table 2) were obtained by Rocha (2004) in late winter 2002.

Particle size and OM content of sediment

Organic matter (OM) content and particle size of sediment differed between rivers (Table 3). OM content in sediment was higher in the Sampaio (0.24 mg mg_{sed}^{-1}), Carnide (0.23 mg mg_{sed}^{-1}) and Cabrunca (0.21 mg mg_{sed}^{-1}) rivers. In the other rivers sediment OM was lower than 0.1 mg mg_{sed}^{-1}. The percentage of gravel, coarse sand, medium sand and fine sand, in sediment differed between rivers. The percentage of silt and clay in sediment of all rivers was lower than 0.55%.

Biotic indices

The Appendix 1 lists BMWP' scores and ASPT' values for all sites. In spring, the BMWP' score and ASPT were higher in all reference rivers than in contaminated rivers (Table 1 and Appendix 1). In this season, BMWP' scores (range: 143 - 203) and ASPT' values (range: 6.0 - 7.1) of reference rivers indicated good water quality, while BMWP' scores (range: 27 - 67) and ASPT values (range: 3.4 - 5.1) of contaminated rivers showed very poor or bad water quality. BMWP' score (79) and ASPT' (56) of the Tinhela river, a low contaminated river, indicated fairly good

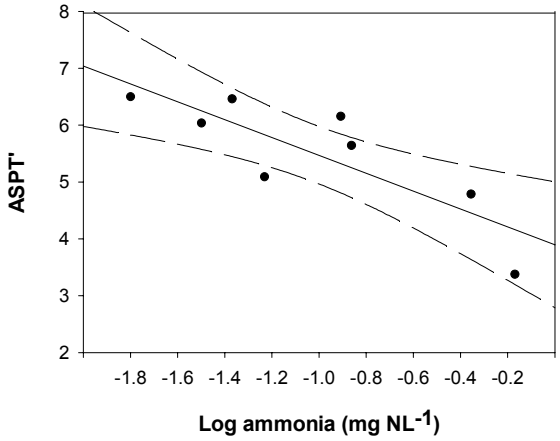

Fig. 3. Relationship of ASPT'values to water concentrations of ammonia in spring. Linear regression: ASPT' = 3.58 – 1.57 Log ammonia, ($P < 0.05$). Dashed lines represent 95 % confidence limits envelopes.

water quality in this season (Table 1 and Appendix 1). In autumn, although the BMWP' scores and ASPT' values were higher in the most of reference rivers than in contaminated rivers, Bestança and Arões, reference rivers, had lower biotic indices than the Tinhela river (Table 1 and Appendix 1). Differences in the BMWP' scores and ASPT values between reference and contaminated rivers were higher in spring than in autumn (Table 1 and Appendix 1). All of the rivers had higher biotic indices in spring than in autumn, except Carnide River (Appendix 1). In autumn, Carnide river had low BMWP' score (47) and ASPT' (3.9), indicating but in spring it had even lower BMWP' score (27) and ASPT' (3.4).

The biotic indices were strongly positively correlated with each other in both seasons (autumn: r = 0.75 $P < 0.05$; spring: r = 0.85 $P < 0.01$), but only ASPT' was significantly ($P > 0.05$) negatively related to water ammonia concentrations in spring ($r^2 = 0.70$) (Fig. 3). No significant ($P > 0.05$) relationships were found between biotic indices and other physical and chemical parameters.

Biological responses

Larval survival in reference rivers varied between 82 and 97% in spring and between 65 and 85% in autumn. In contaminated rivers survival of larvae varied between 71 and 92% in spring and between 61 and 88% in autumn. Survival showed higher differences between seasons than between rivers (Fig. 4A). However, no significant differences were found ($P > 0.05$) in larval survival in each river between seasons and between rivers in both seasons. In autumn, a significant ($P < 0.05$) positive relationship was observed between survival and nitrite concentrations ($r^2 = 0.51$) (Fig. 5A).

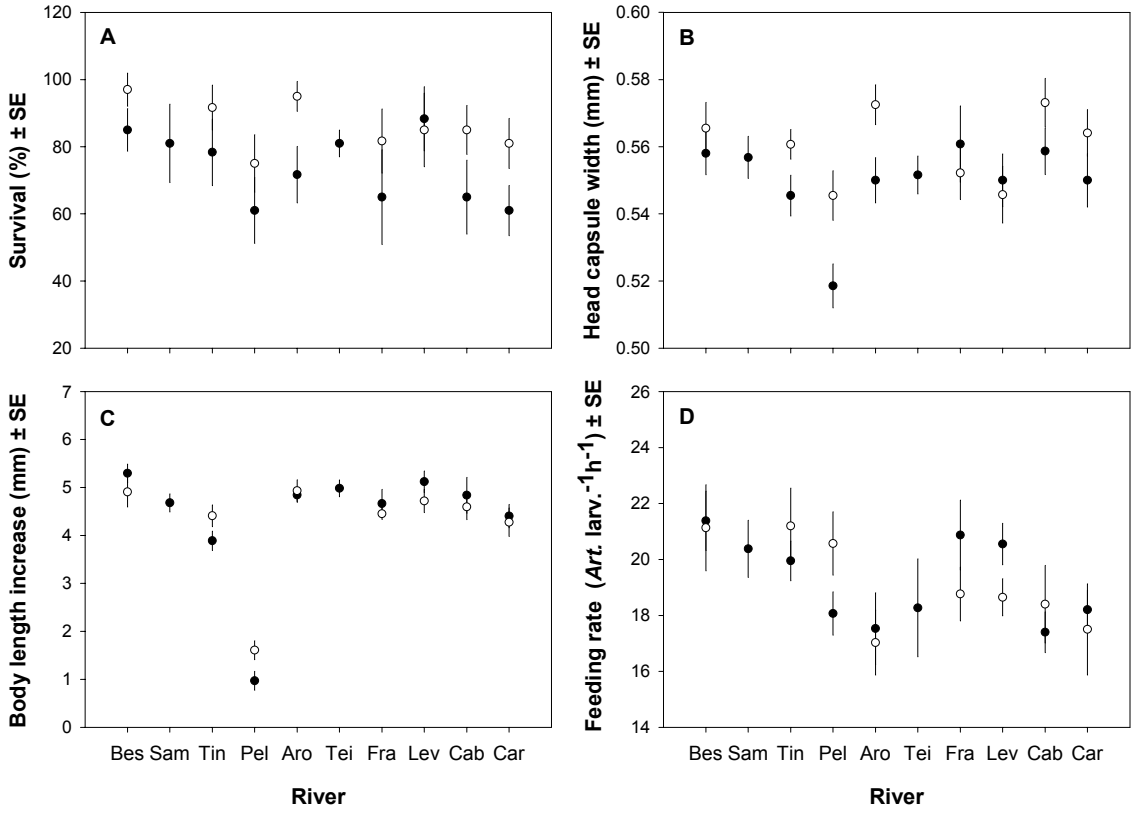

Fig. 4. Mean values and standard errors of biological and functional responses of *Chironomus riparius* larvae in rivers in autumn (●) and in spring (○). A- Survival; B- Development (head capsule width); C- Growth (body length increase); D- Post-exposure feeding rate. The rivers: Bes- Bestança; Sam- Sampaio; Tin- Tinhela; Pel- Peliteira; Aro- Arões; Tei- Teixeira; Fra- Fráguas; Lev- Levira; Cab- Cabrunca; Car- Carnide.

In spring, no significant relationship occurred between this response and physical and chemical parameters.

In all streams, in both seasons, all larvae were in the fourth instar at the end of bioassay. However, differences in larval head capsule width between rivers were found in autumn (Anova: $F_{9,171} = 2.21$, $P < 0.05$) (Fig. 4B). In this season, head capsule width of larvae in the Peliteira stream was significantly lower than in Bestança, Sampaio, Fráguas and Cabrunca rivers (Tukey tests: $P < 0.05$). In spring, no significant ($P > 0.05$) differences were found in larval head capsule width between the rivers. Although an increasing trend in head capsule width was observed from autumn to spring (Fig. 4B), significant differences in head capsule width between seasons were only observed in the Peliteira (ANOVA: $F_{1, 36} = 11.85$, $P < 0.01$) and Arões (ANOVA: $F_{1, 42} = 6.31$, $P < 0.05$) rivers. In autumn, a significant positive relationship was found between head capsule width of larvae and pH of water ($r^2 = 0.60$, $P < 0.01$) (Fig. 5B), but in spring, no significant relationship occurred between this response and environmental parameters.

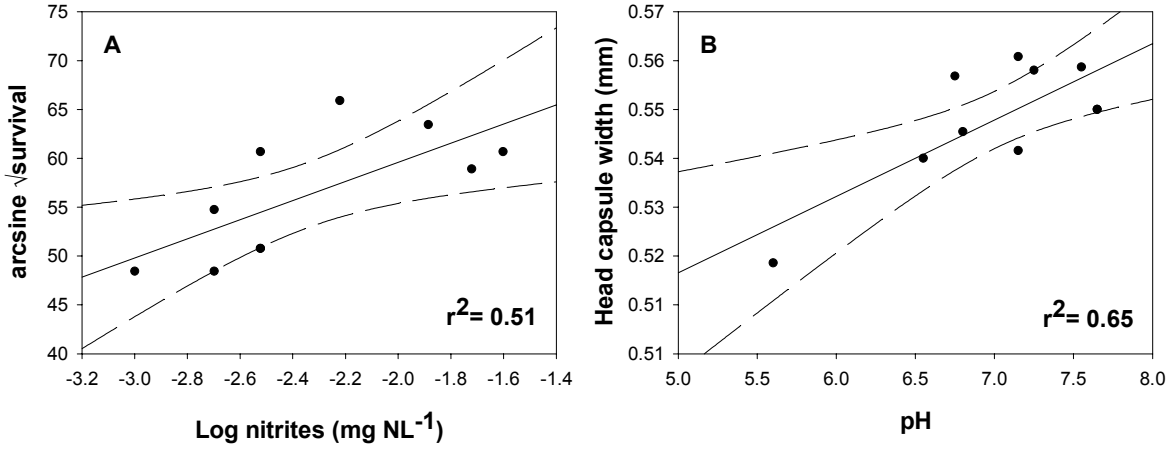

Fig. 5. Relationships of biological responses to physical and chemical parameters of the water, in autumn. Relationship between (A) survival and nitrite concentrations and (B) head capsule width and pH. Linear regression: arcsine survival = 79.2 + 9.79, P < 0.05; head capsule = 0.438 + 0.0156 pH; $P < 0.01$. Dashed lines represent 95 % confidence intervals envelops.

Growth of *C. riparius* larvae differed between rivers in autumn (Anova: $F_{9, 171} = 26.05$, $P < 0.001$) and in spring (Anova: $F_{7, 183} = 20.19$, $P < 0.001$) (Fig. 4C). Larval growth in the Peliteira stream was lower than in all the other rivers, in both seasons (Tukey test: $P < 0.0001$) (Fig. 4C). Differences in this endpoint were also found between the Tinhela river and several rivers (Bestança, Arões, Teixeira and Levira) (Tukey test: $P < 0.05$) in autumn. There were also significant differences in larval growth in the Peliteira stream between both seasons (Anova: $F_{1, 36} = 4.27$, $P < 0.05$) (Fig. 4C). In autumn, growth was significantly positively related to water pH and negatively related to water Mn concentrations (growth = - 5.29 + 1.11 pH - 0.733 Log Mn, $r^2 = 0.96$, $P < 0.01$).

No significant correlation ($P > 0.05$) of the biological responses with biotic indices (BMWP' score or ASPT') was found. The biological responses were also not significantly correlated between them.

Post-exposure feeding rate

Some variation was observed in the post-exposure feeding rate between rivers and in the most rivers between seasons (Fig. 4D) but no significant ($P > 0.05$) differences were found in post-exposure feeding rate of larvae between rivers in both seasons and in each river between seasons (Fig. 4D). In autumn, post-exposure feeding rate was significantly ($P > 0.05$) positively related ($r^2 = 0.56$) to water nitrite concentrations in autumn (Fig. 6). In spring, no significant relationship occurred between this response and physical and chemical parameters.

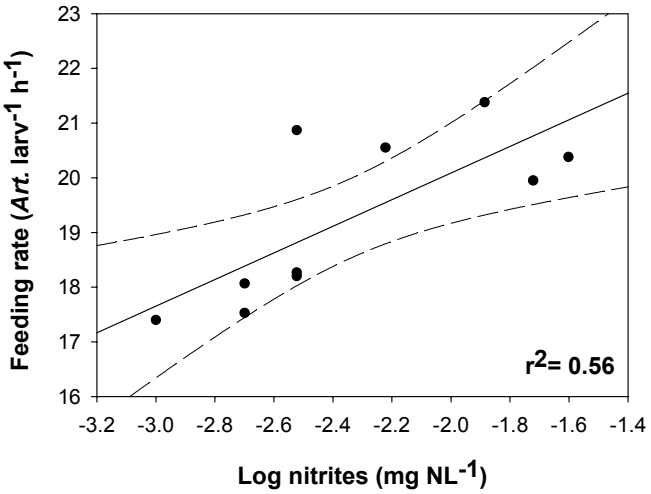

Fig. 6. Relationship of feeding rate to nitrite concentrations, in autumn. Linear regression: feeding rate = 25.0 + 2.43 Log nitrites, $P < 0.05$. Dashed lines represent 95 % confidence intervals envelopes.

No significant correlation was found between post-exposure feeding rate and biotic indices (BMWP' score or ASPT') or between feeding rate and biological responses.

Discussion

Chemical and physical parameters

High concentrations of nitrates and ammonia were found in the Levira and Carnide rivers in autumn and especially in spring, which could reflect organic contamination, although the concentration of phosphates in the water of these rivers was low.

Higher metal contamination was found in the Peliteira stream, as expected, since this river receives the effluent from an abandoned goldmine. Low metal contamination was found in the Tinhela river, which was also expected due to the proximity of the mine. The highest concentrations of Mn in water were found in the Peliteira stream (metal contaminated) but also in the Levira and Cabrunca rivers (organic contaminated), located near rice fields, while the highest concentration of Fe was found in the Carnide river. High concentrations of Mn and Fe in the water of the Carnide river, organic contaminated river, can be explained by the reduction of Mn (IV) and Fe (III) coupled to organic matter oxidation resulting in high concentrations of dissolved Fe^{2+} and Mn^{2+}. Several authors (e.g. Chapelle and Lovley 1992; Lyngkilde and Christensen 1992) verified that organic matter oxidation coupled to Mn (IV) and Fe (III) reduction resulted in the accumulation of high concentrations of dissolved Fe^{2+} and Mn^{2+} in ground water. In spring there was a decrease in heavy metal concentrations in water of the Peliteira and Tinhela rivers, probably due to heavy metal dilution during the high water discharge period (winter-spring).

The lowest levels of pH were found in the Peliteira stream in both seasons. Low levels of pH are usually found in metal contaminated waters (e.g. Godzick and Krywult 1998; DeNicola and Stapleton 2002; Mousavi et al. 2003) and results from the oxidation and/or hydrolysis of Fe and other metals in acid mine drainages (DeNicola and Stapleton 2002). The lowest levels of pH were found in the Peliteira stream in both seasons. Low levels of pH are usually found in metal contaminated waters (e.g. Godzick and Krywult 1998; DeNicola and Stapleton 2002; Mousavi et al. 2003) and results from the oxidation and/or hydrolysis of Fe and other metals in acid mine drainages (DeNicola and Stapleton 2002).

Biotic indices

BMWP' score and ASPT were strongly correlated, but ASPT was more appropriate because it is less sensitive to sampling effort and can be predicted with greater reliability (Armitage et al. 1983; Wright et al. 1993; Chutter 1995).

In autumn, although higher BMWP' scores and ASPT' values were also found in most of the reference rivers, Bestança and Arões rivers (reference rivers) had lower biotic indices than Tinhela river, a low metal contaminated river (Table 1 and Appendix 1). The low biotic indices found in these reference rivers in autumn can be explained by the low ability of these biotic indices to predict water quality of the Mediterranean rivers in low water discharge period (summer-autumn) as was suggested by Coimbra and Graça (1998). In this season the environmental conditions became harsher (e.g. water depth decrease, flow rate approximately zero, aquatic plants decay) and, in same way, more similar between reference and contaminated rivers (Coimbra and Graça 1998). So lower biotic indices in the reference rivers and low differences between reference and contaminated rivers may be due to natural stressing conditions.

In spring the biotic indices showed high ability to assess water quality and detect contamination since higher BMWP' scores and ASPT' values were found in the reference than in the contaminated rivers. In high discharge period (winter-spring) there is an improvement in the environmental conditions and in water quality, resulting in an increase in biotic indices of rivers, mostly of reference rivers, and in a higher ability of these indices to predict water quality (Coimbra and Graça 1998). Low BMWP' and ASPT' in Carnide river in spring can be explained by the increase of nutrients (nitrates, nitrites and ammonia), in the river, that may be due to an increased input of fertilizers from adjacent rice fields.

Lower ASPT' was more observed in organic or diffuse contaminated sites than in metal contaminated rivers (Table 1 and Appendix 1) in both seasons. This can indicate that the macroinvertebrate community is more sensitive to organic contamination than to metal contamination. Furthermore ASPT' was significantly negatively related to water ammonia

concentrations. Chila (1998) also found a strong negative relationship between several biotic indices (Hungarian versions of BMWP and ASPT included) and the concentration of ammonium. But it can also indicate some adaptation of benthic macroinvertebrates to heavy metals in metal contaminated rivers, as was already observed by McWilliam and Baird (2002b) in a stream that received drainage water from a lead mine.

Biological responses

Survival was high in all rivers in spring (> 75%) and at most rivers in autumn (> 70%), indicating that the bioassay was suitable for use *in situ* deployments. In all the rivers survival was higher than 60%. This showed the suitability of the bioassay to evaluate sublethal effects of the contamination in rivers and showed also, the ability of the bioassay to be conducted under field conditions because sufficient live animals were obtained to complete the bioassay and to determine post-exposure feeding rate. So, 25 larvae was an adequate number to deploy per river to compensate the loss of larvae during the exposure period.

Although the lowest values of survival were found in the most contaminated rivers and an increasing trend was observed between autumn and spring but, probably due to high variability within rivers, no significant differences were found in larval survival between rivers and in each river between seasons. So it seems that survival was not affected by organic or metal contamination in rivers. Furthermore, the regression analyses indicated that survival was positively related to water nitrite concentrations, in autumn, and 51% of the variation in survival between sites, in this season, could be explained by the variation in water nitrite concentrations between sites (Fig. 5A). This indicates that survival is high tolerant to nitrites/organic contamination. Thus the increasing trend observed in survival between autumn and spring (Fig. 4A) was probably due to an improvement in the environmental conditions in rivers and not to river contamination.

At the end of the bioassay all larvae were in the fourth instar (developmental stage), so the change of instar was not affected by the contamination or other factor during exposure. However, in autumn, a significant decrease in the head capsule width was found in the Peliteira stream, the highest metal contaminated river. This does not necessarily mean that development was affected by the metal contamination detected in this river, since it could have been affected by water pH since the lowest pH was registered in the Peliteira stream (Appendix 1). The increasing trend in head capsule width observed from autumn to spring (Fig. 4B) was probably due to an improvement in the environmental conditions in rivers. In the Peliteira stream the increase in head capsule width from autumn to spring may be also related to the increase of the water pH (Appendix 1). Although the regression analyses indicated that head capsule width was significantly related to water pH, this relation was only significant due to the inclusion of values from this river (Fig. 5B).

Growth was affected by metal contamination, since impairment in growth was observed in the both metal contaminated rivers (Peliteira and Tinhela) and the lowest growth was found in the Peliteira stream, the most contaminated river. However the impairment in growth might not be due exclusively to metal contamination, but could be also related to water pH since the lowest pH was also registered in the Peliteira stream (Appendix 1). Furthermore growth was positively related with water pH and was negatively related to water Mn concentrations. Regression analyses indicated that 96% of the variation in growth between sites could be explained by the variation in water pH and Mn concentrations between sites, in autumn.

On contrary to what was found for survival and development, an increasing trend in growth from autumn to spring was not observed. An increase in growth from autumn to spring was only observed in Tinhela and Peliteira rivers (Fig. 4C). Furthermore an impairment of growth in Tinhela river was found in autumn but it was not observed in spring. The increase of growth in the Peliteira stream may be due to an improvement in water quality, i.e. decrease on metal contamination, but may be due also to an increase in the water pH. The increase of growth in the Tinhela river can be explained by the improvement of water quality but was not related with the water pH, since similar pH in the Tinhela river water was observed in both seasons. This suggests that growth is more sensitive to metal contamination than to other environmental factors and is highly sensitive to changes (improvement or deterioration) in the water quality of rivers, related to metal contamination. Although regression analyses indicated that head capsule width was significantly related to water pH and Mn concentrations, this relation was only significant due to the inclusion of values from the Peliteira stream (Fig. 5B).

Survival was not sensitive to metal contamination because no significant differences in this response were observed between contaminated and not contaminated rivers. Larval development does not seem to be a sensitive endpoint to metal contamination since all larvae changed instar in all rivers although the sensitivity of the larval head capsule width to this type of contamination was not clear. So, larval survival and development do not seem suitable endpoints to identify metal contamination in the aquatic ecosystems. However, these endpoints can be suitable to indicate higher levels of metal contamination. Leppänen et al. (1998) and Castro et al. (2003) found, respectively, a decrease in survival and a delay in development exposed to high metal contamination in field sediment.

Growth, on the other hand, was sensitive to metal contamination, even to low contamination, discriminated between metal contaminated and not contaminated rivers and was able to detect changes in the metal contamination between seasons in both metal contaminated rivers. This indicates that this bioassay, using growth as the endpoint, can be used to biomonitor metal contamination in lotic ecosystems.

None of the endpoints used were negatively affected by organic contamination, as expected since this species is known to be very tolerant to organic contamination (Gower and Buckland 1978; Pinder and Farr 1987; Arnwine et al. 2003). This indicates that *C. riparius* is not a suitable species to assess organic contamination.

Post-exposure feeding rate

Post-exposure feeding rate was not affected by the river contamination (organic or heavy metal) or by the seasonality. Some variation was observed between rivers and in the most rivers between seasons (Fig. 4D) but, probably due to the high variability within rivers, no significant differences in this endpoint between rivers were. Leppanen et al. (1998) did not found also a decrease in the feeding activity of *C. riparius* larvae exposed to high metal contamination in field sediment. The regression analyses indicated that survival was positively related to water nitrite concentrations, in autumn, and 56% of the variation in post-exposure feeding rate between sites, in this season, could be explained by the variation in water nitrite concentrations between sites (Fig. 6). This can indicate that post-exposure feeding rate is highly tolerant to nitrites/organic contamination. These results show that post-exposure feeding rate is not a suitable endpoint to identify metal and organic pollution or to assess impairment caused by these types of contamination in aquatic ecosystems.

Biotic indices and *in situ* bioassays

In spring (high discharge water period) the biotic indices, BMWP' and ASPT', showed high ability to predict water quality and detect organic and metal contamination. In autumn (low discharge water period), biotic indices showed low ability to predict water quality and failed to detect contamination. The methods based on measures of benthic macroinvertebrates community structure can fail to detect contamination because the macroinvertebrates community structure is affected by other factors besides pollution (e.g. habitat characteristic, percentage of riparian trees and macrophyte cover and hydraulic stress) (Bazzanti et al. 1987; Friday 1987; Wilkinson and Slater 1995; Coimbra and Graça 1998; Bazzanti et al. 2003).

Growth of larvae was more sensitive to metal contamination and less sensitive to other factors than benthic macroinvertebrates community structure. So, the *in situ* bioassay with *C. riparius* larvae using growth as the endpoint was better able to identify metal contamination, since it detected even low levels of metal contamination, and was better able to detected changes in metal contamination within rivers between seasons. These results suggest that the *in situ* bioassay using growth of *C. riparius* larvae as the endpoint can be a more sensitive and suitable tool than the biotic indices to indicate and monitor metal contamination in rivers, at least in the low water

discharge period. The bioassay with *C. riparius* larvae failed to detect organic contamination. A bioassay using a more sensitive species, to this type of contamination, would be a better alternative to indicate and monitor organic contamination in rivers.

Nevertheless, the biotic indices can be a useful tool to use as bioindicator of water quality during high discharge water periods (winter-spring), at least in the Mediterranean rivers. During this period we suggest that *in situ* bioassays can be used in conjunction with biotic indices. This approach can be more reliable and realistic than an approach only based on measures of macroinvertebrate community structure.

Conclusions

The *C. riparius* larvae were successfully deployed in the rivers since high survival was observed. From all the biological responses that were monitored, growth was the most susceptible to metal contamination, proving to be the most suitable endpoint to detect and assess the impact of this type of contamination on rivers. The other responses (Survival, development and feeding rate) did not respond to metal contamination and thus were not suitable and useful responses to evaluate impact of metal contamination. Nevertheless, survival allowed us to evaluate the suitability of the deployment of the *in situ* bioassay.

These findings indicate that the *in situ* bioassay using growth of *C. riparius* larvae can be a suitable tool to biomonitor metal contamination on Mediterranean rivers and can be used in conjunction with other approaches for the assessment of water quality, such as biotic indices, especially in the high water discharge period when biotic indices show high ability to predict water quality.

Acknowledgements

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Appendix 1. Biotic indices and the mean values of physical and chemical parameters observed of rivers. T = temperature; Cond = conductivity; DO = dissolved oxygen. n.d. = Not determined.

Rivers	BMWP'	ASPT'	Tmin (ºC)	Tmáx (ºC)	pH	Cond ($\mu s\ cm^{-1}$)	DO ($mg\ L^{-1}$)	Nitrates ($mg\ L^{-1}$)	Nitrites ($mg\ L^{-1}$)	Ammonia ($mg\ L^{-1}$)	phosphate ($mg\ L^{-1}$)	hardness ($mg\ CaCO_3\ L^{-1}$)
Autumn												
Bestança	57	5.2	16.5	27.0	7.3	47.0	8.7	0.22	0.013	0.05	0.20	4.7
Sampaio	128	6.4	15.5	19.0	6.8	75.8	9.7	0.81	0.025	0.06	0.20	6.4
Tinhela	114	5.7	14.0	17.0	6.6	33.3	6.8	0.14	0.019	0.09	0.04	5.1
Peliteira	32	4.6	12.0	17.5	5.6	210	8.5	0.69	0.002	0.72	0.25	33
Fráguas	146	7.0	11.0	16.0	7.2	66.1	11	0.57	0.003	0.02	0.39	8.7
Arões	55	5.5	11.5	17.0	6.6	31.7	11	0.40	0.002	0.06	1.39	6.1
Teixeira	84	6.0	12.5	16.0	7.2	39.0	9.5	0.55	0.003	0.07	0.33	4.5
Levira	46	3.4	12.0	23.0	7.7	526	10	2.90	0.006	0.83	0.69	92
Cabrunca	185	6.0	15.0	18.0	7.6	379	9.3	2.00	0.003	0.12	0.36	69
Carnide	47	3.9	16.0	20.0	7.7	169	10	3.09	0.001	0.38	0.72	33
Spring												
Bestança	169	6.0	14.0	32.0	7.4	30.7	9.3	0.50	0.003	0.02	0.26	5.7
Sampaio	206	7.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tinhela	79	5.6	14.0	17.0	6.7	34.2	7.3	0.37	0.001	0.09	0.02	9.7
Peliteira	56	5.1	12.0	17.0	6.2	121	8.4	0.48	0.002	0.04	0.02	39
Fráguas	143	6.5	10.0	15.0	7.0	69.8	9.8	1.18	0.003	0.01	0.18	32
Arões	168	6.5	9.0	16.0	6.6	26.8	10	0.18	0.003	0.03	0.01	9.2
Teixeira	205	6.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Levira	67	4.8	12.0	18.0	7.7	451	9.9	7.07	0.027	0.28	0.25	178
Cabrunca	203	6.2	12.0	17.0	7.5	445	9.6	1.16	0.013	0.08	0.14	206
Carnide	27	3.4	12.0	20.0	7.2	297	12	3.62	0.100	0.43	0.26	98

Chapter 2

Biological and functional responses of *Chironomus riparius* larvae to assess water contamination by the insecticide lambda-cyhalothrin

Mafalda Faria, Paula C.L.D. Silva, Jenny Hunt, Rob Wood, Lorraine Maltby, António J.A. Nogueira, Amadeu M.V.M. Soares

Abstract

A mesocosm experiment was conducted to investigate the ability of a bioassay with *Chironomus riparius* larvae using biological (survival, development, growth,) and functional (post-exposure feeding rate) endpoints, previously developed for use in field studies, to assess the impact of the pyrethroid insecticide lambda-cyhalothrin in lotic ecosystems. Third instar larvae of *C. riparius* were deployed in artificial streams dosed with different concentrations of ^{14}C-lambda-cyhalothrin. The relationship between responses and the water lambda-cyhalothrin concentrations was determined as well as correlations between responses. Impairment of larval growth was observed in the low and high dosed streams (100 ng L^{-1} and 250 ng L^{-1}). In high dosed stream was also observed an inhibition of larval development and a decrease in post-exposure feeding rate. These responses were negatively related to water lambda-cyhalothrin concentrations correlated with each other and. No effect on larval survival was found. These results suggest that the bioassay with development, growth and post-exposure feeding rate of *C. riparius* larvae as endpoints can be a sensitive and suitable tool to assess ecological and functional effects of the insecticide contamination in lotic ecosystems. These findings suggest that bioassays with *C. riparius* larvae, using sublethal endpoints (development, growth and post-exposure feeding rates) can be a suitable tool to assess the ecological risk of pyrethroid insecticides to freshwater ecosystems.

Keywords: Lambda cyhalothrin, *Chironomus riparius*, bioassay, biological responses

Introduction

Drift from pesticide spray and runoff events can result in contamination of non-target environments such as surface waters (Leonard et al. 1999; Schulz et al. 2001b). The assessment of the ecological effects of pesticides contamination in running waters is difficult because these compounds degrade very quickly (Barry and Logan 1998), because they can be absorbed onto the sediment (Peterson and Batley 1993; Hamer et al. 1999) and because peak concentrations of pesticides in rivers are rarely measured during storm runoff events since sites are often inaccessible and these peak concentrations may last only a few hours (Cooper 1996). Therefore, the risk and the impact of pesticides to environment are usually assessed by laboratory toxicity tests.

Toxicity testing usually involves the use of bioassays which are performed under static controlled conditions. However, laboratory toxicity testing does not always generate ecologically relevant information for the area in concern (Giesy and Hoke 1989), mainly because field situations may not be accurately simulated in the laboratory. However, the impact of substances can also be assessed using more environmental realistic conditions through the use of mesocosms (e.g. artificial streams) or *in situ* studies (Boxall et al. 2002). Mesocosms can be very valuable to assess the impact of the contaminants that are difficult to assess in *in situ* studies, such as pesticides.

Chironomids (i.e. *Chironomus tentans* and *Chironomus riparius*) are among the test species recognized as useful tools in studies of toxicity (Ankley et al. 1994) because the life stages (aquatic stages: egg, four larvae instars, pupa and an aerial adult stage) are easy to identify and their life history under laboratory conditions is short (Péry et al. 2002). Chironomidae larvae are commonly used for the toxicity analysis of natural sediments, both in laboratory (Giesy et al. 1990; Hoke et al. 1993; Pellinen and Soimasuo 1993; Pery et al. 2003c) and *in situ* (Chappie and Burton 1997; Tucker and Burton 1999; Castro et al. 2003). Most of these studies use biological responses (e.g. survival, growth and development) of larvae as endpoints. These responses are ecologically relevant because are important components of fitness and determinants of population health (Liber et al. 1996; Sibley et al. 1997a), structure and dynamics (Maltby 1994). None of these responses, however, are suitable to be used as indicators of impact on functional integrity of ecosystem although this is essential to determine ecosystem health (European Commission 2000).

Ecological functions in aquatic ecosystems include key processes such as algal production, detritus processing and zooplankton grazer. So, the feeding inhibition of key species can be used to indicate impact on ecosystem functions which depends, however, on the functional redundancy, i.e. occurrence of species in a system that can contribute to the same function. Furthermore, alterations on food intake may affect growth, development, fecundity and survival. Thus, feeding inhibition can be used as a rapid warning response of ecological and functional impairment of ecosystem. Feeding inhibition as bioassay endpoint has been investigated for several functional groups of

organisms such as grazers, like *Daphnia magna* (McWilliam and Baird 2002a, 2002b; Slijkerman et al. 2004), shredding detritivores, like *Gammarus pulex* (Maltby 1994; Maltby et al. 2000, 2002), and predators, like fish (Castro et al. 2004), but only a few studies (Leppänen et al. 1998; Soares et al. 2005) used, as the endpoint, the feeding activity of a collector-gatherer (feed primarily on fine particulate organic material deposited on sediment) (Vos 2001), a basic functional group of aquatic ecosystems, such as *C. riparius* larvae.

The main goal of this study was to investigate the potential ability of a bioassay with C. riparius larvae, an important collector species, using development and functional responses to indicate ecological and functional stress in lotic ecosystems caused by insecticide contamination.

Larvae were exposed to several concentrations of the insecticide ^{14}C-lambda-cyhalothrin in artificial streams, during 6 days. The bioassay was carried out in artificial streams in order to simulate the ecological conditions of natural lotic systems. Lambda-cyhalothrin ($C_{23}H_{19}ClF_3NO_3$) was selected as chemical stressor because is a pyrethroid insecticide widely used in agriculture (USDA 2001; Basa et al. 2003; Benbrook 2003). Pyrethroid insecticides have a high intrinsic toxicity to aquatic invertebrates and may enter aquatic environments through spray drift or runoff (Hill 1985; Hamer et al. 1999).

Materials and methods

Test organisms

Larvae of *C. riparius* used in the bioassay were obtained from laboratory cultures established at the Department of Animal and Plant Sciences, University of Sheffield, UK. The culture unit was an enclosed transparent acrylic box containing all the apparatus necessary to complete the whole life cycle of the chironomids and large enough to allow swarming and copulation of emerged adults (OECD 2000). Cultures were maintained at 20 ± 1ºC, with a 16 h light: 8 h dark photoperiod.

Artificial streams

The artificial streams (six independent stainless steel channels with 3 m long, 20 cm wide and 30 cm deep) were located indoors at the Department of Animal and Plant Sciences in University of Sheffield. Each stream had a 5 cm deep pea gravel substrate (particle size), restricted to the bottom 205 cm length of channel by 600 μm mesh screens, and was covered a 15 cm depth of artificial pond water (APW) (Naylor et al. 1989). The water was continually re-circulated within each channel with a mean flow rate of approximately 0.02 m s^{-1}. A light bank (1.5 m long x 1 m wide), containing twelve 58 W Osram cool white tubes, operated on a 12 h light: 12 h dark photoperiod. 100 mL of the stock solution (94.4 mg KH_2PO_4 and 1.019 g $NaNO_3$ dissolved in 1L

deionised water) was added to each stream and ^{14}C radio-labelled lambda-cyhalothrin was applied in four streams. The lambda-cyhalothrin was mixed with 1 L of APW and was poured slowly (over a period of approximately 2 minutes) into the streams. Two streams were given a nominal dose of lambda-cyhalothrin of 100 ng L^{-1}, two others streams were given a higher nominal dose of 250 ng L^{-1} and two streams were used as control with no dosing given to them. Bioassay chambers for *C. riparius* and, also, cages for *Sericostoma personatum*, *Gammarus pulex*, *Lymnaea peregra*, *Daphnia magna* and *Calamoceras marsupus* were then placed into each stream together with 15 fine and 10 coarse leaf packs for both *Alnus glutinosa* and *Phragmites* sp.

Experimental procedures

Test chambers were the same as used during *in situ* bioassays (see chapter 1) and were fixed at the streams by strings of metal coated with plastic.

On the first day of the bioassay (day 0), 2 chambers with a sea sand (supplied by Merck Co.) layer of 5 cm depth were placed per stream. Five third instar larvae of *C. riparius* were introduced in each chamber and Tetramin (approximately 30 mg in suspension) was added to each chamber. 1 mg larvae-1 day-1 was the dose recommended by Naylor and Rodrigues (1995) as optimal for *C. riparius*. Body length and head capsule width of 20 additional larvae were measured to determine respectively initial body length and initial development stage.

At the end of experiment (day 6), the surviving larvae were counted and used to determine post-exposure feeding rate. The post-exposure feeding rate was adapted from the method developed by Soares et al. (2005). The larvae removed from each chamber were allowed to feed in a glass vial with 150 dead *Artemia* sp. *nauplii* (< 24 h old) for 2 hours in the dark. The number of remaining *Artemia* sp. was counted. Feeding rate was then calculated as the number of *Artemia* sp. eaten by larvae per hour. Finally, all larvae were killed and preserved in Von Törne conservant (Gama 1964) for later measuring. Larvae length and development were determined by measuring body and head capsule width, respectively, using a stereomicroscope fitted with a calibrated eye-piece micrometer. Growth (body length increase) of larvae was calculated by subtracting the average initial length from each individual final length.

Tetramin food was supplied in the beginning of tests because according to several authors (Ankley et al. 1993, 1994; Traunspurger and Drews 1996; Vos and Brink 2002; Hooper et al. 2003; Péry et al. 2003b, 2003c) feeding is necessary in tests with chironomids because they may starve to death, especially when tests are initiated with young larvae and because of the high risk for false positives (e.g. reduced survival, growth, and reproduction due to other reasons than toxicity). Feeding, however, may affect the extent of the exposure and the bioavailability of the toxicants (Harkey et al. 1997; Lyytikainen et al. 2001). According to Muir et al. (1985) and the equilibrium

partitioning theory described by Di Toro et al. (1991), the bioavailability of pyrethroids insecticides to chironomids increase with the decrease of organic content of sediment. In the present study supplying food could have decreased the bioavailability of lambda-cyhalothrin because of the increased organic matter present in sea sand. This increase, however, might result in more a realistic organic matter content than sea sand.

Physical and chemical analysis

Immediately after placing the chambers and larvae in the streams, 3 water samples were taken from each stream and analysed for lambda-cyhalothrin concentration. Further samples were taken from each stream after 24 h, 48 h and 6 d. Hand-held meters were used to measure water temperature (Jenway 9071, Dunmow, Essex UK), pH (Jenway 3150, Dunmow, Essex UK), conductivity (Hanna HI9033 Ronchi di Villafranca, Italy) and dissolved oxygen (Jenway 9071, Dunmow, Essex UK) daily. Nitrate and phosphate concentrations were determined at day 0.

Lambda-cyhalothrin analysis

Analyses to determine lambda-cyhalothrin concentrations were performed by liquid hexane extraction (200 mL of stream water and 20 mL hexane were shaken for 2 minutes in a separating funnel and the hexane lambda-cyhalothrin extract drawn off into a 25mL plastic scintillation vial). Each sample was left uncapped overnight to allow evaporation of hexane, reducing its volume to < 10 mL. Scintillation fluid (10 mL) was added before measuring activity using a Tri Carb 1600TR liquid scintillation counter (Packard, CT, USA).

Lambda-cyhalothrin concentrations were only determined in water because, according to the equilibrium partitioning theory described by Di Toro et al. (1991) and confirmed by other studies (e.g. Ankley et al. 1994; Hoke et al. 1995; Hamer et al. 1999), for organic compounds, such as pyrethroid insecticides, the amount of chemical available to sediment dwelling organisms like chironomids in equilibrated sediment-water systems is represented by the concentration dissolved in aqueous phase.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett multiple comparison tests were conducted to test for significant differences in growth, feeding rate and percentage of larvae in fourth instar between treatments (low and high treatments) and control (Zar 1996). Because no significant differences were found in responses between streams dosed with the same concentration and between the two control channels ($P > 0.05$), pooled data from streams dosed with the same concentration was compared with pooled data from the control channels. Responses were tested for

Table 1. Mean values of physical and chemical parameters observed in artificial streams during larvae exposure to lambda-cyhalothrin. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

Stream	Temp (ºC)	pH	DO (mg L^{-1})	Cond (ms cm^{-1})	Nitrate (mg L^{-1})	Phosphate (mg L^{-1})
Control I	14.8	7.9	8.7	610	0.22	0.05
Control II	16.2	7.9	8.6	650	0.16	0.07
Low I	14.7	8.0	8.7	549	0.07	0.06
Low I	16.2	7.9	8.4	591	0.09	0.06
High I	14.5	7.8	8.8	649	0.09	0.06
High II	14.9	7.9	8.6	643	0.10	0.07

normality using the Kolmogorov-Smirnov test. The percentage of larvae in fourth instar was arcsine transformed to stabilize the variance (Zar 1996).

Relationships between endpoints (percentage of larvae in fourth instar, body length and feeding rate) and day 6 weighted averages concentrations of lambda-cyhalothrin were determined using linear regression analyses (Zar 1996). In these analyses, the concentration of lambda cyhalothrin in control streams was assumed to be 0 ng L^{-1}.

Pearson's coefficients were used to investigate the association between head capsule width within instars, body length and feeding rate (Zar 1996). Statistical analyses were performed using Minitab™ Release 14.

Results

Chemical and physical parameters

During the experiments physical and chemical parameters were similar in all streams (Table 1). Mean values of dissolved oxygen (DO) ranged between 8.4 and 8.8 mg L^{-1}. Temperature varied between 14.5 and 16.2ºC. Measured pH levels were between 7.9 and 8.0. Mean values of conductivity varied from 549 to 650 µs cm^{-1}. Phosphate levels ranged between 0.05 and 0.07 mg L^{-1} and nitrate levels were below 0.3 mg L^{-1} in all streams.

Determined concentrations of ^{14}C-lambda-cyhalothrin in water per stream are shown in Table 2. Aqueous phase concentrations of the insecticide in the different streams varied from < 0.010 to 26.26 ng L^{-1} in day 0 and from 1.4 to 4.97 ng L^{-1} in day 6 of experiment. There is no reason to believe that initial concentrations in the streams were significantly lower than nominal, but in streams dosed with 100 ng L^{-1} of ^{14}C-lambda-cyhalothrin, concentrations declined in approximately 15 minutes to 5.22 and 7.38 ng L^{-1} and in streams dosed with 250 ng L^{-1} ^{14}C-lambda-cyhalothrin concentrations declined to 23.58 and 26.26 ng L^{-1}.

Table 2. Lambda-cyhalothrin concentrations (ng L^{-1}) in water samples collected in artificial streams in the beginning (day 0) and at the end (day 6) of the bioassay.

	Control I	Control II	Low I	Low II	High I	High II
Day 0 (ng L^{-1})	< 0.1	< 0.1	5.22	7.38	23.58	26.26
Day 6 (ng L^{-1})	< 0.1	< 0.1	0.35	0.57	1.42	1.99
WAC[a] (ng L^{-1})	< 0.1	< 0.1	1.4	1.61	4.19	4.97

[a] WAC = day 6 weight averages concentrations.

Biological responses

Exposure to all concentrations of lambda-cyhalothrin used in streams did not affect larval survival since all larvae survived. On contrary to survival, development and growth were affected by the insecticide. Larval development was completely inhibited in the streams dosed with high concentration of lambda-cyhalothrin but was not affected in low dosed streams (Fig. 1A), while larval growth was inhibited in high and low dosed streams (Fig. 1B).

At the end of bioassay, all larvae in control were in fourth instar (mean head capsule width ± SE: 0.550 ± 0.007 mm), while all larvae exposed to high concentration of the insecticide stayed in third instar (mean head capsule width ± SE: 0.293 ± 0.006 mm) as was observed in day 0 (Fig. 1A). All larvae exposed to low concentration of lambda-cyhalothrin were in fourth instar (mean head capsule width ± SE: 0.562 ± 0.007 mm), except two that stayed in third instar, with head capsule of 0. 37 and 0.40 mm. Significant differences were found in larval developmental stage (percentage of fourth instar larvae) (ANOVA: $F_{2, 9} = 171.16$, $P < 0.001$) between high concentration of lambda cyhalothrin and control (Dunnett test: $P < 0.0001$) but no significant differences were found between low concentration of lambda-cyhalothrin and control (Dunnett test: $P > 0.05$).

Mean larval growth was 4.379 ± 0.299 (SE) mm in control, 2.925 ± 0.255 (SE) mm and 1.271 ± 0.110 (SE) mm, respectively, in low and high treatments, at the end of bioassay. There were highly significant differences in growth of larvae (ANOVA: $F_{2, 57} = 43.55$, $P < 0.001$) between treatments and the control (Dunnett tests: high vs control: $P < 0.0001$, low vs control: $P < 0.001$) (Fig. 1B).

Both responses, developmental stage and growth of larvae, were significantly ($P < 0.01$) negatively related ($r^2 = 0.90$ and $r^2 = 0.88$, respectively) with day 6 weighted averages concentration of lambda-cyhalothrin (Fig. 2). A strong significant positive correlation ($r = 0.82$, $P < 0.01$) was observed between body length and the percentage of larvae in the fourth instar. But

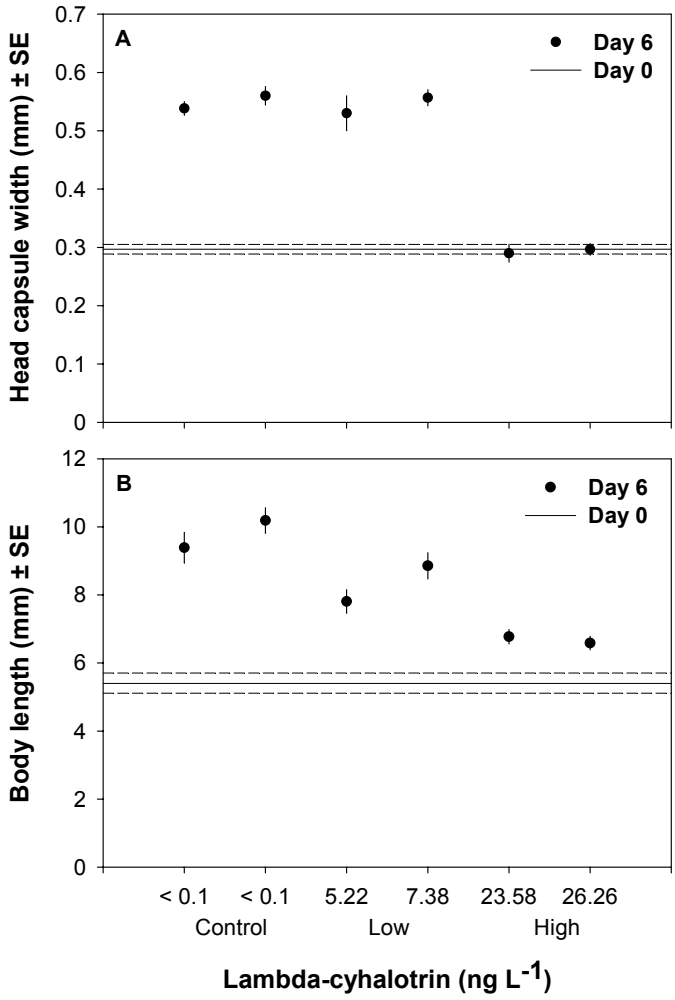

Fig. 1. Mean values and respective standard error (± SE) of larval biometrics (A- head capsule width, B-body length) in control and in low and high concentrations of lambda-cyhalothrin, at the end of bioassay (day 6). The mean and ± SE of larval biometrics in day 0 (measured from 30 larvae) are also shown (horizontal line and dashed line, respectively).

no significant ($P > 0.05$) correlation was found between and body length and head capsule width of larvae within third instar and within fourth instar.

Post-exposure feeding rate

Inhibition of post-exposure feeding rate was observed only for larvae deployed in streams dosed with high concentration of lambda-cyhalothrin (Fig. 3). The mean post-exposure feeding rate of larvae was 16.65 ± 1.05 (SE) mm in control, 14.45 ± 1.83 (SE) mm in low treatment and 10.85 ± 0.64 (SE) mm in high treatment. Significant differences were found in post-exposure feeding rate (ANOVA: $F_{2, 9} = 5.31$, $P < 0.05$) between high concentration of lambda-cyhalothrin and control

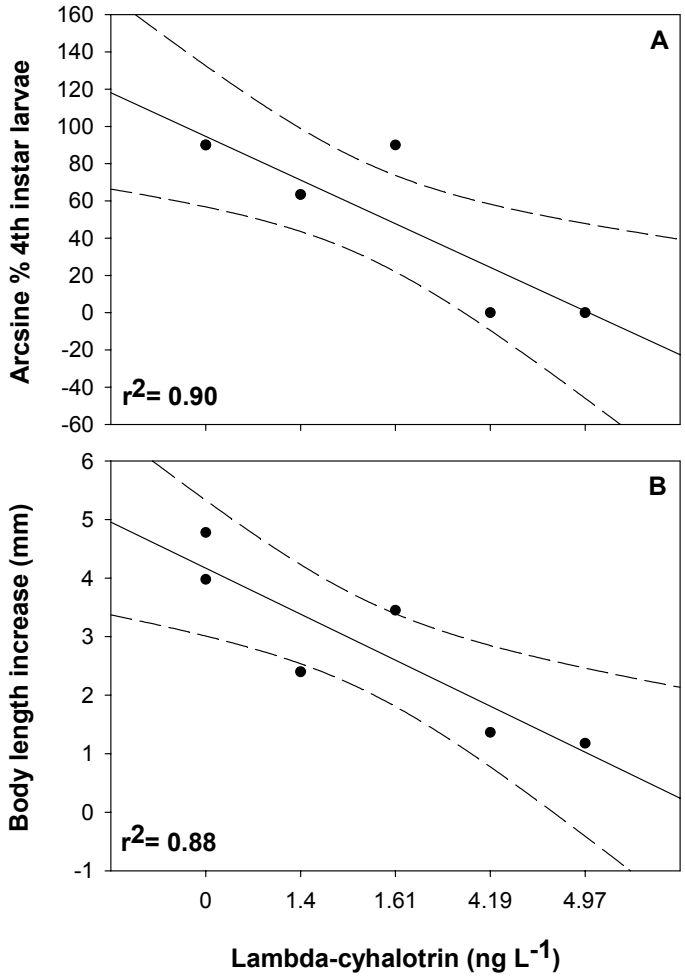

Fig. 2. Relationship of the percentage of fourth instar larvae (A) and growth (body length increase) (B) with day 6 weighted averages concentrations of lambda-cyhalothrin. Regression lines: arcsine percentage of fourth instar larvae = 96.1 – 20.0 conc.; body length = 9.58 – 0.646 conc.; all are significant ($P < 0.01$). Dashed lines - 95 % confidence limit envelopes.

(Dunnett test: $P < 0.05$) but no significant differences were found between low concentration of lambda-cyhalothrin and control (Dunnett test: $P > 0.05$). Nonetheless this functional response was significantly ($P < 0.05$) negatively related ($r^2 = 0.85$) with day 6 weighted averages concentration of lambda-cyhalothrin (Fig. 4).

A significant ($P > 0.05$) positive correlation (r = 0.69) was observed between feeding rate and body length of larvae and between feeding rate and the percentage of larvae in fourth instar (r = 0.63). No significant ($P > 0.05$) correlation was found between feeding rate and head capsule width within instars.

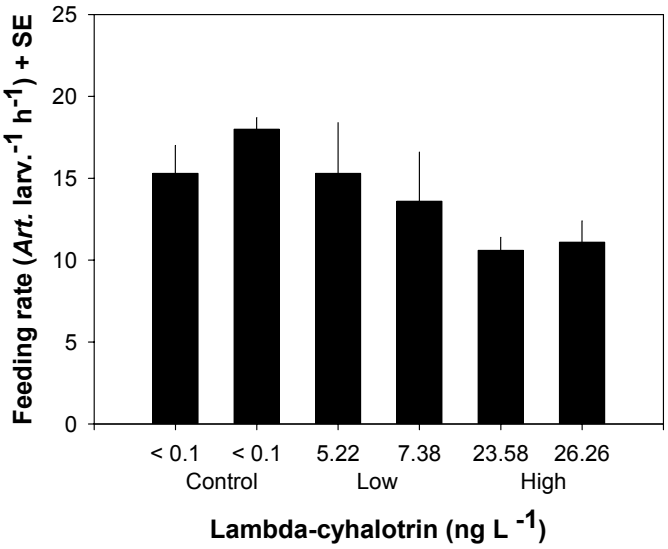

Fig. 3. Mean values and respective standard error (+ SE) of post-exposure feeding rate of larvae in control and in low and high concentrations of lambda-cyhalothrin, at the end of bioassay (day 6).

Discussion

Chemical and physical parameters

During the bioassay the larvae were exposed to similar physical and chemical conditions (Table 1) with the exception of different lambda-cyhalothrin concentrations (Table 2). Consequently we expected that effects on the *C. riparius* larvae responses used as endpoints were related to the exposure of larvae to this insecticide.

The decrease of lambda-cyhalothrin in day 0 was due to adsorption to (1) sediment, according with equilibrium partitioning theory described by Di Toro et al. (1991) and the results of Hamer et al. (1999), (2) test chambers and to other cages present, as observed by Hamer et al. (1999), (3) large amount of leaf material and, probably, and to (4) walls of artificial streams.

On contrary to Hamer et al. (1999), in our study the concentration of lambda-cyhalothrin did not effectively equilibrated in aqueous-phase after 24 hours but gradually declined during the 6 days bioassay. The differences between nominal and determined concentrations on day 0 were much higher (100 to 6.3 ng L^{-1} and from 250 ng L^{-1} to 24.9 ng L^{-1}, average) (Table 2), than in Hamer et al. (1999) (from 225 to 122 ng L^{-1}). However, in our experiment we used artificial streams, instead a static system, which might cause a continuous loss of the insecticide to the environment.

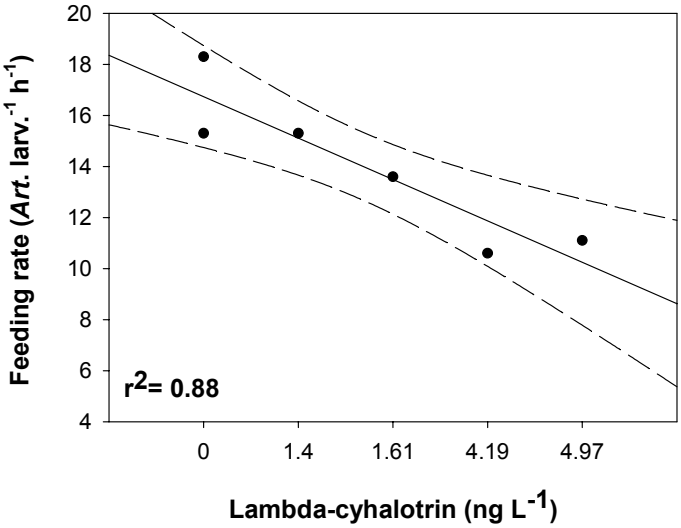

Fig. 4. Relationship of post-exposure feeding rate with day 6 weighted averages concentrations of lambda-cyhalothrin. Regression line: feeding rate = 16.5 – 1.23 conc. (P <0.05). Dashed lines - 95% confidence limit envelopes.

Biological responses

Survival was not affected by the concentrations of lambda-cyhalothrin in the bioassay. In contrast, Hamer et al. (1999) observed a decrease in survival of larvae exposed to a nominal concentration of 225 ng L^{-1} (the number of live larvae diminished from 10 to 6 after 96 hours exposure). This can be explained because determined concentrations were lower in our study (6.3 ng $L^{-1}$and 24.9 ng L^{-1}) than in Hamer et al. (1999) (122 ng L^{-1}) in day 0 and gradually declined during the 6 days bioassay (from 24.9 to 1. 99 ng L^{-1}) while in Hamer et al. (1999) effectively equilibrated after 24 hours (86 ng L^{-1}). More realistic testing environments, such as mesocosms, in comparison to laboratory static tests performed by Hamer et al. (1999), makes our results more accurate and relevant for describing biological effects in streams. The high survival (100%) observed indicates that *C. riparius* larva was successfully deployed in artificial streams and the bioassay is suitable to evaluate sublethal effects of this insecticide.

Development and growth of *C. riparius* larvae were more sensitive endpoints than survival, which was not affected by the insecticide. This was expected because sublethal responses generally precede and are manifested at lower exposure concentrations than lethal responses (Gerhardt 1996). High sensitivity of sublethal responses of Chironomids (*C. riparius* and *C. tentans*) larvae than lethal responses to different types of chemical substances was also observed by several authors (e.g. Pascoe et al. 1989; Maund et al. 1992; Pellinen and 1993; Brown et al. 1996; Bleeker et al. 1998; Watts et al. 2001; Gills et al. 2002; Mäenpää et al. 2003).

Growth and development of larvae were negatively related to lambda-cyhalothrin concentrations (Fig. 2). However, growth was more sensitive to insecticide, because it was inhibited by high and low concentrations of the insecticide (Fig. 1A) while development was only inhibited by high concentrations (Fig. 1B). This suggests that this bioassay can be used to assess insecticide contamination and is able to discriminate between different levels of contamination.

It is known that growth can decrease due to feeding decrease or to additional energy costs due to the presence of toxicants (Péry et al. 2003a). In our experiment, since growth was highly affected by high and low concentrations of the insecticide while post-exposure feeding was only significantly decreased in high concentration, it would appear that growth impairment was due to additional energy costs as a consequence of the toxicant presence and was not related to a decrease in feeding capacity, But, it is most likely the result of an initial feeding depression resulting in reduced growth, followed by recovery as the insecticide dissipated from the water column. Since pyrethroids, such as lambda cyhalothrin, act on sodium channels in insect nerve membranes causing, initially, a knock down and an inhibition in the feeding behaviour, followed by recovery or death (Hill 1985). A decrease on activity of *C. riparius* larvae caused by lindane, an organochlorine insecticide, was found by Hirthe et al. (2001) and Maund et al. (1992). These authors suggested that a reduced adult size of *C. riparius* observed following the exposure period was likely to be related to the reduced activity of larvae during exposure to lindane, since larvae appeared to be paralysed by the insecticide treatment.

Since 90% of the variation in the developmental stage of larvae between treatments could be explained by variation in the lambda-cyhalothrin concentrations between treatments, it would appear that inhibition of development of larvae exposed to high concentration of the insecticide was related to the insecticide. However it could be also related to body length. Since, although head capsule width was not related with body size within instars, development stage was strongly positively correlated with body length. According to Hooper et al. (2003), a delay in juvenile development of *C. riparius* larvae can be attributed to the requirement of a minimum body size and weight. So the inhibition observed in larval development could be the result of an initial feeding depression resulting in reduced growth.

The inhibition of growth and development caused by larvae exposure to the pyrethroid insecticide lambda-cyhalothrin can be the result, at least in part, of feeding depression. This indicates that both endpoints can be used to assess, in an indirect way, the effect of lambda-cyhalothrin in the feeding activity of larvae.

Post-exposure feeding rate

This endpoint was inhibited in the high dosed stream while no effect was observed in the low dosed stream, possibly due to the high variability within this treatment (Fig. 3). Furthermore, post-exposure feeding rate was negatively related with lambda-cyhalothrin concentrations (Fig. 4). So, it would appear that depression in post-exposure feeding rate of larvae of larvae in high dosed stream was directly related with lambda-cyhalothrin concentrations. However, Péry et al. (2002) demonstrated that the quantity of the food ingested by larvae depends on the instar stage, i.e. the minimum food quantity, to normal development of larvae, increases when there is a change in instar stage (from first to second to third and to fourth instar), which is related with a determined body size. So we could expect that larvae in high treatment that stayed in an earlier instar stage, in comparison with larvae in control and low concentration, would have a lower feeding rate in comparison with larvae in control. Furthermore, post-exposure feeding rate was correlated with development stage and body length of larvae.

Since the developmental stage depends on body length, which may depend on feeding, the depression in post-exposure feeding rate of larvae could have been the result of an initial feeding inhibition during larvae exposure, that resulted in reduced growth and development inhibition. Therefore, post-exposure feeding rate may be a less suitable endpoint than growth and development to assess the impact of this insecticide to the feeding activity of larvae during exposure. However, the observed depression of post-exposure feeding rate of larvae in high dosed streams shows that the highest concentration (4.19 and 4.97 ng L^{-1}) of the insecticide caused a persistent negative effect (probably indirectly) on feeding activity of *C. riparius* larvae even after exposure. McWilliam and Baird (2002a) also observed feeding inhibition of *Daphnia magna* after exposure to several concentrations of lambda-cyhalothrin (7.5 - 150 ng L^{-1}). These results suggest that the post-exposure feeding rate bioassays rate with sensitive species can be used to assess the effects of lambda-cyhalothrin after its decline from ecosystems.

Ecological relevance of lambda-cyhalothrin toxicity to *C. riparius* larvae

Pesticides are used in agricultural fields to eliminate target species however pesticides can reach aquatic ecosystems, by spray drift or runoff, where they also affect non-target species. Pesticide toxicity to target prey species in aquatic ecosystems can highly affect other organisms because (1) toxicity observed to prey species can cause a reduction in the food source for organism consuming them and/or because (2) pesticides accumulated in prey species may then cause toxicity in the organisms consuming them or continue to bioconcentrate through the food web (DeLorenzo et al. 2001). Furthermore, pesticides can be toxic to sensitive non-target species. For example, in an

aquatic microcosm study, the organotin insecticide azocyclotin inhibited picoplankton < 2μm and algae of 2 to 10 μm at ≥ 135 μg/L (DeLorenzo et al. 2001).

The impairment of growth and development in chironomids larvae may lead to impairment in the population fitness and subsequently a decrease in the population (Liber et al. 1996; Sibley et al. 1997a). Since chironomids are predominant in freshwater ecosystems (Péry et al. 2002; Ristola 2000) and are important prey items for birds and fish (Prat and Rieradevall 1995; García-Berthou 1999), the observed lambda-cyhalothrin toxicity to development and growth of *C. riparius* larvae, a target species, may affect the population of birds and fish, non-target species, that consume *C. riparius* larvae because their food source is reduced. Furthermore, the lambda-cyhalothrin accumulated in *C. riparius*, which is similar to lambda-cyhalothrin concentrations in aqueous phase according to Hamer et al. (1999), may cause toxicity to the population of birds and fish that consume them or continue to bioconcentrate through the food web. This may also be applied for other pyrethroids pesticides besides lambda-cyhalothrin since they have the same mechanism of action. These findings suggest that growth and development are sensitive and relevant endpoints of the bioassay with *C. riparius* larvae to assess ecological risk of pyrethroid insecticides in freshwater ecosystems.

Since chironomids larvae are a collector-gatherers (feed primarily on fine particulate organic material deposited on sediment) (Vos 2001), recycling organic matter, and are abundant in freshwater ecosystems, thus, they can play an important role in detritus processing (a key process of ecological functioning of aquatic ecosystems) on these ecosystems. Thus, the inhibition of growth and development that was found in larvae exposed to the pyrethroid insecticide lambda-cyhalothrin, as a result of feeding depression, may indicate impairment in detritus processing of aquatic ecosystems. The depression observed in larval feeding rate after larvae exposed to high concentrations of lambda-cyhalothrin (in high dosed streams) may indicate also impairment in detritus processing of aquatic ecosystems and shows that, although pyrethroids insecticides are low persistent in environment, their effects in ecosystem functioning may persist even after their decay. These results suggest that the bioassay, with *C. riparius* larvae, using development, growth and post-exposure feeding rate as endpoints can be an appropriate and relevant tool to assess the ecological risk of pyrethroid insecticides to ecosystem functioning.

Conclusions

The results suggest that the bioassay with *C. riparius* larvae using development and growth as endpoints can be used to assess the effects of pyrethroids insecticides on ecosystem ecology and functioning. The results also suggest that post-exposure feeding rate of this bioassay is an appropriate and relevant endpoint to assess the effects of pyrethroid insecticides on ecosystem

functioning even after their decay. Thus, the results support the use of the bioassay with *C. riparius* larvae in the assessment of ecological risk of pyrethroid insecticides in lotic ecosystems.

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Chapter 3

Pesticide effects on development, growth and biomass of *Chironomus riparius* larvae in a wetland channel adjacent to rice fields

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Abstract

Several pesticides are used worldwide in agriculture. However, the impact of these compounds on river macroinvertebrates is difficult to assess due to their rapid degradation or absorption onto sediments. Therefore, to determine pesticide contamination effects on *Chironomus riparius* larvae, a bioassay was carried out inside a rice field and in the adjacent wetland channel during pesticide (molinate, endosulfan and propanil) treatments. These were located inside the Natural Reserve of Arzila Marsh (Center Portugal). Development, growth and biomass of *C. riparius* larvae were used as endpoints. The bioassay was adapted and performed under laboratory conditions, with water collected in field, to assess whether *in situ* and laboratory bioassays showed comparable results. All tests used natural sediment from the Bestança river, an unpolluted river, previously conditioned in field for 24 hours. In the laboratory, larvae were also deployed in a control treatment with ASTM water and no conditioned sediment from the Bestança river. Larval growth was inhibited by the highest concentration of endosulfan both in field and laboratory exposure. During laboratory exposure, it was also observed a significant inhibition of growth in high and low concentrations of endosulfan when comparing with control treatment. The highest concentration of endosulfan decreased the larval biomass on *in situ* bioassays, but no differences in biomass were observed in laboratory between treatments. No effect on development of larvae exposed to endosulfan was found. The herbicides molinate and propanil did not affect the biological responses of *C. riparius* larvae. The endosulfan concentrations used in the rice fields caused toxicity to *C. riparius* larvae in the wetland channel, this may indicate that pesticide spray drift and runoff from rice fields within the limits of the Natural Reserve can cause a severe

ecological impairment of this protected wetland. The results also suggest that laboratory testing should be complemented with *in situ* bioassays because *in situ* toxicity may not be detected in laboratory.

Keywords: *Chironomus riparius*, bioassays, pesticides, rice field, wetland channels

Introduction

The use of pesticides in agriculture may lead to contamination of surface and ground waters by drift, runoff, drainage and leaching. Pesticides used in Portuguese agricultural areas have been found in surface and ground waters. Insecticides (e.g. endosulfan, lindane, chlorfenvinphos) and herbicides (e.g. propanil, molinate, atrazine) were detected in the surface water, collected in three river basins (Tejo, Sado and Guadiana) from 1983 to 1999, and in the ground water, collected from the wells of seven agricultural areas in Tejo and Sado basins from 1991 to 1998 (Cerejeira et al. 2003). Pesticide contaminated water can cause toxic effects to aquatic flora and fauna, and can highly affect human health (Forney and Davis 1981; Mulla and Mian 1981; Jensen 1983).

Rice, along with wheat and corn, is one of the three crops on which the human species largely subsists. In fact, almost two billion people depend primarily on rice (Ronald 1997). It is also an important crop in terms of pesticide consumption, particularly herbicides, since the use of pesticides during cereal growth may affect the quality of the surrounding aquatic environment. In Portugal rice is an important irrigated crop and water is frequently discharged to the surrounding water bodies (Pereira et al. 2000). Studies with the aim of clarifying the potential toxic effect of the pesticides used in rice fields to the aquatic ecosystems are therefore important and should be performed. However the impact of pesticides on aquatic ecosystems is usually difficult to assess because they degrade very quickly (Barry and Logan 1998), because they can be absorbed onto the sediment (Peterson and Batley 1993; Hamer et al. 1999) and because peak concentrations of pesticides in rivers are rarely measured during storm runoff events, since sites are often inaccessible and these peaks may last only a few hours (Cooper 1996).

The aim of this study was to determine the effect of pesticides used in rice treatments to aquatic macroinvertebrates and their potential risk to aquatic ecosystems. A bioassay with *C. riparius* larvae using biological responses (development, growth and biomass) as endpoints was deployed in a rice field and in the adjacent wetland channel during the pesticide spraying period. Third instar larvae were deployed in rice fields 48 hours after each pesticide (endosulfan, molinate, propanil) treatment. The bioassay with contaminated water from rice fields and conditioned sediment were also run under laboratory conditions, in parallel to the *in situ* bioassay, to assess whether *in situ* and laboratory tests showed comparable results.

Chironomidae larvae have been recorded as pests of rice growing in many temperate countries (Surakarn and Yano 1995). The larvae either attack the seed itself, or feed on the roots or shoots of young seedlings (Helliwell and Stevens 2000; Stevens et al. 2000). Secondary damage can arise through their tunnelling activity in the sediment which can destabilise the root systems of young plants and increase turbidity, reducing photosynthesis and slowing the growth of submerged seedlings (Helliwell and Stevens 2000). But Chironomids are also important macroinvertebrates in the ecology of the aquatic ecosystems. Chironomids is the most widely and often abundant group of insects in freshwater environments (Péry et al. 2002; Ristola 2000) and provide an important link between different trophic levels, recycle organic matter and are important prey for fish and birds (Prat and Rieradevall 1995; García-Berthou 1999).

Endosulfan ($C_9H_6Cl_6O_3S$) is the agricultural pesticide (e.g. rice, cotton, orchards, corn) with the highest potential impact on riverine ecosystems (Leonard et al. 1999; Schulz 2001b). It is an organochlorine insecticide of the cyclodiene subgroup which acts as a contact poison in a wide variety of insects but is also quite toxic to bird species (e.g. *Anas platyrhynchos*, *Colinus virginianus*) (National Library of Medicine 1987), to shellfish and to several fish species (e.g. *Oncorhynchus mykiss*) (Maier-Bode 1968). Endosulfan is highly persistent in environment (Helliwell and Stevens 2000). This insecticide is frequently used, associated with deltamethrin, by farmers in Central Portugal to control Chironomids and crayfish *Procambarus clarkii* infestation in rice fields.

Molinate ($C_9H_{17}NOS$) is a selective herbicide which belongs to the thiocarbamate class and it is used to control weeds in rice fields (Hartley and Kidd 1983; Meister 1991). It is toxic to germinating broad-leafed and grassy weeds (Meister 1991) and is rapidly taken up by plant roots and transported to the leaves where it inhibits leaf growth and development (Meister 1991). Molinate is of low persistence in the soil environment, with a field half-life of 5 to 21 days (Wauchope et al. 1992).

Propanil ($C_9H_9Cl_2NO$) is an acetanilide post-emergence herbicide with a selective mode of action, reducing photosynthesis and consequently dissolved oxygen levels in the water. It is a contact herbicide that within a plant is moved from the leaves to the growing shoots, then back to other leaves (Weed Science Society of America 1994). It is used to control numerous monocotyledonous (narrow leaf) and dicotyledonous (broad leaf) weeds that occur in rice and potato fields. Resistant crop plants such as rice completely metabolize propanil (Weed Science Society of America 1994). Propanil is soluble in water and it adsorbs only weakly to soil particles (Wauchope et al. 1992; Weed Science Society of America 1994). This herbicide rapidly breaks down in water due to microbial activity and is of low soil persistence. Its half-life in the field is 1 to 3 days (Wauchope et al. 1992; Weed Science Society of America 1994).

Material and Methods

Field sites and pesticide treatment

In situ bioassay with *Chironomus riparius* was carried out in rice fields within the limits of a Natural Reserve of Arzila Marsh, a protected wetland located near the Mondego river in Central Portugal. The site selected as high contaminated was located inside a rice field, one of the several rice fields treated with pesticides. The control and low contaminated sites were located in a channel bordering the rice fields. The control site was located upstream and the low contaminated site was located downstream from the point where the channel receives water from the rice fields. Molinate was the first pesticide introduced in rice fields; after three weeks endosulfan was applied and after approximately one month the propanil was applied. Molinate rapidly volatilizes if the soil is wet (Brooks 1980), which is why it is usually ploughed into dry soil before water is added.

Test organisms

Third instar larvae of *Chironomus riparius* used in the bioassays were obtained from laboratory cultures established at the Biology department, University of Aveiro. The culture conditions were the same as described in chapter 1, except for the substrate. Here natural sediment from the Bestança river, considered an unpolluted river (see chapter 1), was used as substrate.

In situ bioassays

Twenty four hours after each pesticide treatment in rice fields, five chambers (described in chapter 1), with a layer of 5 cm depth of natural sediment, previously collected in the Bestança river, were placed in sites, protected by plastic cages, in each site, to condition sediment for *in situ* bioassay. Two additional chambers were deployed, to determine pesticide concentrations in sediment on day 0 and on day 6. Six additional chambers, also protected by plastic cages, were placed in sites to condition sediment during twenty four hours for subsequent laboratory tests.

On the first day of the bioassays (day 0), 2 days after pesticide treatment, five third instar larvae of *Chironomus riparius* were introduced through the plastic tube in each of five chambers and TetraMin® (approximately 30 mg in suspension) was added to each of these chambers. The body length and head capsule width of 30 additional larvae were measured to determine respectively initial body length and development stage. The conditioned sediment of the additionally five chambers was collected to be used in laboratory tests and the sediment of the other additional chamber was collected for pesticide analysis. Water was also collected at each site to use in laboratory tests and for pesticide analysis.

At the end of experiment (day 6) in each site, the surviving larvae were collected from the five chambers, killed and preserved in Von Törne conservant (Gama 1964) for later measurement.

Sediment of the sixth chamber was collected for pesticide analysis. Water samples from each site were also collected for pesticide analysis. Larval biometrics were determined as described in chapter 1. After determining larval biometrics, larvae were placed in foil cups and dried at 60ºC for 24 h, after which they were cooled in a desiccator and weighed on a microbalance. Biomass was determined by dry weight per larva. Differences in dry weight of Chironomids larvae can often be due to differences in larval gut contents that are caused by differences in particle size and organic matter of sediment and not to differences in toxicity (Sibley et al. 1997b). So, sediment toxicity tests, comparing toxicity of sediment with different particle size and organic matter, using dry weight of chironomid larvae as an endpoint, have a high risk of false positives or false negatives (Sibley et al. 1997b). In our study that risk was minimized by the use of the same sediment in all treatments.

Laboratory bioassays

In all treatments, on day 0, field water (300 ml) was introduced into five chambers (glass flasks) with a conditioned sediment layer of 5 cm depth. 5 third instar larvae were introduced into each of the chambers and 30 mg, in suspension, of Tetramin was added to each chamber. The chambers were provided with artificial aeration under a 14 h light: 10 h dark photoperiod with a temperature of approx. 20ºC. At the end of the experiment (day 6), the surviving larvae were collected from the chambers, killed and preserved in Von Törne conservant (Gama 1964) for later measuring (see *in situ* bioassays procedures). Simultaneously, another chamber was also included in each treatment without addition of larvae and its water and sediment were collected for pesticide analysis at the end of the experiment. The treatments used were: (1) water and conditioned sediment brought from each site and (2) ASTM hard water (ASTM 2000), control water, and natural sediment from the Bestança river, used as a control treatment.

Physical and chemical analysis

In field and laboratory tests, hand-held field meters were used to measure water pH, conductivity and dissolved oxygen (DO), and a maximal and minimal thermometer was used to determine the maximal and minimal temperature of water, in day 0 and day 6. During field exposure water samples were collected on the initial day (day 0) and at the end of the experiment (day 6) to determine nitrate, nitrite, ammonia and phosphate concentrations of water.

The sediment and water samples collected for pesticide analysis were placed in dark glass containers and preserved at 4ºC in dark. The analysis of endosulfan and molinate were carried out by organic solvent extraction with cartridges containing Oasis® Hydrophilic-Lipophilic Balance Sorbent (HLB), a reversed-phase sorbent for all compounds, followed by a concentration

determination using liquid chromatography-mass spectrometry (LC-MS). Propanil analysis were carried out by solid phase extraction (SPE), also followed by a concentration determination using liquid chromatography-mass spectrometry (LC-MS). Since endosulfan is frequently used associated with deltamethrin by farmers in Central Portugal, water and sediment samples collected to determine endosulfan concentrations were also used to determined deltamethrin concentrations, but the results of deltamethrin analysis were below the detection limits (0.002 μg L^{-1}). All pesticides concentrations were determined by Innova-lab, Spain.

Statistical analysis

One-way analysis of variance (ANOVA) followed, when necessary, by Tukey HSD multiple comparison tests to test for significant differences in biological endpoints (development, growth and biomass) between treatments and between laboratory and field exposures (Zar 1996). One-way analysis of variance (ANOVA) followed by Dunnett tests was used to compare biological endpoints between contaminated (reference, low and high) treatments to the control treatment (Zar 1996). Responses were tested for normality using the Kolmogorov-Smirnov test. Statistical analyses were performed using Minitab™ Release 14.

Results

Physical and chemical parameters

Physical and chemical conditions were more variable in the field than in the laboratory (Table 1 and Appendix 1). In field tests, physical and chemical parameters of water were similar in reference and low contaminated sites but differed between these sites and high contaminated sites for the three pesticides (Appendix 1). During endosulfan exposure, the maximum temperature was 25ºC in the reference site, 29ºC in low contaminated site and 40ºC in high contaminated site. During molinate exposure, the maximum temperature was 20ºC in the reference site, 29ºC in low contaminated site and 32ºC in high contaminated site. During propanil exposure the maximum temperature was 30ºC in the reference site, 31ºC in low contaminated site and 47ºC in high contaminated site. DO and pH was also higher in high contaminated site than in reference and low contaminated sites, during all pesticide treatments. On contrary, minimum temperature, conductivity, hardness and nutrients (nitrite, nitrate, ammonia, phosphate) concentrations were lower in high contaminated sites than in reference and low contaminated sites.

In laboratory tests, physical and chemical parameters of aqueous phase samples were similar in all pesticide treatments (Table 1). During endosulfan exposure, mean values of DO ranged between 7.0 and 8.4 mg L^{-1}. Minimum temperature was between 17 and 20ºC and maximum

Table 1. Physical and chemical parameters of control, reference (Ref), low and high water determined in days 0 and 6 of laboratory bioassays. Values determined on day 0 in parenthesis.[a] Control = ASTM and not conditioned natural sediment, Ref = water from reference site and conditioned natural sediment, Low = water from low contaminated site and conditioned natural sediment, High = water from high contaminated site and conditioned natural sediment. Temp = temperature; Cond = conductivity; DO =dissolved oxygen.

Treatment [a]	$Temp_{min}$ (ºC)	$Temp_{max}$ (ºC)	pH	Do (mg L^{-1})	Cond (ms cm^{-1})
Endosulfan					
Control	17.8	18.0	7,6 (7.7)	7.0 (7.5)	666 (630)
Ref	17.3	18.8	8.0 (7.7)	7.5 (7.2)	766 (737)
Low	17.3	19.0	8.2 (7.6)	7.3 (7.7)	801(830)
High	17.4	18.7	8.3 (7.9)	7.2 (8.0)	714 (660)
Molinate					
Control	19.1	20.4	8.4 (7.6)	8.1 (8.3)	624 (595)
Ref	19.3	20.3	8.3 (7.8)	8.3 (8.9)	590 (423)
Low	19.0	19.4	8.2 (7.9)	8.0 (8.9)	459 (390)
High	19.0	19.4	8.2 (7.6)	8.5 (8.7)	490 (397)
Propanil					
Control	19.8	20.5	8.0 (7.7)	7.5 (8.0)	667 (615)
Ref	19.6	20.3	8.1 (7.9)	7.5 (7.3)	710 (733)
Low	19.4	19.8	8.2 (8.0)	7.2 (8.0)	704 (730)
High	19.7	20.0	8.0 (7.9)	7.9 (8.2)	675 (798)

temperature varied between 18 and 21ºC. Measured pH levels were between 7.6 and 8.4. Mean values of conductivity were similar within each pesticide treatment.

Pesticides concentrations

Molinate and propanil concentrations and total endosulfan concentrations, the sum of α and β isomers and endosulfan sulphate, in water and sediment during field and laboratory exposure are shown in Table 2. In day 0, the endosulfan concentration in water varied from 0.91 to 2.78 μg L^{-1}, while in sediment was 0.62 μg Kg^{-1} in the rice field and was not detected in sites of the wetland channel. In day 6, the endosulfan concentrations in water were higher in field (0.62 to 1.82 μg L^{-1}) than in laboratory (0.19 and 0.94 μg L^{-1}) while in sediment endosulfan concentrations were similar in field (0.11 μg Kg^{-1}) and in laboratory tests (0.10 μg Kg^{-1}). Molinate concentration, in day 0, varied between 0.21 and 4.63 μg L^{-1} in water and between 1.10 and 2.64 mg Kg^{-1} in sediment. In day 6, molinate concentrations in water and in sediment were higher in field than in laboratory. Propanil was not detected in sediment samples, but was detected in the water from the rice field in day 0 (2.58 μg L^{-1}) and in laboratory in day 6 (0.14 μg L^{-1}).

Table 2. Pesticide concentrations in water (μg L^{-1}) and sediment (μg Kg^{-1}) during field and laboratory tests. (d.l.) = Detection limit. n.d. = Not detected.

Treatment (d.l.)	Water			Sediment		
	day 0	day 6 (field)	day 6 (lab)	day 0	day 6 (field)	day 6 (lab)
Endosulfan (0.002 μg L^{-1})						
Reference	0.91	0.62	0.19	n.d.	n.d.	n.d.
Low contaminated	1.18	0.81	0.33	n.d.	n.d.	n.d.
High contaminated	2.78	1.82	0.94	0.62	0.11	0.10
Molinate (0.03 μg L^{-1})						
Reference	0.31	0.81	0.21	1.10	1.09	0.10
Low contaminated	1.48	0.97	0.60	1.28	1.14	0.57
High contaminated	4.63	1.31	1.03	2.64	1.43	0.99
Propanil (0.03 μg L^{-1})						
Reference	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Low contaminated	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
High contaminated	2.58	n.d.	0.14	n.d.	n.d.	n.d.

Biological responses

The herbicides molinate and propanil did not cause biological responses of *C. riparius* larvae (Fig. 1), since no significant ($P > 0.05$) differences were observed in endpoints between treatments in laboratory and field exposures. The insecticide endosulfan, however, affected negatively larval growth and biomass in field and laboratory exposure, but did not affected larval development, since all larvae changed from the third to the fourth instar and no significant ($P > 0.05$) differences were observed in head capsule width of larvae between treatments (Fig. 2), although a decrease trend in head capsule width was observed from the highest to the lowest (reference) contaminated sites in field exposure (Fig. 2).

In situ, there were clear and significant differences in larval growth and biomass between the sites (ANOVA, growth: $F_{2, 53} = 9.61$, $P < 0.001$, biomass: $F_{2, 53} = 5.56$, $P < 0.01$) (Fig. 2). However, larval growth was only inhibited in the rice field, the highest insecticide contaminated site (Tukey post hoc test: reference vs low: $P > 0.05$, reference vs high: $P < 0.001$, low vs high: $P < 0.05$). A significant decrease in biomass was, also, only observed in this site (Tukey post hoc test: high vs low: $P > 0.05$, high vs control: $P < 0.05$, low vs control: $P > 0.05$).

In the laboratory, there were also clear and significant differences in larval growth between treatments (ANOVA: $F_{3, 80} = 10.69$, $P < 0.001$) (Fig. 2). As observed in field exposure, there were also significant differences in growth between the treatment with water and conditioned sediment from the rice field (high endosulfan concentration) and the treatment with water and conditioned

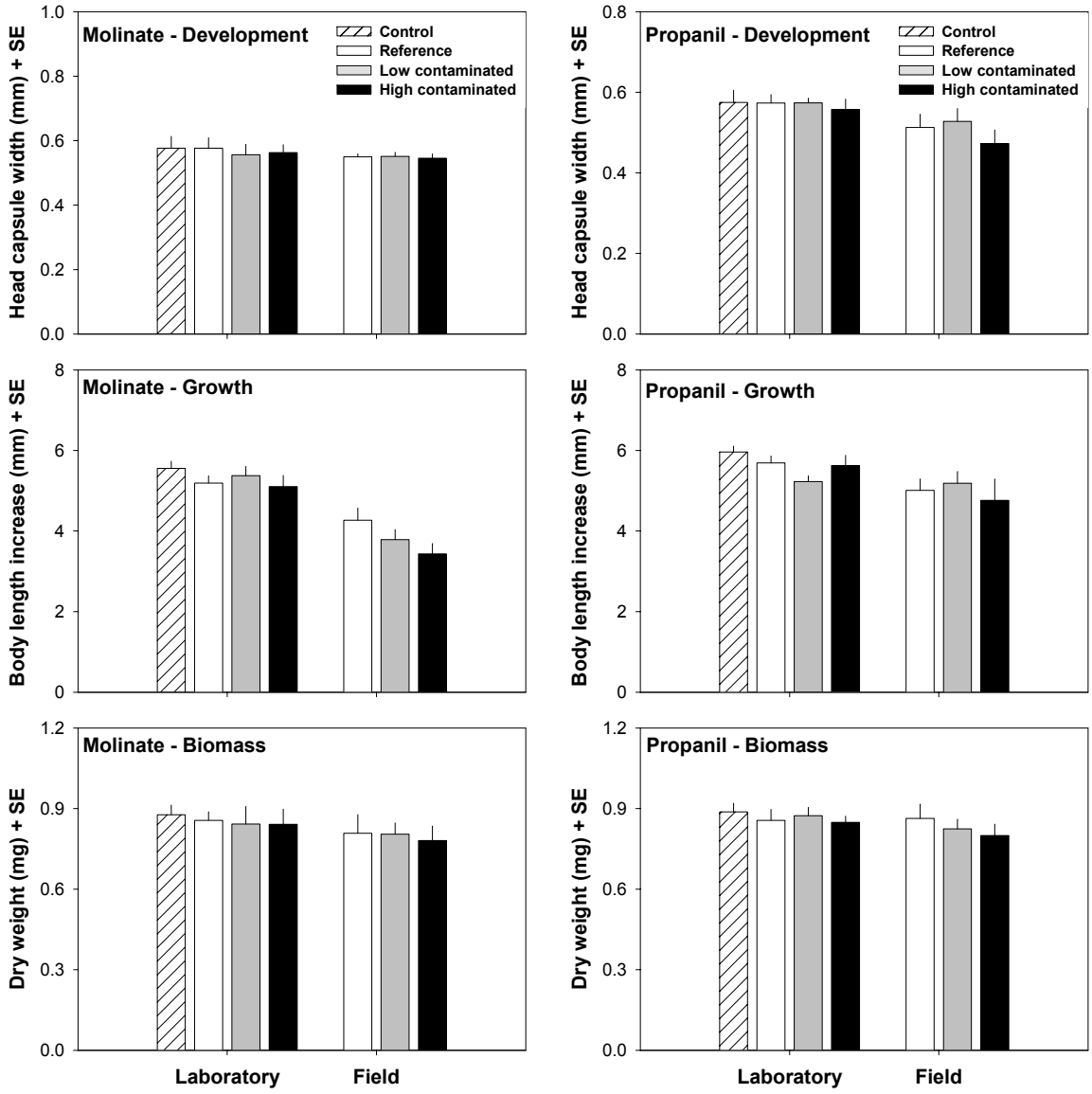

Fig. 1. Mean values and standard errors of biological responses, development (head capsule width), growth (body length increase) and biomass (dry weight) of *C. riparius* larvae exposed to molinate and propanil water contamination, in laboratory and in field tests. Control - ASTM, Reference - water from reference site, Low contaminated - water from low contaminated site, High contaminated - water from high contaminated site (see Table 3).

sediment from the wetland channel (low and reference endosulfan concentrations) (Tukey post hoc test, reference vs high: $P < 0.05$, reference vs low $P > 0.05$; low vs high: $P < 0.05$). However, in laboratory was also observed an inhibition in growth in high, low and reference treatments of the insecticide when comparing with control (Dunnet test, control vs reference: $P < 0.05$, control vs low: $P < 0.05$, control vs high: $P < 0.0001$) (Fig. 2). In the laboratory no significant ($P > 0.05$) differences in larval biomass were observed between treatments.

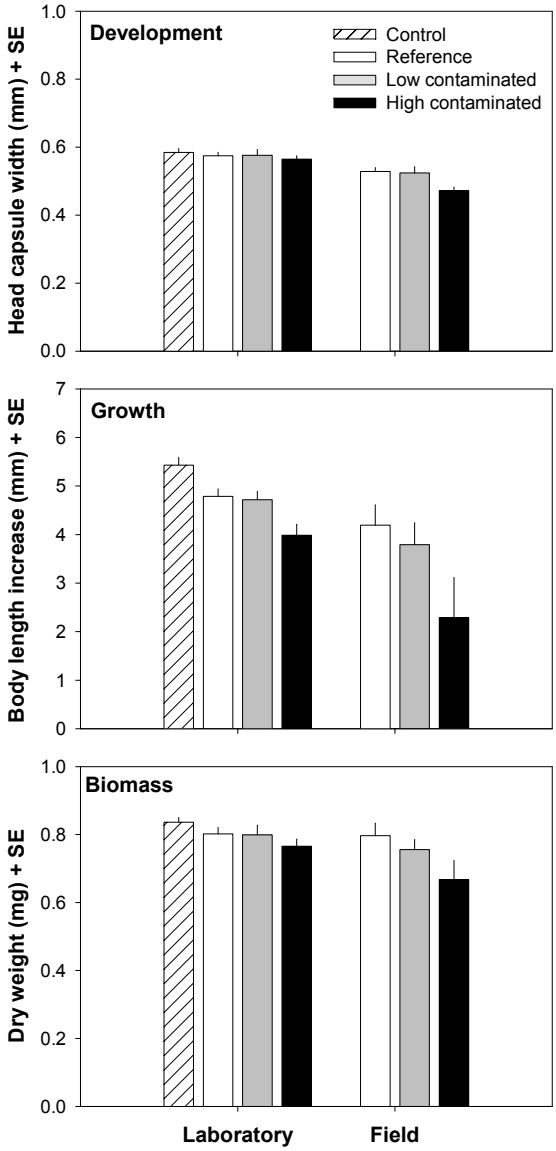

Fig. 2. Mean values and standard errors of biological responses, development (head capsule width), growth (body length increase) and biomass (dry weight) of *C. riparius* larvae exposed to molinate and propanil water contamination, in laboratory and in field tests. Control - ASTM, Reference - water from reference site, Low contaminated - water from low contaminated site, High contaminated - water from high contaminated site (see Table 2).

Higher values and lower variability of endpoints were observed in laboratory exposure than *in situ* exposure to insecticide endosulfan (Fig. 2). Significant differences in all endpoints of *C. riparius* larvae, exposed to high concentration, were found between laboratory and field (Appendix 2). There were also differences in larval growth in low concentration and in larval development in low and reference concentrations between the two exposure environments (laboratory and field) (Appendix 2). Lower values of biological endpoints were also observed *in situ* exposure of

herbicides (Fig. 2). Similarly to endosulfan, in propanil exposure significant differences in larval development were found between laboratory and field in all treatments, but also in larval growth between two types of exposure in reference treatment (Appendix 2). In molinate exposure, significant differences in larval growth were found between laboratory and field in all treatments (Appendix 2).

Discussion

Pesticides concentrations

On day 0 of the *in situ* bioassay, 2 days after endosulfan and molinate spray in rice fields, the wetland channel was also contaminated due the spray drift or runoff of pesticides from rice field. The highest pesticide concentrations were found in the rice field, as expected, since was the site where the pesticides were applied (Table 2). The lowest pesticide concentrations were found in the reference site, located in the wetland channel. Although pesticide concentrations in reference site were lower than in the low contaminated site, the differences in the pesticide concentrations between the two sites were lower than we expected, since reference site and low contaminated site were located upstream and downstream, respectively, from the place where the channel receives water from the rice fields. This may be explained by the occurrence of a reflux of water between the two sites, which are located very near to each other, in the same channel. This effect may be higher when water from rice fields is discharged to the channel. High differences in pesticide concentrations between these sites were observed for molinate treatment on day 0 and in laboratory on day 6, but on day 6 molinate concentration was similar at both sites in the field.

During bioassay, propanil was only detected in the rice field. So although would appear that wetland channel was not contaminated, this channel may be contaminated with propanil metabolites that were not determinated during pesticide analysis. This herbicide rapidly breaks down in water due to microbial activity and its half-life in the field is 1 to 3 days (Wauchope et al. 1992; Weed Science Society of America 1994). Cerejeira et al. (2003) frequently detected 3,4-dichloroaniline, a propanil metabolite, in Sado river throughout the rice crop season reaching a maximum level of 9.37 μg L^{-1}. In rice field, propanil was only detected in the water of rice field on day 0 and also on day 6 in the laboratory, but was not detected on the conditioned sediment.

It was expected that pesticides concentrations at the end of bioassay (on day 6) were higher in laboratory than in field because higher loss rate of pesticides to the environment is mostly likely to occur in field. In an experiment, previously conducted in artificial streams (Chapter 2), it was observed a higher decrease of the insecticide lambda-cyhalothrin concentration than in the experiment conducted by Hamer et al. (1999) in laboratory conditions. But, on contrary, the

molinate and endosulfan concentrations in water and conditioned sediment on day 6 were lower in laboratory than in field (Table 3), indicating that a higher loss rate of pesticides had occurred in laboratory. However, there is the possibility that on day 0 pesticide concentrations of laboratory samples were lower than in field conditions, due to the storage and transport of water and sediment from field to the laboratory.

Biological responses

Herbicides did not affect *C. riparius* larvae, which was expected, since this is not a target species for herbicides. Several authors (Hartley and Kidd 1983; Meister 1991; Burdett et al. 2001; Maenpaa et al. 2003) found negative effects of herbicides (e.g. propanil, molinate) on biological responses of non-target organisms, including chironomids (*C. riparius* and *Chironomus tepperi*). Most of these studies, however, used high and unrealistic water and/or sediment concentrations of herbicides. For example, Maenpaa et al. (2003) observed lethal effects on *C. riparius* larvae exposed to water concentrations of 1.79 and 2.79 mgL^{-1} of ioxynil and 79.11 and 62.31 mgL^{-1} of bentazone, and observed sublethal effects on *C. riparius* larvae exposed to sediment concentrations of 15.46 $mgkg^{-1}$ of ioxynil and 1160 and 4650 $mgkg^{-1}$ of bentazone. Studies that use unrealistic concentrations of chemicals do not accurately describe impacts on natural ecosystems and thus are less relevant than studies that use realistic concentrations.

Contrary to herbicides, the insecticide endosulfan negatively affected biomass and, especially, growth of *C. riparius* larvae. Negative effects of endosulfan treatments on *C. riparius* larvae were expected since this is a target species for the insecticide. However, larval development was not affected by the insecticide. Larval development seemed to be more affected by physical and chemical conditions of exposure than by the insecticide endosulfan. No significant differences were observed in larval head capsule width between treatments in laboratory and between sites *in situ* exposure although the lowest head capsule width was observed in rice field, the highest contaminated site (Fig. 2), which may be explained by higher maximum temperature in that site (40ºC) comparing with other sites (26 and 29ºC) (Appendix 1). Similar results were observed for head capsule with during *in situ* exposure to propanil (Fig. 1 and Appendix 1). Frouz et al. (2002) observed and that development rate of *Chironomus crassicaudatus* increased rapidly with increasing temperature up to 20ºC, slowed between 20 and 27.5ºC, and decreased at temperatures higher than 27.5ºC.

During exposure to endosulfan, no toxicity effect of contaminated treatments with endosulfan to larval biomass was observed in laboratory while *in situ* a decrease in larval biomass was observed in the rice field. This decrease could be due to the higher concentration of endosulfan observed in that site comparing to sites in wetland channel (Table 2), but it can be also explained

by the high maximum temperature observed in rice field (40ºC), comparing with maximum temperature in the sites of the wetland channel (26 and 29ºC), that may have enhanced the toxicity of this insecticide. Landrum et al. (1999) tested several organophosphate insecticides (e.g. azinphos methyl, disulfon, fensulfothion, terbufos) on *C. riparius* larvae and verified that their effect varied with pH and temperature. Lydy et al. (1999) found a positive correlation between temperature and toxicity of the organophosphate insecticides, chlorpyrifos and m-parathion to *Chironomus tentans*.

Larval growth was the most sensitive response to the endosulfan insecticide. Toxicity of endosulfan contamination of rice field to growth was observed in laboratory and *in situ*. But, while the inhibition of growth in laboratory can be directly linked to the presence of high endosulfan concentrations, since physical and chemical conditions were similar between treatments, the differences of growth between the rice field and the wetland channel during *in situ* exposure could also due to differences in physical and chemical conditions, in special high maximum temperature. This may have caused an additional stress to *C. riparius* larvae. Frouz et al. (2002) observed that the larval size of *C. crassicaudatus* was greatest at 20ºC and decrease with increase temperature. Toxicity to growth of endosulfan contamination from the wetland channel site was also observed in laboratory. This was not possible to assess *in situ* because it would be necessary to use a site without endosulfan contamination (control site), to compare with the other sites. In contaminated areas (e.g. agricultural areas, mining areas) it can be difficult to find a site with none or minor contamination.

Relevance of the *C. riparius* larvae bioassay to assess ecological impact of pesticides

Growth of *C. riparius* larvae was highly affected by endosulfan in the rice field and in the wetland channel. The toxicity of endosulfan contamination in wetland channel to growth of *C. riparius* larvae could also affect the organisms that consume these larvae (e.g. birds and fish) because it seemed that their food source was reduced, but this may not occur because the biomass of larvae in that channel was not affected. However, since this level of endosulfan contamination caused sublethal effects on larvae, endosulfan accumulated in *C. riparius* larvae may cause toxicity to fish, birds or other organisms that consume them, or may continue to bioconcentrate through the food web in wetland channel. There are several evidences (DeLorenzo et al. 2001) that pesticides accumulated in organisms may cause toxicity to organisms that consuming them or may continue to bioconcentrate through the food web. Thus, the endosulfan spray drift and runoff from rice fields within the limits of the Natural Reserve of Arzila Marsh may cause a severe ecological impairment of this protected wetland channels or at least may constitute an ecological risk to this protected area. The toxicity of endosulfan contamination to growth of *C. riparius* larvae observed in wetland channel may also be indicative of impairment in rivers where similar contamination is observed,

since the aqueous endosulfan concentrations (0.55 mg L^{-1} , average) in reference site of this channel observed in laboratory was similar to an endosulfan concentration (36 mg L^{-1}) detected by Cerejeira et al. (2003) in the Sado river water shortly after application of the insecticide in rice fields.

Although the molinate did not affect *C. riparius* larvae in the wetland channel, is possible that molinate from rice crop treatments can cause an impact on ecology of wetland channels. Herbicides can reduce photosynthesis and consequently dissolved oxygen levels in the water. Furthermore, the toxicity of herbicides to phytoplankton may result in a reduced source for grazers (DeLorenzo et al. 2001).

No effects of endosulfan contamination to biomass were observed in the wetland channel, which most likely means that the consumption of energy by larvae was not affected, indicating that feeding activity of larvae in this channel was not affected. The decrease in biomass and growth observed in the rice field (high endosulfan contaminated) caused high metal contamination or as a result of enhanced toxicity by the maximum temperature, may not accurately describe toxicity of endosulfan to biomass and growth in freshwater ecosystems because this level of temperature is not usually found in these ecosystems, at least in Portugal. In this study the maximum temperature found in wetland channel was 31ºC and in a previous study (Chapter 1) the maximum temperature observed for several Portuguese rivers was lower than 25º C except for Bestança river that was 32ºC.

Laboratory versus *in situ* bioassays

Higher values of the biological responses of *C. riparius* larvae were found in laboratory than in *in situ* exposure for all pesticide treatments (Fig. 1 and 2). This can be explained by the occurrence of differences and a higher variability in physical and chemical conditions observed *in situ*. This could have caused higher stress to larvae comparing with the controlled laboratory optimal conditions. When in presence of contaminants, this probably generated even more stress. Higher body length and dry weight of *C. riparius* larvae in laboratory than *in situ* were also observed by Castro et al. (2003).

Nevertheless, a similar pattern of larval growth was found in laboratory and field exposure to endosulfan (Fig. 2). Furthermore, the effects of endosulfan on larval growth that were observed in the rice field were also detected in the laboratory bioassay, providing evidence for comparability of the two designs. DeWitt et al. (1999) also found comparable results in survival of amphipods (*Chaetocorophium* cf. *lucasi*) exposed to cadmium, between field and laboratory tests. In the laboratory it was possible to detect toxicity effects in larval growth of the endosulfan contamination present in the wetland channel, by comparing the growth of larvae in treatments with water and

conditioned sediment from the wetland channel with the control treatment. *In situ* this was not possible due to absence of a control site, which can be difficult to find in contaminated areas (e.g. agricultural areas, mining areas).

On contrary to what was observed on larval growth, the decrease of larval biomass observed in the rice field was not detected in the laboratory bioassay. This can be due to the presence of lower endosulfan concentrations of rice field water in laboratory (1.86 mg L^{-1} average) than *in situ* (2.30 mg L^{-1} average). But it also possible that the maximum temperature (40ºC) in the rice field (much higher than the maximum temperature (29ºC) of the wetland channel), may have enhanced the toxicity of endosulfan to larval biomass *in situ*, while in laboratory this did not occur since physical and chemical conditions of water were similar between treatments. Tucker and Burton (1999) also found higher lethal effects on *Chironomus tentans* larvae exposed to contaminated rivers surrounded by an agricultural area than to larvae exposed to water and sediment from that rivers in laboratory and, according to these authors, this was probably due to sampling-related artefacts associated to laboratory testing, such as handling and storage of samples (Chappie and Burton 1997). These findings suggest that laboratory-to-field extrapolations can be biased because differences between laboratory and field exposure can be occur, due to (1) different and higher variable physical and chemical conditions in the field, which may cause additional stress to organisms and/or affect toxicity of contaminants and due to (2) alteration in contamination of the water and sediment during sample collection and storage that may affect toxicity of contaminants to organisms. Thus, laboratory-to-field extrapolations may be not indicative what actually occur in aquatic ecosystems.

In situ bioassays have several advantages: (1) they eliminate laboratory-to-field extrapolations; (2) reduce sampling-related artefacts; (3) allow stressor concentrations to fluctuate naturally. However, laboratory bioassays with water and sediment collected *in situ* provide an effective way to discriminate effects caused by stress contamination from effects caused by stress of other physical and chemical conditions. Laboratory bioassays can also be very valuable to assess low levels of contamination by comparison with a control treatment (no contaminated treatment), which may not be possible to assess *in situ* because in contaminated areas (e.g. agricultural areas, mining areas) it can be difficult to find a site with none or minor contamination to compare with contaminated sites. Thus, when *in situ* toxicity testing is used in combination with laboratory toxicity testing and physicochemical characterization, a more realistic assessment of pesticide spray drift and runoff effects, or of other contaminants effects, can be made.

Conclusions

Growth of *C. riparius* larvae was affected by insecticide contamination in the wetland channel adjacent to rice fields, suggesting that insecticide spray in rice fields may cause ecological impairment in freshwater ecosystems located nearby rice fields. This lends to a positive support to the use of the bioassay with *C. riparius* larvae using growth as the endpoint in the assessment of ecological impairment in lotic ecosystems caused by insecticide treatments in rice fields.

Bioassays conducted in laboratory are suitable and valuable tools to evaluate effects of contamination in aquatic ecosystems. However, laboratory should be complemented with *in situ* bioassays to integrate biological and physical and chemical processes in the toxicity assessment, that can not reproduced in laboratory.

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Appendix 1. Physical and chemical parameters in water in reference (Ref), low and high contaminated sites in days 0 and 6 of *in situ* bioassays. Values determined on day 0 in parenthesis. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

Treatment	$Temp_{min}$ (ºC)	$Temp_{max}$ (ºC)	pH	Do (mg L^{-1})	Cond (ms cm^{-1})	Nitrate (mg L^{-1})	Nitrite (mg L^{-1})	Amonia (mg L^{-1})	Phosphate (mg L^{-1})	Hardness (mg Ca CO_3 L^{-1})
Endosulfan										
Ref	15.0	26.0	7.9 (7.9)	7.0 (6.8)	790 (730)	2.20 (2.13)	0.10 (0.05)	0.26 (0.59)	0.57 (0.37)	26.3 (34.5)
Low	14.0	29.0	7.8 (7.9)	7.3 (6.9)	810 (733)	2.12 (2.23)	0.12 (0.05)	0.27 (0.50)	0.50 (0.42)	25.9 (40.9)
High	12.5	40.0	8.8 (8.0)	9.7 (10.7)	667 (507)	0.95 (1.7)	0.02 (0.07)	0.20 (0.28)	0.01 (0.04)	15.8 (18.6)
Molinate										
Ref	12.0	20.0	7.7 (6.8)	8.7 (8.5)	315 (389)	1.14 (1.32)	0.02 (0.02)	0.40 (1.14)	0.24 (0.15)	47.5 (44.0)
Low	13.0	29.0	7.8 (6.5)	9.2 (8.6)	442 (352)	0.72 (1.54)	0.005 (0.01)	0.93 (1.10)	0.04 (0.26)	23.86 (32.8)
High	7.0	32.0	7.2 (8.3)	10.2 (10.7)	397 (386)	0.43 (1.51)	0.002 (0.02)	0.38 (0.92)	0.02 (0.08)	16.2 (20.8)
Propanil										
Ref	12.0	30.0	8.4 (7.9)	7.4 (7.8)	716 (751)	2. 03 (2.20)	0.02 (0.03)	0.28 (0.49)	0.11 (0.08)	22.6 (27.2)
Low	17.0	31.0	8.2 (7.9)	(7.3) 8.02	713 (781)	1.93 (2.11)	0.02 (0.04)	0.26 (0.45)	0.10 (0.07)	27.0 (27.7)
High	18.0	47.0	8.9 (8.0)	10.53 (10.71)	636 (831)	0.12 (0.43)	0.02 (0.04)	0.51 (0.22)	0.03 (0.02)	15.1 (24.3)

Appendix 2. One way analysis of variance (ANOVA) to comparing effects of pesticides on biological endpoints between laboratory and field exposure.

Treatment	Ecological endpoints	*df*	*F*	*p*
Endosulfan - Reference				
	Development	1, 40	11.49	< 0.01
	Growth	1, 40	1.16	n.s.
	Biomass	1, 38	0.00	n.s.
Endosulfan - Low contaminated				
	Development	1, 32	12.11	< 0.001
	Growth	1, 32	7.00	< 0.05
	Biomass	1, 32	1.70	n.s.
Endosulfan - High contaminated				
	Development	1, 37	20.10	< 0.001
	Growth	1, 37	36.64	< 0.001
	Biomass	1, 34	17.19	< 0.001
Molinate - Reference				
	Development	1, 43	3.16	n.s.
	Growth	1, 43	6.59	< 0.01
	Biomass	1, 43	0.40	n.s.
Molinate - Low contaminated				
	Development	1, 30	0.12	n.s.
	Growth	1, 30	20.55	< 0.001
	Biomass	1, 28	1.64	n.s.
Molinate - High contaminated				
	Development	1, 30	1.66	n.s.
	Growth	1, 30	17.86	< 0.001
	Biomass	1, 28	4.20	n.s.
Propanil - Reference				
	Development	1, 45	36.22	< 0.001
	Growth	1, 45	4.52	< 0.05
	Biomass	1, 43	0.02	n.s.
Propanil - Low contaminated				
	Development	1, 43	23.37	< 0.001
	Growth	1, 43	0.02	n.s.
	Biomass	1, 38	0.80	n.s.
Propanil - High contaminated				
	Development	1, 33	15.32	< 0.001
	Growth	1, 33	2.84	n.s.
	Biomass	1, 30	1.59	n.s.

Chapter 4

Biological responses of *in situ* bioassays with *Chironomus riparius* larvae to biomonitor metal pollution in rivers

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Abstract

The midge *Chironomus riparius* was used to study the impact of metal contamination in streams near an abandoned goldmine in Portugal. A bioassay with *C. riparius* larvae was carried out in spring, in three consecutive years (2002-2004) throughout an environmental rehabilitation process of the abandoned goldmine. A laboratory bioassay was also performed in 2003 and 2004 (1) to verify if the *in situ* bioassay responded in the same way as in laboratory conditions, (2) to compare the sensitivity of biological endpoints to different metal contamination in water and sediment and (3) to determine toxicity of heavy metals that enter the organism through ingested material. During the environmental rehabilitation process a decrease of heavy metals in water and sediment was observed. This trend was also observed for the inhibition of growth. High metal contamination significantly inhibited growth in both laboratory and *in situ* bioassays. In the laboratory bioassay, growth was inhibited by high and low contamination on the sediment but was only inhibited by high concentrations of heavy metals in water, while biomass was only affected by metal contamination in sediment. The lowest effect of heavy metals was observed in the treatment that prevented ingestion of sediment by larvae. The results indicate that growth response of the *in situ* bioassay with *C. riparius* larvae can be used to biomonitor metal contamination in rivers, although *C. riparius* larvae are more affected by heavy metals that enter in the organism through ingested material associated with sediment particles than by dissolved metals in water. The results also indicate that laboratory-to-field extrapolations can be made if field water and sediment is used in laboratory testing.

Keywords: *Chironomus riparius*; Bioassays; Abandoned goldmine; Heavy metals

Introduction

The chemical impact of drainage from abandoned mines into the environment throughout the world is large. Studies of effects of mine drainage on benthic macroinvertebrates (Armitage 1980; Winner et al. 1980; Clements et al. 1988; Clements 1994; Kiffney and Clements 1994; Rutherford and Mellow 1994; Winterbourn and McDiffet 1996) and fish (Rutherford and Mellow 1994) generally revealed reduced diversity and abundance in impacted areas. In addition to the impact that metal contamination in rivers cause to benthic macroinvertebrates and fish communities, it causes a potential health risk for human populations living near metal contaminated rivers (Albering et al. 1999).

Chironomus riparius larva is a collector-gatherer from the dipteran family Chironomidae. This family is the most widely distributed group of insects, having adapted to almost every type of aquatic or semi-aquatic environment (Armitage et al. 1995; Batzer and Wissinger 1996; Silver Botts 1997), and can play an important role as biological indicators of metal contamination (Mousavi et al. 2003). Negative effects of single metal contaminants in genetic (Mattingly et al. 2001; Gills et al. 2002), morphological (Janssens de Bisthoven et al. 1998, 2001; Groenendijk et al. 1999) and, mainly, biological parameters such as growth and survival, of *Chironomus* larvae are well documented (e.g. Palawski et al. 1989; Pascoe et al. 1989; Timmermmans et al. 1992; Postma et al. 1994; Leppänen et al. 1998; Bervoets and Blust 2000; Milani 2000). However, there are only a few studies on metals effects to *Chironomus riparius* conducted *in situ* (e.g., Castro et al. 2003; den Besten et al. 2003), although it is known that interactions between metals can produce synergistic or antagonistic effects, depending on metals involved and the concentrations of metals (Fargašová 2001, 1997). Therefore, single metal laboratory testing is not sufficient to understand the effects of contamination by metal mixtures as they occur in natural environments, resulting from mine drainage. To understand the impact of heavy metal pollution to *Chironomus* species, more research on *in situ* tests is needed, particularly if site specific information is important, since laboratory toxicity testing is not generating information biologically relevant for the area of concern (Giesy and Hoke 1989).

Many pollutants that enter aquatic environments either bind to the surface of suspended particles in the water column and settle in the sediment (Simkiss et al. 2001) or, in the case of metals from mine drainage, precipitate when they reach the streams with higher pH and deposit in the substrate (DeNicola and Stapleton 2002). As a result of these processes, the concentration of contaminants in the sediment may be hundreds of times higher than in water (Simkiss et al. 2001). Eventually they can be released into surrounding water and assimilated by organisms (Burton

1991; DeNicola and Stapleton 2002). Thus, contaminants in sediments can be a threat to organisms either when released back into water or when absorbed directly from the sediment (Simkiss et al. 2001). Understanding how chemicals in water and in substrate from mine drainage affect benthic organisms is critical to successful remediation of impacted streams (DeNicola and Stapleton 2002). Accumulation of metals in aquatic organisms is accomplished by transport of dissolved metals across external membrane, adsorption on body surfaces, or by intake of particulate forms of metals that enter organism through ingested material (sediment or food) (Knezovich et al. 1987; Luoma, 1989; Hare 1992). In aquatic organisms, according to Boese et al. (1990) and Harkey et al. (1994), ingested material plays an important role in bioaccumulation of sediment-associated contaminants, but none of these studies was focused on *C. riparius*. This implies the importance of functional feeding groups of organisms based on food acquisition mechanisms.

To evaluate the impact of abandoned mines in rivers an *in situ* bioassay using *C. riparius* larvae was performed in two streams (Tinhela and Peliteira), located near the abandoned Jales goldmine in northern Portugal (Fig. 1). Gold mining in Jales dates back to Roman times and ended in 1992. It comprises underground galleries and a 14.4 ha open air deposit of more than five millions tons of mine residues, containing high concentrations of arsenic (As), cadmium (Cd), lead (Pb), and zinc (Zn) (Santos Oliveira and Ávila 1995; Bleeker et al. 2003). Until June 2002, wind and water erosion resulted in the spreading of contaminated particles into adjacent ecosystems (Bleeker et al. 2003). This has also affected the adjacent human population. High levels of Pb and Cd in the blood were reported and also a high prevalence of respiratory symptoms, eye irritation and anosmia (Gomes 1999; Mayan et al. 2005). Between June 2002 and June 2003, an environmental rehabilitation program of the open air mine residue deposit took place. The main goals were to resolve the erosive and structural instability of open air deposits of the mine residues and to reduce the air, water and soil contamination (http://www.exmin.pt/proj_jales.htm). This program consisted mainly in the embankment of mine residues and its cover with an impermeable geomembrane of high density polystyrene to avoid spreading of contaminated particles, the entrance of water and the heavy metals lixiviation. Two drainage systems, one superficial and one underground, were built, which made possible the treatment of lixiviated water (http://www.exmin.pt/proj_jales.htm). Finally, the area was enclosed, reforested and integrated in the landscape.

The *in situ* bioassay was carried out during spring, in three consecutive years (2002-2004). Development, growth and biomass were used as endpoints in order to evaluate the performance of the bioassay throughout the environmental rehabilitation process. Simultaneously, laboratory bioassays were also performed, at 15º C and 20º C, with sediment and water from the same rivers. The objectives of this study were to: a) verify that the heavy metal concentrations were altered

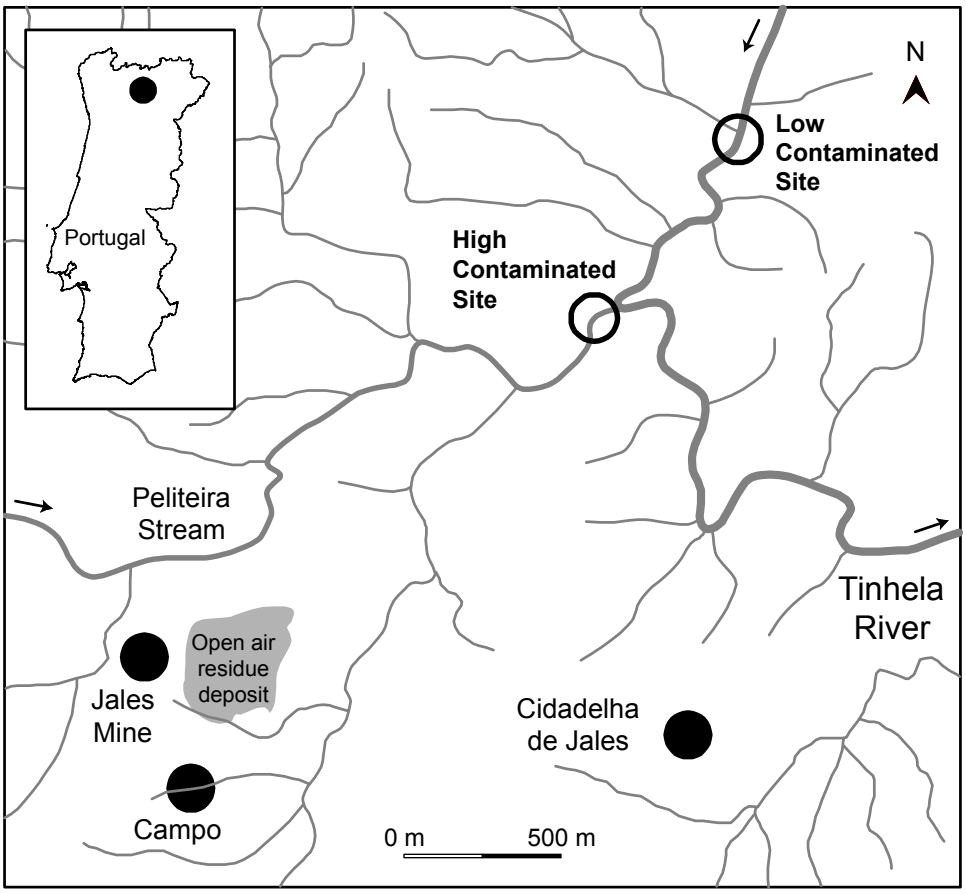

Fig. 1. Study area and selected sites on Peliteira stream and Tinhela river.

throughout the environmental rehabilitation program; b) evaluate the toxicity of metal contaminated sites to *C. riparius* larvae, throughout this program; c) verify if *in situ* bioassays responded in similar way in laboratory conditions; d) analyse the relative toxic effect of metals in water and sediment on *C. riparius* larvae; and, e) evaluate the toxicity of metals that enter *C. riparius* larvae through ingested material (sediment particles or organic matter).

Materials and methods

Study sites

The *in situ* bioassays of *Chironomus riparius* were carried out in two streams (Tinhela and Peliteira), located near Jales goldmine (Fig. 1). The site selected in the Tinhela river was considered low metal contaminated since underground water and atmospheric contamination can occur, although it is located upstream of any contaminated tributary stream. On the other hand, the site selected in Peliteira stream was considered to be high metal contaminated since the drainage basin of this stream receives the drainage from the Jales mine and inputs mine residue from open

air deposit. Furthermore, in other study (Chapter 1), it was shown that in spring 2002, Peliteira stream was ecologically impaired.

Test organisms

Chironomus riparius larvae used in the bioassays were obtained from laboratory cultures established at the Biology department, University of Aveiro. The culture conditions were the same as described in chapter 1. There were only differences in the substrate origin. In 2003 and 2004, instead of sea sand (supplied by Merck Co.) it was used organic matter free (ignited in a muffle furnace for 6 hours at 450ºC) natural sediment from Bestança river. This river was considered unpolluted because was located where no significant sources of pollution were observed and due to the high biotic score (BMWP'=169) determined in spring 2002 (see Chapter 1). Cultures were maintained at 20 ± 2ºC, with a 14 h light: 10 h dark photoperiod.

In situ bioassay

The bioassays with *C. riparius* larvae were conducted in spring, in three consecutive years (2002-2004). The *in situ* bioassay procedures were the same as described in Chapter 1. On initial day of the bioassay (day 0), 25 third instar larvae were introduced in five chambers (5 larvae per chamber), with sediment previously collected and conditioned *in situ*, placed in the river bed (see Chapter 1 for more details). At the end of bioassay (day 6), all larvae alive were removed from chambers and were killed and preserve in Von Törne conservant (Gama, 1964) for later measuring. Initial and final length and development of larvae were determined by measuring body length and head capsule width, respectively, using a stereomicroscope fitted with a calibrated eye-piece micrometer. Two water and sediment samples were collected from the rivers in plastic containers, on day 0 and day 6 of the bioassay, to determine heavy metal concentrations.

In 2003, on day 0, twenty five third instar larvae were also introduced in five additional chambers, with sediment previously collected in the Bestança river (control sediment) and conditioned *in situ*. One additional chamber with control sediment without larvae was placed in the river to determine heavy metal concentration of control sediment on day 6. On day 6, after determining larval biometrics, larvae were placed in foil cups and dried at 60ºC for 24 h, after which they were cooled in a desiccator and weighted on a microbalance. Biomass was quantified as dry weight per larva.

In 2003 and 2004, an additional chamber with field sediment was deployed by site, without *C. riparius* larvae, to compare heavy metal concentration in sediment inside chambers with the sediment collected in the river. Water and sediment from rivers were collected in plastic containers to use in the laboratory bioassay.

Laboratory bioassay

Laboratory bioassays were carried out in spring, in 2003 and 2004. The general procedures were the same as described in chapter 3. On initial day (day 0) of the bioassay, 25 third instar larvae were introduced in five plastic flasks (5 larvae per flask) with water and sediment, provided with artificial aeration, in each treatment. Two treatments were used: (1) water and sediment from Tinhela river and (2) water and sediment from Peliteira stream. The finishing of the bioassay and larval biometrics procedures and, also, dry weight determination were the same as described for the *in situ* bioassays. The results of the *in situ* bioassay were compared with the laboratory bioassay in 2003 and 2004. For this purpose, the laboratory bioassay was performed in 2003 at 15ºC, which was approximately the average temperature in field conditions; and at 20ºC, which is the optimal temperature for larval development and growth (McCahon and Pascoe 1988). In 2004, the laboratory bioassay was only performed with 20ºC since in this year the average temperature in field conditions was also 20ºC. All laboratory bioassays were conducted under a 14 h light: 10 h dark photoperiod.

In 2003, differences between water and sediment toxicity were also evaluated and, for this aim, the treatments with field water and sediment were compared with three additional treatments: (1) field water and control sediment; (2) control water and field sediment, to determine relative sensitivity of biological endpoints to water and sediment contamination; and, (3) ASTM water (ASTM 2000) and sediment from Bestança river, used as control treatment. The laboratory test was performed 24 hours after the *in situ* test to allow the sediment to settle down after the water was introduced. Two additional flasks without larvae were used, per treatment, to determined heavy metal concentrations of the water and sediment on day 0 and day 6.

In 2004, the toxicity of metals that enter the organisms through ingested material (associated with sediment particles or associated with organic matter) was also evaluated. For this purpose, the treatments with field water and sediment, not treated (NT), were compared with four additional treatments: (1) field water and dry and sieved (≥ 250 μm) sediment (DS); (2) field water and sediment with no added food (NTWF); (3) field water and organic matter free sediment (OMF); and, (4) ASTM hard water and sediment from Bestança river, used as a control treatment. In sieved sediment, only sediment particles with size superior to 250 μm were used to avoid ingestion of sediment particles by larvae. This grain size was determined before by measuring, with stereomicroscope, the mentum width of fifty fourth instar larvae from the laboratory culture. The average of mentum width was 150 ± 18 μm (Mean ± SD). *C. riparius* larvae are mainly deposit feeders that eat whole sediments with a particle size limit determined by the mentum width (Vos 2001). Prior to be sieved, sediment was dried for 24 hours at 60ºC. Organic matter free sediment (ignited in a muffle furnace for 6 hours at 450ºC, after dried) was used to avoid larvae feeding on

sediment. Since *C. riparius* larvae are detritivores of the collector–gatherer type, they use organic matter as a food source in sediment (Vos 2001). Since organic matter of sediment is very important when larvae are not fed (Pery et al. 2003c), comparisons between Peliteira stream and Tinhela river for larvae in NTWF sediments were possible because both stream sediments had similar organic matter contents (0.03 mg mg_{sed}^{-1}), which was determined in a previous study (see chapter 1). The laboratory test was performed 36 hours after the *in situ* test due to sediment treatment and to allow the sediment, after be treated, to settle down after the water was introduced. One additional flask without larvae was used, by treatment, to determined heavy metal concentrations of the water and sediment on day 6. During the sediment treatment, the water samples and sediment that was not treated were kept in airtight plastic containers and placed in the dark at 4ºC.

Physical and chemical analysis

Water physical and chemical parameters were determined in laboratory and *in situ* tests. On day 0 and day 6 of the bioassays, pH, conductivity and dissolved oxygen (DO) were measured with hand-held meters and water samples were collected to determine nitrate and phosphate levels. The minimum and maximum temperature throughout each bioassay was determined using a maximum-minimum thermometer.

Heavy metal analysis

Water samples were acidified (pH < 2.0) and sediment samples were frozen until analysis. Heavy metal analyses were performed on water and sediment samples to determine concentrations of zinc (Zn), cadmium (Cd), lead (Pb), nickel (Ni), manganese (Mn), chromium (Cr), iron (Fe), copper (Cu) and arsenic (As). Sediment was dried at 60ºC during 48 hours and digested in a mixture 1:3 of nitric acid (HNO_3 (70%)) and hydrochloric acid (HCL (37%)) on a hot (100-150ºC) plate to determine heavy metal concentrations (Clesceri et al. 1995). Heavy metal analyses were performed with inductively coupled plasma atomic emission spectrometry (ICP-AES) (Clesceri et al. 1995) at the Central Laboratory of Analysis in University of Aveiro.

Statistical analysis

One-way analysis of variance (ANOVA) followed, when necessary, by Tukey HSD multiple comparison tests, were used to compare larval head capsule width, body length increase and dry weight between years and/or between treatments (Zar 1996). One-way analysis of variance (ANOVA) followed by Dunnett tests was used to compare biological endpoints between different treatments and the control treatment (Zar 1996). Stepwise multiple regressions and linear regression analyses were used to investigate significant relationships between responses of *C. riparius* and heavy metal concentrations in sediment, during *in situ* bioassays, and between larval

Table 1. Physical and chemical parameters observed in control, Tinhela river and Peliteira stream water in the beginning (day 0) and at the end (day 6) of laboratory bioassay, in spring 2003. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

		Temp (ºC)		pH		DO (mg L^{-1})		Cond (µs cm^{-1})	
Water	Sediment	Min	Max	day 0	day 6	day 0	day 6	day 0	day 6
Laboratory - 20ºC									
Control	Control	17.8	19.8	7.6	8.1	8.5	8.7	624	657
Tinhela	Control	17.8	19.9	7.3	8.0	8.3	8.2	43	47
Peliteira	Control	18.0	19.7	7.1	7.9	8.8	9.0	80	97
Control	Tinhela	18.0	19.6	7.4	8.2	8.2	8.4	519	590
Tinhela	Tinhela	17.8	19.7	7.2	7.9	8.0	8.6	28	33
Control	Peliteira	17.6	19.7	7.3	8.1	8.4	8.0	515	610
Peliteira	Peliteira	17.7	19.3	6.9	7.6	8.0	8.2	77	93
Laboratory - 15ºC									
Tinhela	Tinhela	14.7	15.7	7.0	6.9	8.0	8.2	33	37
Peliteira	Peliteira	14.8	15.7	6.4	6.6	8.1	8.3	69	83

responses and heavy metal concentrations in water and sediment during laboratory bioassays conducted in 2003 (Zar 1996). The relationships between larval responses and heavy metal concentrations in water from the streams during *in situ* bioassays were not performed because the low number (n = 2) of water treatments used. Statistical analyses were performed using Minitab™ Release 14.

Results

In situ bioassays

The physical and chemical parameters of water in Tinhela river and Peliteira stream were similar in all years (2002-2004), with some exceptions (e.g. maximum temperature in 2004 was 9-10ºC higher in both streams) (Appendix 1). Generally, Peliteira stream showed lower values of pH and minimum temperature and higher conductivity than Tinhela river (Appendix 1).

In all years, several heavy metals were detected in water and mainly in sediment from the Peliteira stream (Appendix 2). In sediment high concentrations of Fe, As, Mn, Pb, and Zn and the presence of several other metals (Cd, Ni, Cr, and Cu) where observed, while in water moderate concentrations of Fe, As, Mn, and Zn and low concentrations of Cd and Ni were detected (Appendix 2). On the other hand, the concentrations of the most heavy metals (As, Zn, Pb, Ni, Cr, and Cu) detected in sediment from the Tinhela river, were very low (< 1.0 mg Kg^{-1}), except for Fe (161 ± 88 mg Kg^{-1}) and Mn (1.27 ± 0.72 mg Kg^{-1}), while in water, low concentrations of Mn and Fe were detected (Appendix 2).

Table 2. Physical and chemical parameters in control, Tinhela river and Peliteira stream water during laboratory bioassay, in spring 2004. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

	Temp (ºC)		pH		DO (mg L^{-1})		Cond ($\mu s\ cm^{-1}$)	
Treatments	min	max	day 0	day 6	day 0	day 6	day 0	day 6
Control								
NT	17.8	19.8	7.8	8.0	8.6	9.0	606	671
Tinhela								
NT	18.3	20.2	7.6	7.9	8.8	8.9	43	47
NTWF	18.6	19.8	7.6	8.0	8.4	8.2	28	37
DS	18.0	19.9	7.4	8.1	8.2	8.7	36	45
OMF	18.2	19.7	7.5	8.0	8.6	8.8	33	42
Peliteira								
NT	18.6	20.0	7.4	7.5	8.1	8.9	96	102
NTWF	18.2	19.8	7.3	7.6	9.0	9.2	91	97
DS	18.1	19.7	7.2	7.8	8.2	8.8	90	100
OMF	18.2	20.0	7.2	7.6	8.9	8.9	87	93

However, a decrease in the heavy metal concentrations was observed from 2002 to 2004 in the two rivers, but was higher in the Peliteira stream (Appendix 2). In the peliteira stream, it was observed a high decrease in the concentration of all heavy metals in sediment, while in water was only observed a decrease in the concentrations of As, Zn and Mn and an increase of Fe concentration from 2002 to 2003, followed by a decrease from 2003 to 2004 (Appendix 2). In the Tinhela river, the decrease of heavy metal concentrations was only observed in sediment (Appendix 2). The negative trend observed in heavy metal concentrations of the sediment was more pronounced between 2002 and 2003 than between 2003 and 2004 (Appendix 2).

Heavy metal concentrations in test chamber sediments, as determined from the additional chamber with field sediment, were similar to average metal concentrations found in the sediment from each stream, so we can assume that the caged larvae were exposed to the heavy metal concentrations of rivers sediment.

During *in situ* bioassays, development of *C. riparius* larvae was not affected by metal contamination, since all larvae change instar (from third to fourth instar) and there were no significant differences ($P > 0.05$) in head capsule width of larvae between the two streams and in each stream between years (Fig. 2).

On contrary, larval growth was strongly inhibited by high metal contamination (Fig. 2). High significant differences in growth of *C. riparius* larvae were found between Peliteira stream and Tinhela river in all years (ANOVA, 2002: $F_{1, 46} = 90.02$, $P < 0.001$; 2003: $F_{1, 42} = 71.58$, $P < 0.001$;

Table 3. Heavy metal concentrations determined in water and sediment at the end of laboratory bioassay, in spring 2004. n.d. = not detected.

Treatments	As	Zn	Cd	Pb	Ni	Mn	Cr	Fe	Cu
Water (mg L^{-1})									
Tinhela									
NT	n.d.	0.03	n.d.	n.d.	n.d.	0.08	n.d.	0.46	n.d.
NTWF	n.d.	0.02	n.d.	n.d.	n.d.	0.08	n.d.	0.69	n.d.
DS	n.d.	0.01	n.d.	n.d.	n.d.	0.05	n.d.	0.43	n.d.
OMF	n.d.	0.01	n.d.	n.d.	n.d.	0.04	n.d.	0.69	n.d.
Peliteira									
NT	1.54	0.11	n.d.	0.02	n.d.	1.48	n.d.	2.15	n.d.
NTWF	0.85	0.39	n.d.	0.04	n.d.	1.03	n.d.	3.26	n.d.
DS	0.74	0.11	n.d.	0.03	n.d.	0.96	n.d.	2.75	n.d.
OMF	1.63	0.24	n.d.	0.03	n.d.	1.10	n.d.	2.82	n.d.
Sediment (mg Kg^{-1})									
Tinhela									
NT	0.19	0.25	n.d.	0.05	0.07	0.66	0.12	143	0.04
NTWF	0.17	0.27	n.d.	0.06	0.08	0.75	0.09	128	0.03
DS	0.18	0.31	n.d.	0.06	0.08	0.84	0.09	127	0.03
OMF	0.19	0.28	n.d.	0.05	0.04	0.69	0.03	126	0.03
Peliteira									
NT	12.5	1.08	0.02	1.33	0.06	2.35	0.07	131	0.13
NTWF	12.7	0.91	0.02	1.67	0.05	2.16	0.08	132	0.13
DS	11.7	0.92	0.01	1.55	0.05	2.12	0.08	139	0.12
OMF	11.2	0.80	0.02	1.50	0.07	2.04	0.09	128	0.09
Detection limit (mg L^{-1})	0.025	0.005	0.001	0.015	0.005	0.001	0.005	0.005	0.01

2004: $F_{1, 47} = 73.12$, $P < 0.001$). From 2002 to 2004 it was observed a significant increase in larval growth in the Peliteira stream (ANOVA: $F_{2, 69} = 32.00$, $P < 0.001$) and in the Tinhela river (ANOVA: $F_{2, 69} = 4.29$, $P < 0.05$) (Fig. 2). In the Peliteira stream there were clearly significant differences in growth between all years (Fig. 2) (Tukey post hoc test: 2002 vs 2003: $P < 0.0001$, 2002 vs 2004: $P < 0.0001$, 2003 vs 2004: $P < 0.05$), while in the Tinhela river there were only significant differences in growth between 2002 and 2004 (Tukey post hoc test: $P < 0.05$). The increase in larval growth from 2002 to 2004 resulted in a significant decrease in growth inhibition (ANOVA: $F_{2, 69} = 21.57$, $P < 0.001$) between all years (Tukey post hoc test: 2002 vs 2003: $P < 0.001$, 2002 vs 2004: $P < 0.0001$, 2003 vs 2004: $P < 0.05$) (Fig. 2).

Laboratory versus *in situ* bioassays

As expected, the mean temperature in the *in situ* bioassays (Appendix 1) was similar to laboratory conditions at 15ºC, in 2003 (Table 1), and at 20ºC, in 2004 (Table 2), although its

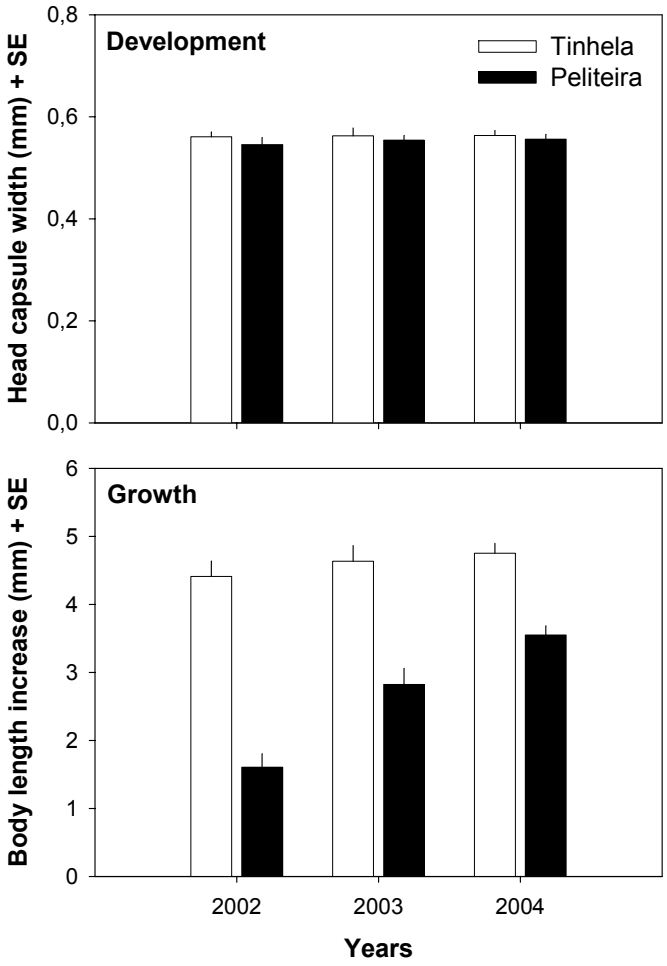

Fig. 2. Mean values and standard error of larvae biometrics in low (Tinhela) and metal contaminated (Peliteira) sites, during *in situ* bioassay in spring 2002, 2003 and 2004.

variability was always higher in field. Conductivity and DO values of water were similar during laboratory and *in situ* bioassays but pH values were higher during laboratory bioassays (Tables 2 and 3 and Appendix 1). As was observed in field, in the laboratory pH values were higher and conductivity values were lower in water of Tinhela stream than in water of Peliteira stream in 2003 (Table 1) and 2004 (Table 2).

In 2003 and 2004, the average concentrations of the most heavy metals in water were slightly higher during laboratory bioassays (Table 3 and Appendix 3) than during *in situ* bioassays (Appendix 2) in both streams. During laboratory bioassays some heavy metals, not detected during *in situ* tests, were detected in very low concentrations (Appendix 2), Zn in Tinhela river water, Pb and Cu in Peliteira stream water (Table 3 and Appendix 3). In sediment from both streams the average concentrations of the most heavy metals were slightly different during laboratory bioassays (Appendix 3 and Table 3) comparing with *in situ* bioassays (Appendix 2). As was observed for *in*

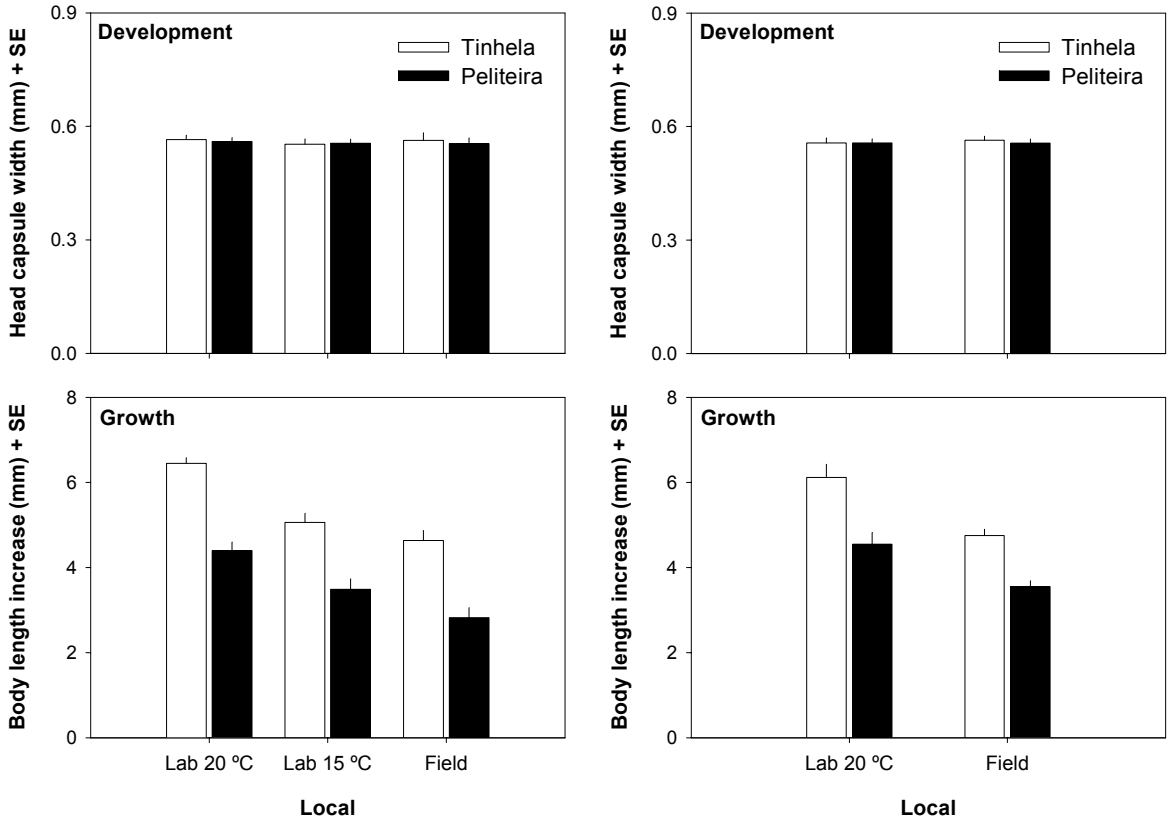

Fig. 3. Mean values and standard error of larvae biometrics in low (Tinhela) and high (Peliteira) metal contaminated treatments, during laboratory and *in situ* bioassays in spring 2003 (left) and 2004 (right).

situ tests (Appendix 2), in laboratory conditions higher concentrations of heavy metals were found in sediment than in water from both streams (Table 3 and Appendix 3).

In both years, in laboratory all larvae change instar (from third to fourth instar) and there were no significant differences ($P > 0.05$) in head capsule width of larvae between Peliteira stream and Tinhela river, as was observed *in situ*. No significant differences in head capsule width were found between field and laboratory conditions for both streams (Fig. 3).

A similar pattern of the differences in larval growth between Tinhela river and Peliteira stream was found in laboratory and *in situ* bioassays in 2003 and 2004, with lower growth found in the Peliteira stream (Fig. 3). As observed in the *in situ* bioassays, differences in larval growth between the two streams were highly significant in laboratory at 20ºC in both years (ANOVA: 2003: $F_{1, 41} = 68.65$, $P < 0.001$; 2004: $F_{1, 47} = 18.05$, $P < 0.001$) and in laboratory at 15ºC in 2003 (Anova: $F_{1, 48} = 41.88$, $P < 0.001$). In both years, however, higher larval growth was observed in laboratory at 20ºC than *in situ* tests (Fig. 3) in Tinhela river (ANOVA, 2003: $F_{1, 41} = 85.55$, $P < 0.001$; 2004: $F_{1, 48} = 20.64$, $P < 0.001$) and in Peliteira stream (ANOVA, 2003: $F_{1, 42} = 36.53$, $P < 0.001$; 2004: $F_{1, 46} = 26.44$, $P < 0.001$). Higher larval growth was also found in the laboratory

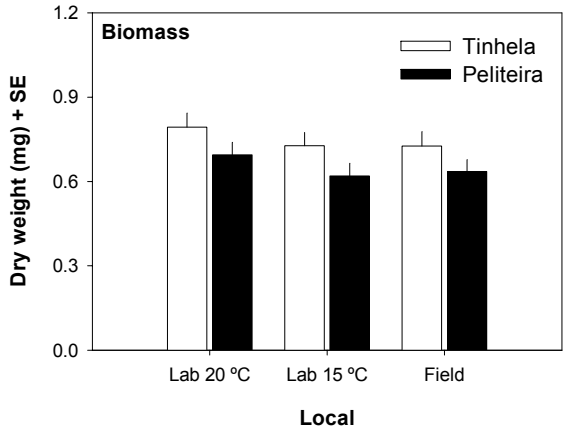

Fig. 4. Mean values and respective standard error of larval biomass (dry weight) in low (Tinhela) contaminated and high (Peliteira) metal contaminated treatments, during laboratory and *in situ* bioassays in spring 2003.

at 15º C than in the *in situ* bioassays, in 2003, in both streams (ANOVA, Tinhela river: $F_{1, 43} = 4.13$, $P < 0.05$; Peliteira stream: $F_{1, 44} = 6.07$, $P < 0.05$).

In 2003, as was observed with growth, a similar pattern of biomass of *C. riparius* larvae between Tinhela river and Peliteira stream was found in laboratory and *in situ* bioassays, with lower biomass found in the Peliteira stream (Fig. 4). The differences in biomass between the two streams were highly significant *in situ* (ANOVA: $F_{1, 42} = 10.78$, $P < 0.01$), in laboratory at 20ºC (ANOVA: $F_{1, 41} = 7.98$, $P < 0.01$) and in laboratory at 15ºC (ANOVA: $F_{1, 44} = 15.22$, $P < 0.001$). Larval biomass was significantly higher in the laboratory at 20ºC than in the *in situ* bioassays in both streams (ANOVA, Tinhela river: $F_{1, 41} = 24.46$, $P < 0.001$; Peliteira stream: $F_{1, 42} = 46.85$, $P < 0.001$) (Fig. 4), but no significant differences ($P > 0.05$) in larval biomass were found between the laboratory at 15ºC and *in situ* bioassays, for both streams (Fig. 4).

Relative effect of metals associated to water and sediment

During the laboratory bioassay at 20ºC in 2003, the physical and chemical conditions of the water were similar in all treatments, with the exception of higher values of pH and conductivity in all treatments with control water (Table 1).

In the laboratory bioassay, the highest heavy metal concentrations were found in Peliteira stream sediment and the lowest were observed in all treatments with control sediment. However, for each type of sediment, heavy metal concentration was lower with control water and higher with Peliteira stream water (Appendix 3). The highest concentrations of metal in water were found in treatments with the highest concentrations of metal in sediment (Appendix 3). *In situ*, higher heavy

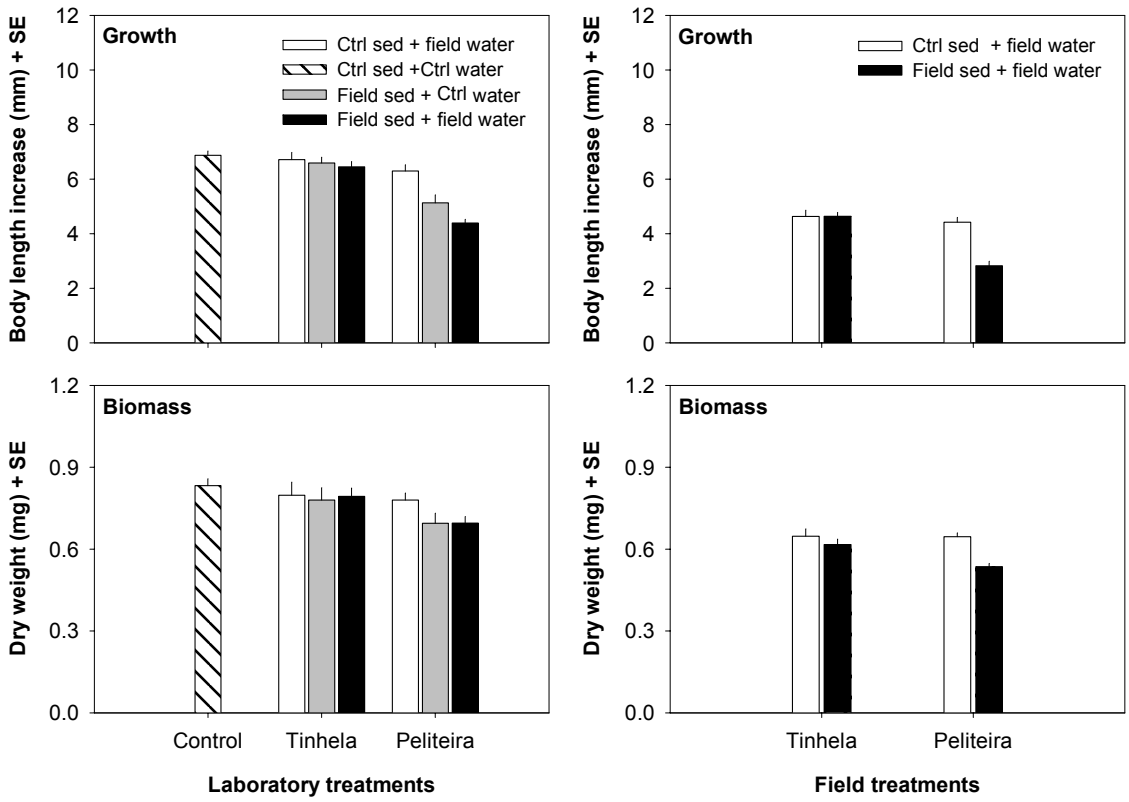

Fig. 5. Mean values and standard error of larvae growth and biomass in control, Tinhela (low contaminated) and Peliteira (high contaminated) treatments during laboratory (left) at 20 ºC and *in situ* (right) bioassays, in spring 2003. Ctrl = control; sed = sediment.

metal concentrations were observed in control sediment in Peliteira stream than in Tinhela river at the end of bioassay (Appendix 2).

In Peliteira stream, larval growth and biomass were clearly and significantly higher in the control sediment than in the stream sediment (ANOVA, growth: $F_{1, 44} = 44.76$, $P < 0.001$; biomass: $F_{1, 44} = 33.95$, $P < 0.001$) (Fig. 5). In contrast, in Tinhela river no significant differences ($P > 0.05$) were found in both endpoints between the control sediment and the river sediment (Fig. 5). Although growth and biomass were significantly (growth: $P < 0.001$; biomass: $P < 0.01$) lower in Peliteira stream than in Tinhela river when the streams sediment was used, no significant differences ($P > 0.05$) were found in these endpoints between the two streams when control sediment was used (Fig. 5).

Similar results were observed in the laboratory bioassay. Growth and biomass were significantly (growth: $P < 0.001$; biomass: $P < 0.01$) lower in Peliteira stream than in Tinhela river when the sediment from the streams was used, but no significant differences ($P > 0.05$) in these endpoints were found between the two streams when control sediment was used (Fig. 5). In the laboratory, growth was higher in the control treatment than in all other treatments (Fig. 5), but

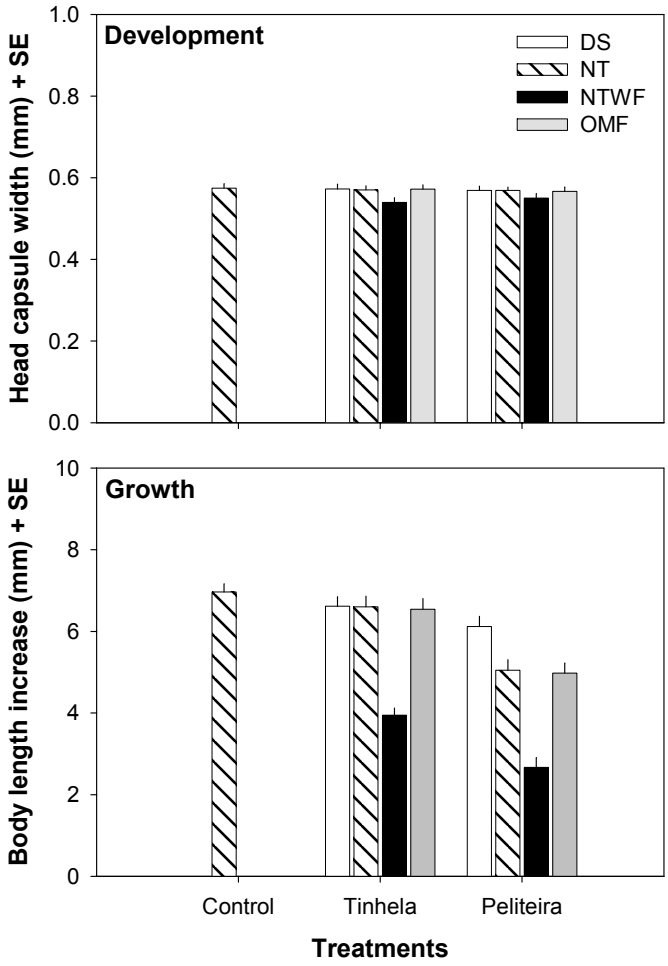

Fig. 6. Mean values and standard error of larvae biometrics in control, low (Tinhela) and high (Peliteira) metal contaminated treatments with dry and sieved (DS), not treated (NT), not treated without added food (NTWF) and organic matter free (OMF) sediments, during laboratory test in spring 2004.

significant differences in this endpoint (ANOVA: $F_{6, 158} = 20.27$, $P < 0.001$) were only found between the control treatment and the treatments with Peliteira stream sediment (with control water or Peliteira stream water) (Dunnett post hoc tests: $P < 0.0001$) and between control treatment and the treatment with control sediment and Peliteira stream water (Dunnett post hoc test: $P < 0.05$). Growth was also significantly higher in control water than in Peliteira stream water in treatments with Peliteira stream sediment (ANOVA: $F_{1, 45} = 6.69$, $P < 0.05$). Biomass was also higher in the control treatment than in all other treatments (Fig. 5), but significant differences in this endpoint (ANOVA: $F_{6, 154} = 3.15$, $P < 0.01$) were only found between the control treatment and the treatments with Peliteira stream sediment (Dunnett post hoc tests: $P < 0.01$).

Linear regression analyses indicated that both endpoints, growth and biomass, were significantly ($P < 0.05$) negatively related ($r^2 > 0.80$) to As, Zn, Cd, Pb, Mn and Cu concentrations

in the treatments sediment and were highly significantly negatively related ($r^2 > 0.91$, $P < 0.05$) to the Cd concentration in the sediment of the Peliteira stream during laboratory bioassay. Stepwise multiple regressions indicated that 99.9% of growth and biomass variation between treatments could be explained by variation in Mn and Cu concentrations (body length increase = 5.22 - 1.20 Mn + 2.48 Cu, $P < 0.01$) and in Fe and Cu concentrations (dry weight = 0.779 - 0.000615 Fe - 0.299 Cu, $P < 0.05$), respectively, in sediment between treatments during *in situ* bioassay. Stepwise multiple regressions indicated that 97.9% of growth and biomass variation between treatments could be explained by variation in As concentrations (body length increase = 6.53 - 0.140 As, $P < 0.001$) and in As and Mn concentrations (dry weight = 0.810 - 0.00331 As - 0.0375 Mn, $P < 0.001$), respectively, in sediment between treatments during laboratory bioassay.

Linear regression analyses indicated that growth and biomass, were significantly ($P < 0.05$) negatively related ($r^2 \geq 0.80$) to As and Mn, and growth also to Fe, concentrations in the treatments water and were highly significantly negatively related ($r^2 > 0.91$, $P < 0.01$) to the Pb concentration in the water of the Peliteira stream during laboratory bioassay. Stepwise multiple regressions indicated that 99.4% of growth variation between treatments could be explained by variation in As concentrations (body length increase = 6.61 - 1.98 As, $P < 0.001$) in water between treatments and 97.6% of biomass variation between treatments could be explained by variation in As and Mn concentrations (dry weight = 0.787 + 0.220 Mn - 0.265 As, $P < 0.01$) in water between treatments, during laboratory bioassay.

Importance of ingested material in metal toxicity

As was observed in 2003, in the laboratory bioassay in 2004, physical and chemical conditions were very similar, with the exception of higher values of pH and conductivity in all treatments with control water (Table 2).

Heavy metal concentrations in water and sediment of Tinhela river were similar on all treatments (Table 3). The same was observed in water and sediment of Peliteira stream, with the exception of As, which was slightly higher in water with NT sediment and OMF sediment, and for Mn that was slightly higher in water with NT sediment (Table 3). However, heavy metals concentrations in water and sediment of Peliteira stream were higher than in water and sediment of Tinhela river (Table 3).

In the laboratory bioassay in 2004, all larvae changed from the third instar to the fourth instar in all treatments. However, significant differences in head capsule width of larvae were observed between different treated sediments from Tinhela river (ANOVA: $F_{3, 96} = 6.72$, $P < 0.001$) and Peliteira stream (ANOVA: $F_{3, 95} = 6.26$, $P < 0.01$). Larval development (head capsule width) was significantly lower in NTWF sediments than in the other (NT, DS, OMF) sediments (Tukey post

hoc tests: $P < 0.001$), but no significant ($P > 0.05$) differences in larval development were found between NT, DS and OMF sediments (Fig. 6). No significant ($P > 0.05$) differences were found in larval development in all treated sediments between Tinhela river and Peliteira stream (Fig. 6). Compared to the control (Anova, $F_{8, 215} = 5.37$, $P < 0.001$), larval development was significantly lower in NTWF sediment from the Tinhela river (Dunnett post hoc test, $P < 0.001$) and from the Peliteira stream (Dunnett post hoc test, $P < 0.001$) (Fig. 6).

As was observed with development, significant differences were found in larval growth between different treated sediments from Tinhela river (ANOVA: $F_{3, 96} = 31.94$, $P < 0.001$) and Peliteira stream (ANOVA: $F_{3, 95} = 34.65$, $P < 0.001$) Growth was significantly lower in NTWF sediments than in NT, DS and OMF sediments (Tukey post hoc tests: $P < 0.0001$) (Fig. 6). Growth in DS sediment from Peliteira stream was significantly higher than in NT sediment (Tukey post hoc tests: $P < 0.05$) and in OMF sediment (Tukey post hoc tests: $P < 0.01$) from the same stream (Fig. 6). However, growth in NT, DS, OMF sediments from Tinhela river were not significantly different from each other ($P > 0.05$). There were similar high significant differences in larval growth in NT, OMF and NTWF sediments between Tinhela river and Peliteira stream (ANOVA, NT: $F_{1, 47} = 18.05$, $P < 0.001$; OMF: $F_{1, 48} = 19.06$, $P < 0.001$; NTWF: $F_{1, 48} = 18.47$, $P < 0.001$), with lower values found in Peliteira stream, but there were no significant differences in larval growth in DS sediment between the two streams ($P > 0.05$) (Fig. 6). Compared to the control (Anova: $F_{8, 215} = 37.83$, $P < 0.001$), growth was significantly lower in all sediments from the Peliteira stream (Dunnett post hoc tests, NT: $P < 0.0001$; NTWF: $P < 0.0001$; OMF: $P < 0.0001$; DS: $P < 0.05$) and in NTWF sediment from the Tinhela river (Dunnett post hoc test, $P < 0.0001$) (Fig. 6).

Discussion

In situ bioassays

In the Peliteira stream were observed higher concentrations of heavy metals than in Tinhela river, as was expected, since its drainage basin receives the acid mine drainage from the Jales mine and until 2002 received input residues from the open air deposit, while Tinhela river is located upstream of any contaminated tributary stream, although underground water and atmospheric contamination can occur. The higher concentrations of heavy metals found in sediment than in water in rivers was also expected because heavy metals dissolved in the water of acid mine drainage may precipitate when they reach aquatic environments, due to the increase of pH (DeNicola and Stapleton 2002), and because heavy metals that enter aquatic environments can bind to the surface of suspended particles in the water column or may settle into the sediment (Simkiss

et al. 2001). As a result of this, the concentration of contaminants in the sediment is expected to be much higher than in water (Simkiss et al. 2001; DeNicola and Stapleton 2002).

A decrease in heavy metal concentrations in sediment in the Peliteira stream and, to a lesser extent, in the Tinhela river, was observed from spring 2002 to spring 2004. This decrease was probably due to a decrease input of heavy metals to these rivers, as a result of the environmental rehabilitation program of the open air mine residue deposit in Jales (June 2002 - June 2003). More precisely, the decrease of input of heavy metals in rivers was due to the embankment of mine residues and its cover with an impermeable geomembrane of high density polystyrene, performed to avoid spreading of contaminated particles as was mencioned above, the entrance of water and the heavy metals lixiviation, and also due to the drainage systems that made possible the treatment of lixiviated water. The decrease in heavy metals concentrations was higher in Peliteira stream than in the Tinhela river because the Peliteira stream is directly affected by open air mine residue deposits. So it was expected that the environmental rehabilitation program of the open air mine residue deposit had more impact on the Peliteira stream than on the Tinhela river.

The decrease input of heavy metals to streams initially improved water quality, which was followed by a release of heavy metals from sediment to water resulting in an improvement of sediment quality. DeNicola and Stapleton (2002) suggested that precipitate deposited on the substrate, from acid mine drainages inputs, could be the source of aqueous metals. This could explain why it was only observed an improvement in chemical quality of sediment while no improvement in chemical quality of water in the streams was observed. The decrease in heavy metals concentration in rivers sediment was higher between spring 2002 and spring 2003 than between spring 2003 and spring 2004, probably due to the fact that in December 2003 the embankment of mine residues and its cover with an impermeable geomembrane was already finished, resulting in the reduction of the input of heavy metals to environment (including Peliteira stream and Tinhela river), and after that, the reducing of heavy metals in sediment was probably due to an improvement of water quality, followed by a release of metals from sediment to water.

The significant increase in growth of *C. riparius* larvae in the Peliteira stream observed from 2002 to 2004, which resulted in a significant decrease in the larval growth inhibition (Fig. 2), was mostly likely due to the decrease of the metal contamination that was observed during this period (Appendix 2), since the decrease in the inhibition of larval growth had a similar pattern that the decrease of heavy metal concentrations in the Peliteira sediment (Appendix 2) during the study period, were higher from 2002 to 2003 than from 2003 to 2004 (Fig. 2). Furthermore, the increase in larval growth observed in the Tinhela river (where the decrease of heavy metals concentration was smaller) was lower and less significant than in Peliteira stream.

The decrease in the growth inhibition of *C. riparius* larvae in the Peliteira stream as a consequence of decrease of metal contamination in this river indicates that the improvement in chemical quality of the sediment resulted in an improvement in biological quality of the river. However, at the end of the study an inhibition in larval growth was still observed, probably due to the metal contamination in sediment that persisted in the stream, suggesting that in 2004 there was still an ecological impairment of the Peliteira stream. These results suggest that growth of *C. riparius* larvae was able to detect differences in metal contamination between rivers and also detected alterations in metal contamination of rivers during and after the rehabilitation program. Furthermore, the effects of metal contamination in rivers on the growth of *C. riparius* larvae allows the assessment of the biological effects of this contamination and, thus, can indicate the water quality of the rivers.

The results suggested that the environmental rehabilitation program of the open air mine residue deposit in Jales resulted in an improvement on the chemical quality of the aquatic environment principally in the Peliteira stream, but also in Tinhela river, which in turn resulted in some improvement of water quality in the Peliteira stream. However, the rehabilitation of open air mine residues deposits was not sufficient to eliminate contamination of the Peliteira stream sediment and the ecological impairment of the Peliteira stream, since the source of sediment contamination in this river was also the acid mine drainage input from mine galleries. So, to achieve the chemical and biological recovery of sediment and the ecological recovery of Peliteira stream, other remediation efforts, such as the treatment of the acid mine drainage from the mine galleries, are also needed. Treatment of the acid mine drainage can be performed by passive treatment systems described by DeNicola and Stapleton (2002). According to DeNicola and Stapleton (1999) and Milavec (2000), the passive treatment systems of acid mine drainage can be extremely effective in improving the chemical quality of water of the aquatic ecosystems.

Laboratory versus *in situ* bioassays

The overall physical and chemical conditions in water were similar during laboratory and *in situ* bioassays in 2003 and 2004, but lower values of pH and high variability in physical conditions, principally in temperature, were observed during *in situ* bioassays for all treatments. In 2003, the average temperature in field was lower than the average temperature during laboratory bioassay at 20º C. The small differences in the heavy metal concentrations of the sediment and water between laboratory and *in situ* bioassays, possibly caused by sampling-related artefacts (handling, transport and storage of samples) or by the occurrence of higher pH values in laboratory, indicate that the bioavailability of heavy metals was slightly altered. In spite of these differences in physical and chemical conditions and in metal contamination, a similar pattern of biological responses of *C.*

riparius larvae was found in laboratory and in *situ* bioassays and no differences in toxicity of metal contamination to *C. riparius* larvae were observed between the two exposure environments in both years (2003 and 2004) (Fig. 3 and 4), which gives evidence for comparability of the two designs. DeWitt et al. (1999) also found comparable results between field and laboratory tests in survival of the amphipods *Chaetocorophium* cf. *lucasi* exposed to cadmium. On contrary, in Castro et al. (2003), the toxicity of metal contamination to body length and dry weight *C. riparius* larvae observed during *in situ* bioassays was not detected in laboratory bioassays. However, in our study water and sediment samples collected *in situ* were immediately used or were used 24 hours after their collection in laboratory bioassay, while in Castro et al. (2003) study, the water and sediment samples collected *in situ* were stored for a week before they were used in the laboratory bioassay, which could cause higher alteration in bioavailability of heavy metals. These findings suggest that bioassays conducted in laboratory can indicate with high reliability the *in situ* toxicity of metal contamination to *C. riparius* larvae, if water and sediment collected from study sites are used shortly after their collection in laboratory bioassays. However, the *in situ* bioassays integrate biological and physical and chemical processes that can not be reproduced in the laboratory (Chappie and Burton 1997). So, if site-specific ecotoxicological information is needed, the *in situ* bioassays should preferable be used.

Higher and significant growth and biomass of *C. riparius* larvae was observed during laboratory bioassays at 20ºC than during *in situ* bioassays, in 2003 and 2004. Similar results were found by Castro et al. (2003) that also found lower body length and dry weight *in situ* than in laboratory bioassays at 20ºC. In 2003, higher growth, but less significant, was also observed during laboratory at 15ºC than during *in situ* exposure, while no differences in biomass was observed between the two exposure environments. The high significant growth and biomass found during laboratory bioassays at 20ºC comparing with *in situ* bioassays, can be explained by the optimal conditions (e.g. higher average temperature in 2003 and pH values in 2003 and 2004) in laboratory for growth of *C. riparius* larvae (Tables 1 and 2 and Appendix 1). The similar biomass between laboratory at 15ºC and *in situ* bioassay can be explained by the similar average temperature, pH values, dissolved oxygen and conductivity found in both exposure environments, while higher growth observed in laboratory at 15ºC (2003) than *in situ* exposure could be a consequence of lower fluctuation of physical and chemical conditions in laboratory (Table 1 and Appendix 1). It is clear that the more similar the conditions in laboratory are to *in situ* conditions more similar would be the biological responses of *C. riparius* larvae between the laboratory and *in situ* tests. But, since the water physical and chemical parameters of rivers are highly variable and since the differences in biological responses between the two environments do not seem to implicate differences in toxicity, the standard temperature of 20ºC is preferable to compare results between studies.

Relative effect of metals associated to water and sediment

In the experiments conducted in 2003, higher concentrations of heavy metals in water were observed in the treatments with, also, higher concentrations of heavy metals in sediment (Appendixes 2 and 3). This can be explained by the release of heavy metals from sediment to the water as was suggested by DeNicola and Stapleton (2002).

The toxicity of metal contaminated sediment to *C. riparius* larvae was clearly evidenced by significantly lower larval growth and biomass in Peliteira stream sediment (high contaminated) compared with Tinhela river (low contaminated) and control sediments, for either type of water (field or control water), in laboratory and *in situ* bioassays. Differences in biomass of larvae between treatments with different sediment could be due to differences in larval gut contents, caused by differences in particle size and organic matter of sediments, and not to differences in contamination in sediments. Sibley et al. (1997b) verified that differences in dry weight of chironomids larvae can be due to differences in larval gut contents, caused by differences in particle size and organic matter of sediments, and not to differences in toxicity. However, linear regression analyses and stepwise multiple regressions indicated that both biomass and growth were significantly negatively related to the concentrations of almost all heavy metals in the treatments sediment, and growth was mostly affected by Mn and Cu concentrations *in situ* and by As concentrations in laboratory, while biomass was mostly affected by Fe and Cu concentrations *in situ* and As and Mn concentrations in laboratory. Both endpoints were also highly affected by the Cd concentration in the sediment of the Peliteira stream. These results indicate that both endpoints, growth and biomass, are highly affected by metal contamination in sediment.

Larval growth seemed to be less sensitive to metal contamination in water than in sediment, since no significant differences were found between Peliteira stream and Tinhela river, when control sediment was used, in both laboratory and in *situ* bioassays, although low but significant differences in growth were found in laboratorial conditions between treatments with Peliteira stream water and control water when Peliteira sediment or control sediment were used, growth was only negatively related to As, Mn and Fe concentrations in the treatments water, but was mostly affected by As concentrations in treatments water and was also highly affected by Pb concentration in water of Peliteira stream. This confirms that growth was also affected by metal contamination in water but was less sensitive to water contamination than to sediment contamination. Larval biomass did not seem to be affected by metal contamination in water, since no significant differences were found for this endpoint between treatments with the same sediment and different types of water (control, low and high contaminated). However, biomass was highly affected by As and Mn concentrations in treatments water and by Pb concentration in water of Peliteira stream. These results suggest that although larval biomass was affected by heavy metals in water was low

sensitive to metal contamination in water.

The high sensitivity of *C. riparius* larvae to metal contamination in Peliteira stream sediment was expected since chironomids larvae are benthic (bottom-dwelling) organisms (i.e. live in tubes that they build in the sediments) and are collector-gatherers (feed on detritus deposited on sediment) (Vos 2001). Furthermore, due to its high sensitivity to contaminants in sediment, chironomids (i.e. *Chironomus tentans* and *Chironomus riparius*) are among the test species recognized as useful tools in studies of sediment toxicity (Ankley et al. 1994).

The lower but significant sensitivity of *C. riparius* larvae to metal contamination in the Peliteira stream water could be expected because chironomids can be found in the surface sediment layer (van de Bund 1994) and, thus, could be in contact with contaminants in water. However, the tubes that chironomids larvae build for protection against predators, apart their role in feeding, respiration and as anti-predation shelter (Hershey 1987; Macchiusi and Baker 1992), serve as chemical shields, protecting the larvae from water contaminants, such as copper or chloramine (Halpern et al. 2002). The degree of protection of shelter against contaminants, however, depends on sediment characteristics. Halpern et al. (2002) found that silt tubes have higher protective value than sand tubes to *Chironomus luridus* when exposed to copper. Since the sediment of Peliteira stream has 64.1% sand and only 0.2% of silt and clay, as determined in other study (Chapter 1), the tubes of *C. riparius* larvae had a low protective value against heavy metals and so the larvae was probably highly exposed to heavy metals dissolved in water. Stuijfzand et al. (2000) observed that in organic enriched rivers, organic matter stimulated growth of *C. riparius* larvae at such an extent that effects of high toxicant concentrations of metals in water to larval growth were reduced or neutralized. These findings suggest that the bioavailability and/or the toxicity of heavy metals to chironomids are influenced by organic matter and particle size of sediment, which can be confounding factors to assess metal contamination in water.

These findings suggest that growth and biomass of the *C. riparius* larvae bioassay can be used to assess metal contamination in the rivers sediment and its toxicity effects. Since the results showed that growth of the *C. riparius* larvae is also affected by metal contamination in water, we can assume that growth of the *C. riparius* larvae bioassay can also be used to assess metal contamination in the rivers water, which, however, depends on organic matter and particle size of sediment.

Importance of ingested material in metal toxicity

In the experiments conducted in laboratory in 2004, significantly higher growth was found in the treatments with dry and sieved sediment (DS), where ingestion of material (except added food), was prevented and since no significant differences were found in growth of larvae in this treatment

between high (Peliteira) and low (Tinhela) contaminated streams, we can assume that the most important toxicity to *C. riparius* larvae was caused by metals that enter the organism through ingested material from sediment. Nevertheless, dissolved metals in water, that enter organism by transport of dissolved ions across external membranes and/or adsorption on body surfaces, although less important, seem to contribute to overall metal toxicity since low but significant differences were found between treatment with water and DS sediment from Peliteira stream and control treatment. These results confirm and explain why *C. riparius* larvae are more sensitive to metal contamination in rivers sediment than in rivers water.

The fact that no differences were found in larval growth between the treatments with not treated sediment (NT) and organic free sediment (OMF), in both rivers, seems to indicate that heavy metals do not enter the organism associated with organic matter. However, since food is better assimilated by chironomids if it is available in the surface than if it is mixed with the sediment (Naylor and Rodrigues 1995), the food that was provided to *C. riparius* larvae in surface of NT sediment could be preferably consumed by larvae than the organic matter mixed with sediment, as suggested by Pery et al. (2003c). Consequently, if heavy metals entered the organism associated to organic matter they would had a low effect on the larval growth, due to its low bioavailability to larvae. This could explain the lack of significant differences between treatments and, thus the results do not necessarily mean that metals do not enter the organism associated with organic matter. However, given that no differences in toxicity were observed between the two streams for treatments with not treated sediment with added food (NT) and for treatments with not treated sediment without added food (NTWF), where the only source of food was the organic matter of sediment, the toxicity of heavy metals that enter the organism associated with organic matter (food) did not seem important. Therefore, the main toxicity to *C. riparius* larvae was caused by metals that enter the organism through ingested sediment associated with sediment particles.

These results imply that in sediments with high percentage of particles bigger than 250 μm, which are not ingested by *C. riparius* larvae, such as medium sand, coarse sand or gravel (Doeglas 1968; Larsonneur 1977), the ingestion of sediment along with organic matter and, consequently, ingestion of metals associated to sediment would be low. This would result in a low toxicity effect of heavy metals to *C. riparius* larvae due to low bioavailability of heavy metals and not because the heavy metal concentrations in sediment are not toxic to *C. riparius* larvae. So, in that type of sediments the toxicity of metal contamination to *C. riparius* larvae can be underestimated due to sediment characteristics. These results also imply that in metal contaminated and organic enriched sediment the effects of metal contamination on *C. riparius* larvae may be reduced because organic matter may reduce the inhibitory toxicity effects of heavy metals to *C. riparius* larvae by reducing the bioavailability of heavy metals associated to sediment particles, but also by serving as superior

(high nutrient value) food resource, since the mineral particles of sediment ingested along with organic matter is reduced. This may stimulate larval growth, for example, as was observed by Stuijfzand et al. (2000) in water-only toxicity tests, mentioned above. Furthermore, since collectors-gatherers, such as *C. riparius* larvae, maximize ingestion rate in order to maximize food intake (Cummins and Klug 1979), in more organic enriched sediments, food resources with higher high nutrient value, it could be expected a lower larval ingestion rate and, thus, a lower ingestion of sediment particles and heavy metals associated to them, resulting in a lower toxicity of metal contaminated sediments to *C. riparius* larvae. Castro et al. (2003) observed that growth of *C. riparius* larvae was less reduced in metal contaminated sediments with high organic matter than with low organic matter content. Thus the bioavailability and/or toxicity effects of heavy metals to *C. riparius* larvae depends of organic matter and particle size of sediment, which can be confounding factors to assess metal contamination in sediment as was observed for contamination in water. Ristola et al. (1999) and Pery et al. (2003c) measured growth and emergence of *C. riparius* larvae exposed to four unpolluted natural sediments, in laboratory, and also found some evidences that organic mater content and size particle of sediment may be confounding factors in sediment toxicity tests with *C. riparius* larvae.

Lower growth and development in treatments with no added food compared with treatments in which food was added, was expected and can be explained by the food limitation. During bioassays with *C. riparius* larvae food privation inhibit larval growth and development (Ristola 2000; Pery et al. 2003c), principally if the organic matter content of sediment is low, as the case of Peliteira stream and Tinhela rivers, or have a low nutrient value (Pery et al. 2003c).

Conclusions

During the environmental rehabilitation of the open air mine residue deposit a decrease of metal contamination in rivers was observed. This trend was also observed on the inhibition of growth of *C. riparius* larvae. This supports the use of the bioassay with *C. riparius* larvae using growth as the endpoint to biomonitor metal contamination in rivers.

The results indicate that growth and biomass of *C. riparius* larvae bioassay were affected mostly by metal contamination on sediment because the most important route of metal entrance in the organism is through ingested material associated to sediment particles. This suggests that these endpoints can be used to assess biological quality of river sediments. To a lesser extent, growth of *C. riparius* larvae was also affected by metal contamination in water and thus, may be used to assess quality of water column in metal contaminated rivers. However, the organic matter content and particle size of the sediment need to be taken into account because they can be confounding factors on the assessment of water and sediment quality in metal contaminated rivers.

Laboratory bioassays can indicate with high reliability the *in situ* toxicity of metal contamination to biological parameters of *C. riparius* larvae, but *in situ* bioassay should be used when site-specific ecotoxicological information is needed.

Acknowledgements

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Appendix 1. Physical and chemical parameters observed in Tinhela river and Peliteira stream water in the beginning (day 0) and at the end (day 6) of *in situ* bioassay in spring 2002- 2004. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

		Temp (ºC)		pH		DO (mg L^{-1})		Cond (μs cm^{-1})		Nitrate (mg L^{-1})		Phosphate (mg L^{-1})	
	Rivers	min	max	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6
2002													
	Tinhela	14	17	6.7	6.6	8.0	6.6	34	34	0.20	0.53	0.02	0.02
	Peliteira	12	17	6.2	6.2	8.5	8.6	115	128	0.30	0.67	0.02	0.02
2003													
	Tinhela	14	17	6.4	6.7	8.6	8.0	20	27	0.3	0.3	0.06	0.06
	Peliteira	12	18	6.3	6.2	8.8	9.4	84	73	0.3	0.4	0.06	0.07
2004													
	Tinhela	13	26	6.7	6.9	8.7	8.5	34	29	0.4	0.3	0.04	0.06
	Peliteira	12	27	6.3	6.6	8.2	7.5	90	81	0.2	0.2	0.06	0.07

Appendix 2. Mean concentrations and standard error (in parenthesis) of heavy metals in streams during field exposure in spring 2002-2004, and heavy metals concentrations in field and control sediment (sed) from chambers at the end of bioassays. n.d. = not detected; n.a. = not analysed.

	As	Zn	Cd	Pb	Ni	Mn	Cr	Fe	Cu
Water (mg L^{-1})									
Tinhela									
2002	n.a.	n.a.	n.d.	n.a.	n.d.	0.03	n.d.	0.22	n.d.
2003	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 (0.001)	n.d.	0.24 (0.03)	n.d.
2004	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 (0.01)	n.d.	0.43 (0.13)	n.d.
Peliteira									
2002	n.a.	1.16	0.01	n.a.	0.01	1.11	n.d.	0.18	n.d.
2003	0.24 (0.16)	0.95 (0.03)	0.01 (0.0003)	n.d.	0.01 (0.0003)	0.86 (0.03)	n.d.	1.26 (0.96)	n.d.
2004	0.09 (0.06)	0.06 (0.01)	0.01 (0.0003)	n.d.	n.d.	0.43 (0.004)	n.d.	0.59 (0.41)	n.d.
Sediment (mg Kg^{-1})									
Tinhela									
2002	0.35 (0.1)	0.49 (0.08)	n.d.	0.17 (0.2)	0.21 (0.06)	2.07 (1.91)	0.20 (0.04)	256 (19.9)	0.10 (0.02)
2003	0.21 (0.04)	0.25 (0.04)	n.d.	0.07 (0.02)	0.07 (0.01)	1.04 (0.55)	0.09 (0.01)	146 (7.77)	0.05 (0.02)
2003-chamber (field sed)	0.18	0.22	n.d.	0.04	0.06	0.55	0.06	84.1	0.03
2003-chamber (control sed)	n.d.	0.02	n.d.	n.d.	n.d.	0.50	n.d.	48.6	n.d.
2004	0.13 (0.01)	0.24 (0.03)	n.d.	0.07 (0.01)	0.06 (0.01)	0.69 (0.09)	0.06 (0.01)	82.0 (6.10)	0.03 (0.002)
2004-chamber (field sed)	0.13	0.24	n.d.	0.06	0.07	0.74	0.09	130	0.05
Peliteira									
2002	29.1 (3.36)	3.64 (0.08)	0.07 (0.02)	4.12 (1.51)	0.38 (0.03)	4.13 (0.77)	0.36 (0.06)	492 (37.0)	0.20 (0.01)
2003	17.7 (1.93)	1.21 (0.03)	0.02 (0.002)	2.05 (0.39)	0.07 (0.01)	2.31 (0.18)	0.08 (0.01)	156 (22.0)	0.15 (0.003)
2003-chamber (field sed)	17.9	1.40	0.02	1.90	0.10	2.35	0.08	150	0.17
2003-chamber (control sed)	0.09	0.33	n.d.	n.d.	n.d.	0.66	n.d.	58.1	n.d.
2004	11.4 (0.99)	0.96 (0.12)	0.02 (0.002)	1.22 (0.08)	0.05 (0.01)	2.56 (0.59)	0.07 (0.01)	173 (22.50)	0.11 (0.01)
2004-chamber (field sed)	11.6	1.17	0.02	1.15	0.06	2.53	0.08	166	0.08
Detection limit (mg L^{-1})	0.025	0.01	0.005	0.025	0.01	0.001	0.01	0.01	0.01

Appendix 3. Mean concentrations and standard error (in parenthesis) of heavy metals in water and sediment during laboratory exposure in spring 2003. n.d. = not detected.

Water	Sediment	As	Zn	Cd	Pb	Ni	Mn	Cr	Fe	Cu
Water (mg L^{-1})										
Tinhela	Control	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 (0.01)	n.d.	0.48 (0.15)	n.d.
Peliteira	Control	0.17 (0.04)	0.79 (0.17)	0.004 (0.004)	n.d.	0.01 (0.0003)	0.60 (0.18)	n.d.	0.59 (0.28)	n.d.
Control	Tinhela	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 (0.01)	n.d.	0.44 (0.31)	n.d.
Tinhela	Tinhela	0.03 (0.01)	0.01 (0.004)	n.d.	n.d.	n.d.	0.02 (0.01)	n.d.	0.56 (0.47)	n.d.
Control	Peliteira	0.72 (0.45)	0.41	n.d.	0.02 (0.01)	0.01 (0.001)	0.16 (0.01)	n.d.	0.97 (0.15)	n.d.
Peliteira	Peliteira	1.13 (1.01)	0.91 (0.17)	0.01 (0.003)	0.02 (0.02)	0.01 (0.001)	0.93 (0.60)	n.d.	3.18 (2.53)	0.01 (0.001)
Sediment (mg Kg^{-1})										
Tinhela	Control	n.d.	n.d.	n.d.	n.d.	n.d.	0.54 (0.02)	n.d.	60.4 (4.35)	n.d.
Peliteira	Control	0.09 (0.01)	0.03 (0.01)	n.d.	0.001 (0.0004)	n.d.	0.56 (0.06)	n.d.	60.1 (4.13)	n.d.
Control	Tinhela	0.10 (0.02)	0.29 (0.03)	n.d.	0.06 (0.02)	0.07 (0.02)	0.60 (0.01)	0.07 (0.01)	98.2 (13.1)	0.04 (0.01)
Tinhela	Tinhela	0.16 (0.02)	0.33 (0.02)	n.d.	0.08 (0.01)	0.10 (0.003)	0.62 (0.001)	0.09 (0.01)	98.4 (14.2)	0.05 (0.01)
Control	Peliteira	10.0 (1.49)	0.80 (0.11)	0.02 (0.003)	1.15 (0.18)	0.05 (0.01)	2.18 (0.64)	0.05 (0.01)	153 (4.42)	0.10 (0.01)
Peliteira	Peliteira	15.1 (0.91)	1.44 (0.07)	0.02 (0.01)	1.78 (0.36)	0.04 (0.002)	2.73 (0.05)	0.06 (0.004)	160 (5.84)	0.12 (0.01)

Chapter 5

Application of an *in situ* bioassay with *Chironomus riparius* larvae to assess ecological impact of metal contamination in rivers. The case of a tungsten mine in Portugal

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Abstract

The mining industry often causes environmental damage due to its chemical impact. In this study we evaluate the ability of an *in situ* bioassay with *Chironomus riparius* larvae, using larval development and growth as endpoints, to assess ecological impact in metal contaminated rivers caused by a tungsten mine in Portugal. For this purpose the bioassay was performed in specific sites in rivers along the contamination gradient, allowing us to analyse also the performance of the acid mine drainage (AMD) treatment station. During the *in situ* bioassay high concentrations of several heavy metals were found in the stream that receives acid drainage from the mine and treated water from the AMD treatment station, even in sites far downstream from the mine and the treatment station. Low level of contamination was observed in a main river near an open air mine residue deposit. Growth and development of *C. riparius* larvae were highly inhibited by the high metal contamination in the stream sites located upstream and downstream the AMD treatment station. Low metal contamination tested in laboratory conditions only caused impairment of growth. These results suggest that the bioassay with *C. riparius* larvae using growth and development as endpoint can be used to assess ecological impact caused by mining activities and to evaluate the efficiency of techniques used to diminish the impact of mining activity to environment.

Keywords: *Chironomus riparius*, bioassays, tungsten mine, heavy metals

Introduction

Mining activity can cause serious environmental problems due to chemical impacts caused by production of exploitation residues and mineral processing, resulting in solubility and diffusion of heavy metals. This has eliminated several invertebrate species and significantly changed the community composition in many metal contaminated areas (e.g. Clements 1994; Kiffney and Clements 1994; Winterbourn and McDiffet 1996). However, it is now understood that to achieve a sustained development of mining industry is essential to take into account environmental issues (Allan 1995).

Portugal is the third-leading world producer of tungsten, which is all extracted from the Panasqueira mine (Costa 1998; Romão 2001). This mine has produced tungsten and tin since the the end of the X1X century (http://www.igm.ineti.pt/edicoes_online/boletim.htm) and later started to produce copper minerals as well. In 2000, this mine produced 1269 tons of tungsten, 37 tons of copper and, together with the Neves-Corvo mine, produced 1228 tons of tin (Romão 2001). Panasqueira mine is located in central Portugal, in the drainage basin of Zezere river. Further downstream in this river is the biggest water reservoir for public supply in Portugal (Aprisco et al. 2003). The Panasqueira mine comprises twelve thousand kilometres of underground galleries (http://urbi.ubi.pt/010130/edicao/52minaspanasqueira.html) and several open air deposits of mine residues, some of them forested with pine trees, *Pinus* spp. The mine also comprises a treatment station where acid mine drainage (AMD) is mechanically treated with chemicals. This treatment consists, basically, in diverting AMD through an artificial channel into an artificial pound where heavy metals are neutralized, using calcium carbonate ($CaCO_3$), and dissolved metals are allowed to precipitate (Nuno Alves, personal communication). After this, the treated water is released into Casinhas stream (tributary of Zezere river) and the metal-laden sludge resulted of heavy metals precipitation is placed in an impermeable sedimentation reservoir (Nuno Alves, personal communication). Sometimes there is a over flow of AMD from the divert channel near the mine entrance galleries into the Casinhas stream and the $CaCO_3$ solution is released directly in the stream. However, the impact of mining activities in Panasqueira mine in Casinhas stream and, particularly, in Zezere river was not yet evaluated. The efficiency of the treatment station in reducing the impact of mine drainage to streams was not evaluated as well.

In this study, we evaluate the ability of *in situ* bioassay with *Chironomus riparius* larvae to assess the impact of mining activity in Zezere river and in Casinhas stream and to evaluate efficiency of the water treatment station in reducing the impact caused by AMD. A laboratory bioassay adapted from the *in situ* bioassay was also used to assess impact of a contaminated site that was not possible to assess *in situ*.

Chironomus genus is relevant in ecology (e.g. Vos 2001), is distributed through North America and Europe in a wide variety of freshwater habitats (e.g. Bazzanti et al. 1997; Diggins and Stewart 1998; Craig et al. 1999; Batzer et al. 2000), and can play an important role as biological indicators of metal contamination (Mousavi et al. 2003). In previous studies (see chapters 1 and 3), development and, principally, growth of *C. riparius* larvae showed to be sensitive endpoints to metal contamination and could discriminate between metal contaminated and not contaminated sites.

Material and Methods

Study sites

In situ bioassays were carried out in Zezere river and in a tributary stream, Casinhas stream, located near the Panasqueira tungsten mine in Barroca Grande, central Portugal. The study area and selected sites are shown in Fig. 1.

Site 1, selected as reference site (REF), was located in Zezere river more than 1 km upstream from Panasqueira mine and from any open air residue deposit. Nevertheless, we can not rule out the possibility of contamination of this site by depositing of heavy metals dispersed by the air. The low metal contaminated site (site 2) was located in the margin of Zezere river, where an open air deposit of mine residues reached the river. The high metal contaminated sites (sites 3, 4, 5 and 6) were located in Casinhas stream, that receives acid mine drainage (AMD) directly from the mine galleries and treated water from the AMD treatment station. Site 3 was located far downstream of the water treatment station, site 4 and 5 were located near the treatment station (site 4 downstream and site 5 upstream of the discharge site of treated water and $CaCO_3$ solution from the treatment station). Site 6 was located more upstream, but near, from the mine galleries entrance.

Bioassays

Third instar larvae of *Chironomus riparius* used in the bioassays were obtained from laboratory cultures established at the Biology department, University of Aveiro. The culture conditions were the same as described in chapter 1, except for the substrate. Here we used organic matter free (ignited in a muffle furnace for 6 hours at 450ºC) natural sediment from Bestança river, considered an unpolluted river (see chapter 1), as substrate.

The *in situ* bioassay procedures were the same as described in Chapter 1. On initial day of the bioassay (day 0), 25 third instar larvae were introduced in five chambers (5 larvae per chamber), with sediment previously collected and conditioned *in situ*, placed in the river bed (see Chapter 1 for more details). At the end of bioassay (day 6), all larvae alive were removed from chambers and were killed and preserved in Von Törne conservant (Gama, 1964) for later

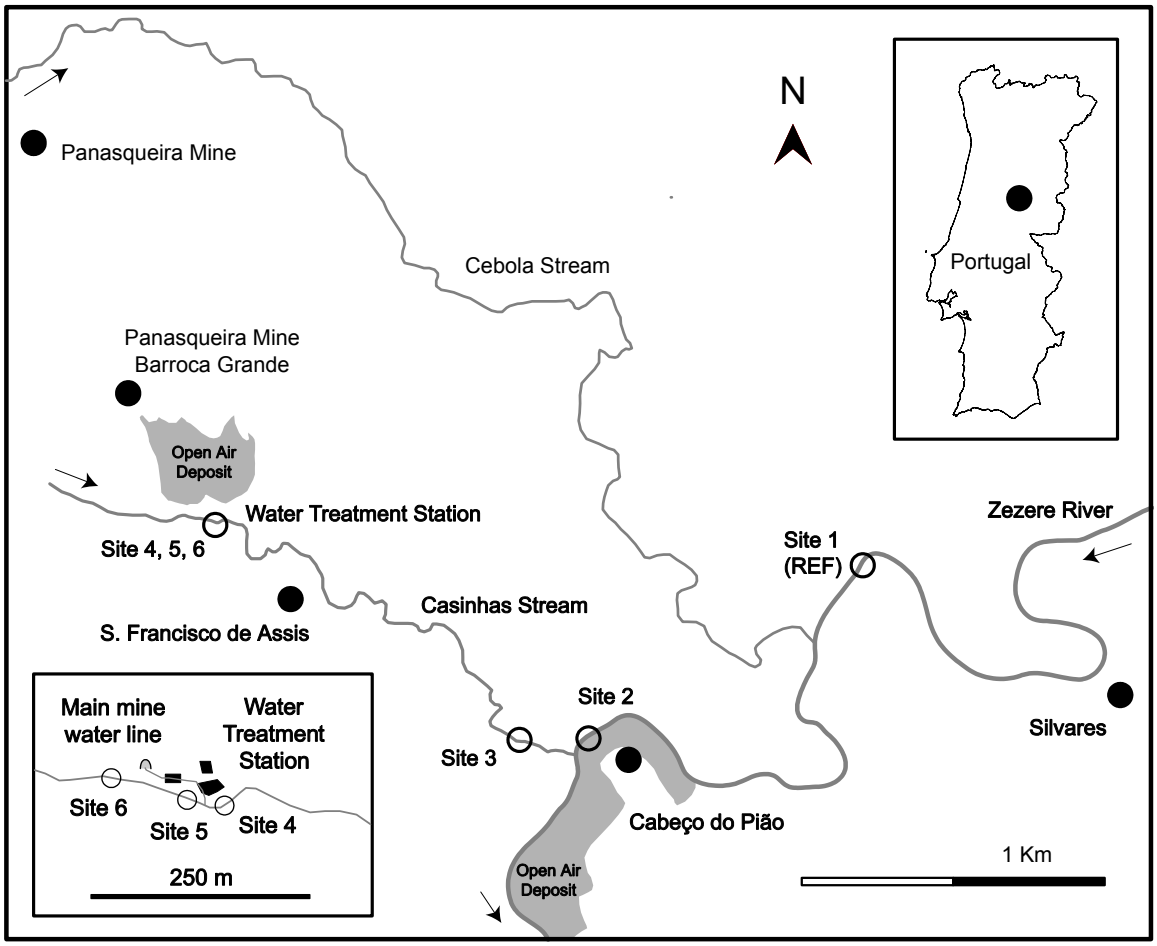

Fig. 1. Study area and selected sites.

measuring. Initial and final body length and development of larvae were determined by measuring body and head capsule width, respectively, using a stereomicroscope fitted with a calibrated eye-piece micrometer. At site 2 the exposure chambers were vandalized, so water and sediment was collected in this site and used in a laboratory bioassay. Water and sediment was also collected at REF site for use in the laboratory bioassay as the reference treatment.

According to previous results (see chapter 4), laboratory tests (with river water and sediment) respond in similar way to *in situ* tests for metal contamination, despite the higher values of biological responses (e.g. growth) of *C. riparius* larvae observed in laboratory possibly due to optimal laboratory conditions for larval development and growth.

The general procedures of the laboratory bioassay were the same as described in chapter 3. On initial day (day 0) of the bioassay, 25 third instar larvae were introduced in five plastic flasks (5 larvae per flask) with water and sediment, provided with artificial aeration, in each treatment (see chapter 3 for more details). Three treatments were used: (1) water and sediment from REF site (reference site), (2) water and sediment from site 2 (low contaminated), and, (3) ASTM hard water

and sediment from Bestança river, used as a control treatment. Two additional flasks without larvae were used, per treatment, to determined heavy metal concentrations of the water and sediment on day 0 and day 6. The finishing of the bioassay and larval biometrics procedures were the same as described for the *in situ* bioassays. The laboratory bioassay was conducted under 20º C and 14 h light: 10 h dark photoperiod.

Physical and chemical analysis

Physical and chemical parameters were determined in water for each site. Hand-held field meters were used to determine the maximum and minimum temperature of water during bioassays and also to measure pH, conductivity and dissolved oxygen (DO) of water in initial day (day 0) and in the final day (day 6) of the bioassays. Water samples were collected in day 0 and day 6 of the experiments to determine nitrate and phosphate water levels.

Heavy metal analysis

Water samples were acidified (pH < 2.0) and sediment samples were frozen until analysed. Heavy metal analyses were performed on water and sediment samples to determine concentrations of arsenic (As), zinc (Zn), cadmium (Cd), lead (Pb), nickel (Ni), manganese (Mn), chromium (Cr), iron (Fe) and copper (Cu). Since Casinhas stream is treated with a solution of $CaCO_3$ and NaOH to neutralized inputs of acid mine drainage in the stream, the concentration of the alkaline earth metal calcium (Ca) in water and sediment samples was also determined. Sediment was dried at 60ºC during 48 hours and digested in a mixture 1:3 of nitric acid (HNO_3 (70%)) and hydrochloric acid (HCL (37%)) on a hot (100-150ºC) plate to determine heavy metal concentrations (Clesceri et al. 1995). Heavy metal analyses were performed with inductively coupled plasma atomic emission spectrometry (ICP-AES) (Clesceri et al. 1995) at the Central Laboratory of Analysis in University of Aveiro.

Statistical analysis

One-way analysis of variance (ANOVA) followed, when necessary, by Tukey HSD multiple comparison tests, were conducted to test for significant differences biological endpoints, the percentage of larvae in fourth instar (development) and body length increase (growth), between sites (Zar 1996). One-way analysis of variance (ANOVA) followed by Dunnett tests was used to compare biological endpoints between reference and low contaminated treatments and the control treatment (Zar 1996). Stepwise multiple regressions and linear regression analyses were used to investigate significant relationships of biological responses of *C. riparius* to heavy metal concentrations in water and sediment (Zar 1996). Responses were tested for normality using the Kolmogorov-Smirnov test. The percentage of larvae in fourth instar was arcsine transformed to

stabilize the variance (Zar 1996). Pearson's coefficients were used to investigate the association between development (the percentage of larvae in fourth instar or the head capsule width within instars) and the body length (Zar 1996). Statistical analyses were performed using Minitab™ Release 14.

Results

Chemical and physical parameters in water

During *in situ* bioassays, mean values of DO in water were higher than 8.6 at all sites and minimum temperature was between 11 and 15ºC and maximum temperature ranged between 24 and 26ºC (Appendix 1). Physical and chemical conditions of water were similar between sites 5 and 6 and between sites 3 and 4 (Appendix 1). Water at sites 5 and 6 was acidic (varied between 5.1 and 5.3), with a moderate conductivity and low levels of nitrates and phosphates (Appendix 1). Conductivity of water at sites 3 (1112 μs cm^{-1} average) and 4 (1140 μs cm^{-1} average) was very high and phosphate levels were low (Appendix 1). However, nitrate levels at site 3 were very high (5.3 mg L^{-1} average). At site 3, pH values were higher than in sites 5 and 6 and, on day 0, pH of site 3 was higher than pH at site 4. However, at site 4 pH values showed a great variation from 5.6 on day 0 to 9.1 on day 6 (Appendix 1) due to irregular discharge of $CaCO_3$ from treatment station. Physical and chemical parameters of water at REF site highly differed from the observed values at the other sites, except for DO and temperature. At this site, water showed higher mean values of pH (6.9) and lower values of conductivity (68 μs cm^{-1} average). The physical and chemical parameters of water at site 2, determined on day 0 of the bioassays, and nitrate and phosphate concentrations in the water samples collected from that site, in the same day, are also shown in Appendix 1. The water pH (6.1) was lower and conductivity (360 μs cm^{-1}) was higher at site 2 than at REF site.

During laboratory testing, mean values of dissolved oxygen and temperature of water were similar in all treatments. Mean values of DO ranged between 9.0 and 9.8 mg L^{-1} and temperature varied between 21 and 22.5ºC (Table 1). Conductivity was 74 ms cm^{-1} in REF site, 364 in site 2 and 847 ms cm^{-1} average in the control (Table 1) and pH was lower in site 2 (7.4 average) than in REF site (7.9 average) and in the control (7.8 average), as we would expect from *in situ* results (Table 1).

Heavy metal concentrations

Concentrations of heavy metals in water and sediment from the selected sites during *in situ* bioassays are shown in Appendix 2. High heavy metal concentrations were observed in sediment from sites 4, 5 and 6, especially As (mean values 138, 124 and 139 mg Kg^{-1}), Zn (mean values

Table 1. Physical and chemical parameters of water observed in reference (Ref), low metal contaminated (Low) and control treatments during laboratory bioassay, in day 0 and day 6. Temp = temperature; Cond = conductivity; DO = dissolved oxygen.

	Temp ºC		pH		DO (mg L^{-1})		Cond ($\mu s\ cm^{-1}$)	
Treatments	Min	Max	day 0	day 6	day 0	day 6	day 0	day 6
Control	21.5	22.0	7.8	8.0	9.8	9.8	810	883
Ref (REF site)	21.0	22.0	7.6	8.0	9.7	9.6	67	81
Low (site 2)	21.0	22.5	7.3	7.4	9.0	9.7	347	381

22.0, 15.0 and 22.5 mg Kg^{-1}), Fe (mean values 696, 587 and 553 mg Kg^{-1}) and Cu (mean values 26.4, 20.8 and 26.0 mg Kg^{-1}). Comparing sediment from site 3, concentrations of heavy metals were lower, except for Ni (mean value 0.7 mg Kg^{-1}), Mn (mean value 6.7 mg Kg^{-1}), Cr (mean value 0.33 mg Kg^{-1}) and for the metal Ca (mean value 28.7 mg Kg^{-1}). In sediment from REF site, the concentration of most metals detected (As, Zn, Pb, Ni, Mn, Cr, Cu) was < 0.5 mg Kg^{-1}, except for Fe (mean value 36.4 mg Kg^{-1}) and Ca (mean value 2.4 mg Kg^{-1}). The water concentrations of all metals were higher at sites 3 and 4, principally Zn (mean values 8.5 and 4.5 mg L^{-1}), Mn (mean value 9.3 and 6.0 mg L^{-1}) and Ca (mean value 106 and 132 mg L^{-1}). At site 5 and 6, heavy metals present in water were the same as those found in water from sites 3 and 4, but their concentrations were lower (Appendix 2). In water from REF site we observed only low concentrations of the detected metals Mn (mean value 0.045 mg L^{-1}), Fe (mean value 0.45 mg L^{-1}) and Ca (mean value 13.05 mg L^{-1}) (Appendix 2). The heavy metals Pb and Cr were not detected in water samples.

During laboratory bioassays, metal concentrations in water and sediment from REF site were similar to those observed in water and sediment collected in days 0 and 6 in field (Appendixes 2 and 3). Heavy metal concentrations in water and sediment from site 2 were higher than those from REF site (Appendix 3). Additionally, at site 2 were found some heavy metals (water: As, Zn, Ni, Cu; sediment: Cd) that were not detected at REF site. Heavy metal concentrations in water and sediment from site 2, during the laboratory bioassay, were lower than heavy metal concentrations in water and sediment from sites 3, 4, 5 and 6, during *in situ* bioassays, except Fe concentrations (mean value 5.5 mg L^{-1}) in water that were higher at site 2.

Biological responses

At the end of the *in situ* bioassays, all larvae in reference site (REF) were in fourth instar (mean head capsule width ± SE: 0.55 ± 0.01 mm), while all larvae in high metal contaminated sites (sites 3, 4, 5 and 6) stayed in third instar (mean head capsule width ± SE: 0.33 ± 0.01 mm, pooled data from the four sites) (Fig. 2A), meaning that larval development was completely inhibited in these sites. No significant differences in head capsule width of larvae between sites 3, 4, 5 and 6

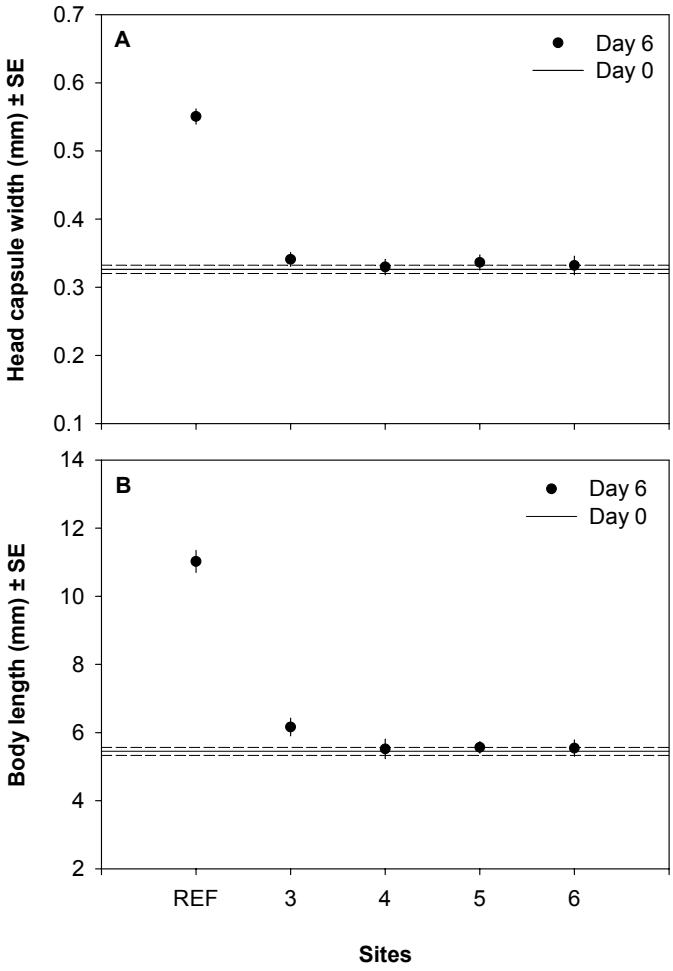

Fig. 2. Mean values and respective standard error (± SE) of larval biometrics (A - head capsule width, B - body length) in reference (REF) and metal contaminated (3, 4, 5, 6) sites, at the end of the *in situ* bioassays (day 6). The mean and ± SE of larval biometrics in day 0 (measured from 30 larvae) are also shown (horizontal line and dashed line, respectively).

($P > 0.05$) were found, although slightly higher larval head capsule width was observed at site 3 (Fig. 2A). During these bioassays there were significant differences in larval growth between sites (ANOVA: $F_{4, 96} = 230.45$, $P < 0.001$). Comparatively to the reference site, a significant inhibition in larval growth was found in sites 3, 4, 5 and 6 (Tukey post hoc tests: $P < 0.0001$) (Fig. 2B). There were also observed differences in larval body length between the initial (day 0) and the end (day 6) of the bioassays in reference site and site 3 (Fig. 2B). Those differences (ANOVA: $F_{5, 125} = 248.19$, $P < 0.001$) were highly significant in reference site (Dunnett test: $P < 0.0001$) and were lower but significant in site 3 (Dunnett test: $P < 0.01$). On the contrary, no significant differences ($P > 0.05$) between day 0 and day 6 of the bioassays were found in body length of

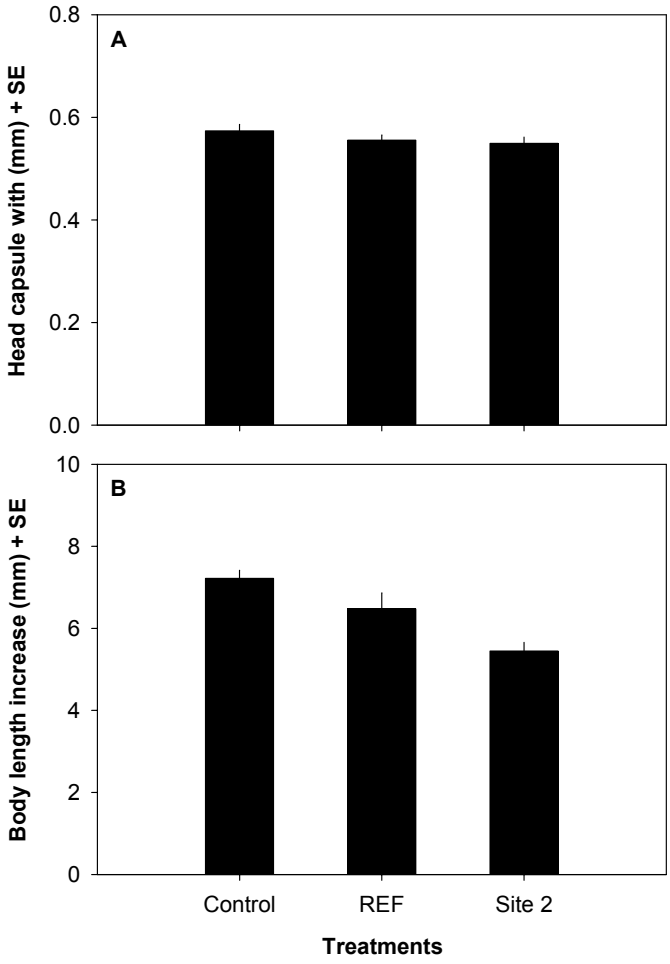

Fig. 3. Mean values and respective standard error (+ SE) of larvae biometrics: A - development (head capsule width), B - growth (body length increase); in control, reference (REF) and metal contaminated (site 2) treatments, at the end of the laboratory bioassay.

larvae in sites 4, 5 and 6, the most contaminated sites, meaning that growth of larvae in these sites was completely inhibited.

During the *in situ* bioassays, linear regression analyses indicated that growth was highly negatively related ($r^2 = 0.90$, $P < 0.05$) to Zn, Cr, Fe, Cu concentrations in sediment of all sites, and stepwise multiple regressions indicated that 98% of variation in growth between sites could be explained by variation in Cu and Fe concentrations (body length increase = 5.90 - 0.00545 Fe - 8.43 Cr, $P < 0.05$) in sediment between the sites. Stepwise multiple regressions also indicated that 94% of larval development between sites could be explained by variation in Cr concentrations (percentage of larvae in fourth instar = 93.4 - 309 Cr, $P < 0.01$) in sediment between the sites. Developmental stage (percentage of larvae in fourth instar) and growth were not related to ($P > 0.05$) to heavy metal concentrations in water during the *in situ* bioassays. The percentage of

larvae in fourth instar and larval body length were strongly positively correlated (r = 0.99, $P < 0.01$). The Larval body length was also positively correlated with head capsule width of larvae in fourth instar (r = 0.61, $P < 0.01$) but was not correlated with head capsule width of larvae in third instar ($P > 0.05$).

During the laboratory bioassay, all larvae change to fourth instar and no significant differences were observed between head capsule width of larvae between treatments ($P > 0.05$) (Fig. 3A). On the contrary, some significant differences were observed in larval growth between treatments (ANOVA: $F_{2, 53} = 10.45$, $P < 0.001$) (Fig. 3B). Growth of larvae was significantly lower in treatment with water and sediment from site 2, low metal contaminated site, than in treatment with water and sediment from reference site (ref treatment) (Tukey post hoc test: $P < 0.05$) and in control treatment (Dunnett test: $P < 0.001$). No significant differences ($P > 0.05$) were found in larval growth between the reference and the control treatments (Fig. 3B). The Larval body length was only weakly positively correlated with head capsule width of larvae in fourth instar (r = 0.44, $P < 0.01$).

Discussion

Physical and chemical and parameters

The reference site was the site with the highest pH and lower conductivity and phosphate levels, although the phosphate levels were higher at this site, which was expected since is located upstream great sources of pollution.

The water acidity found in sites 4, 5 and 6 could be explained by the high concentration of heavy metals in those sites. Low levels of pH are usually found in metal contaminated waters (e.g. Godzick and Krywult 1998; DeNicola and Stapleton 2002; Mousavi et al. 2003) and may result from the oxidation and/or hydrolysis of Fe and other metals (DeNicola and Stapleton 2002). Low levels of pH in those sites can also be explained by the occasional input of AMD from mine galleries in Casinhas stream near site 4, resulting in a decrease of pH in that site and in the nearest downstream sites (sites 5 and 4).

The slightly higher pH value found in site 4 on day 0 of the *in situ* bioassay, comparing with sites 5 and 6 (Appendix 1), can be explained by the discharge of AMD treated water and the occasional discharge of $CaCO_3$ solution from the treatment station into Casinhas stream near the site 4, which increases pH of that site. The high pH value found in site 4 on day 6 (9.1) of the *in situ* bioassay was the result of the release of $CaCO_3$ solution from the treatment station into Casinhas stream, just before the measure of pH and the finalization of the bioassay *in situ*. The treatment station activities resulted in a high pH average pH at site 4 (7.4).

The average pH found at site 3 was lower than the average pH observed at site 4. This can be explained by the input of acidic water (from an unknown source) into Casinhas stream, downstream of the site 4, which greatly decreases the pH, which can reaches 3.0 (Alves, personal communication). More downstream there is an increase of pH, that can reaches 6.0 at site 3, as was observed, probably due to the input of untreated domestic sewage from the S. Francisco the Assis population (Fig. 1) in the stream (Alves, personal communication), since domestic sewage can have high pH values (e.g. 12.3) (Marciano 1999). This could also explain the high levels of nitrates in water at site 3 (Appendix 1).

The higher conductivity found in site 4 and 3, comparing with other selected sites of Casinhas stream (Appendix 1) could be due to the high concentration of $CaCO_3$ dissolved in the stream at those sites, which is indicated by high concentrations of Ca in water (Appendix 2), as a result of its release from the treatment station into the stream. But, it could also be due to the higher concentration of dissolved metals (metal ions) in water at those sites (Appendix 2).

Heavy metal concentrations

The metal contamination in sites 2, 3, 4, 5, and 6 is due to the mining activities from the Panasqueira tungsten mine. Sites 4, 6 and 5 were the most metal contaminated sites, as we would expect, since they are located in Casinhas stream near the place where, for many years, the stream received, and occasional still receives, inputs of AMD. The high contamination of those sites is due to very high concentrations of heavy metals in sediment, which is the result of precipitation of dissolved heavy metals of AMD by the increase of pH when AMD reached the stream.

The heavy metal concentrations in the stream sediment was higher at site 6 than at site 5, probably because site 6 is located nearest the mine galleries entrance and receives higher inputs of the AMD. However, from site 5 to site 4 there is an increase of heavy metals concentration in water and in sediment. The increase of heavy metal concentrations in water can be explained by the discharge of treated water into the stream between the two sites, from the treatment station, that still has dissolved metals after the treatment with $CaCO_3$, indicating that the treatment with $CaCO_3$, performed in the treatment station, to eliminate the heavy metals from AMD is not completely effective. The increase of heavy metal concentrations in sediment is probably due to the precipitation of heavy metals dissolved in water as a result of increased pH caused by input of $CaCO_3$ solution, from the treatment station, into the stream at the same place. Thus, the treatment station is not efficient to eliminate, although it reduced, the contamination in Casinhas stream caused by the AMD.

The lower heavy metal concentrations in sediment of site 3, comparing with sites 4, 5 and 6 was expected because this site is located downstream from sources of heavy metals (mine galleries

entrance and treatment station). However, in site 3 was observed higher heavy metal concentrations in water than in the other sites. The high heavy metals concentration in stream water at site 3, may be due to the release of metals from sediment upstream the site 3, where the pH in stream water is very low, as was mentioned above, although some precipitation of those metals probably occur when they reach the site 3 due to the increase of pH in that site.

The highest concentrations of Ca in stream water were observed at sites 3 and 4 that are located downstream of treatment station, so the high concentrations of Ca in those sites was the result of the release of $CaCO_3$ solution from the treatment station into stream. At sites 5 and 6, located upstream the treatment station, the Ca concentrations in stream water was low.

Site 2, located in Zezere river, was less contaminated than sites 3, 4, 5 and 6, although Fe concentration in the water from this site, during the laboratory bioassay, was higher than in the water from the other sites, during *in situ* bioassays, which could be due to sampling-related artefacts, i.e. alteration of bioavailability of Fe in water as a consequence of storage during transportation and handling before the laboratory bioassay. Although site 2 receives directly the mine residues from an open air deposit, the low contamination found at this site can be explained by the runoff or dilution of metals in Zezere river due to the large volume of water that flows in this river. This second hypothesis was formulated by Ristola et al. (1996) for explaining the low concentrations of contaminants in Ladoga lake, including metals, surrounded by many sources of contamination. Nevertheless, the site 2 had higher concentrations of heavy metals than the reference site (REF), as was expected since the REF site is also located in Zezere river but far upstream from Panasqueira mine and from any open air residue deposit. This site can be considered an uncontaminated site, since in the water was only detected Mg and Fe, in low concentrations, and Ca, in moderate concentrations, and the sediment had only low concentrations of heavy metals.

These results show that the chemical impact of Panasqueira mine activities in Zezere river and especially in Casinhas stream is clear. The water treatment station should have prevented that all heavy metals from AMD were released to Casinhas stream, but this was not confirmed. What was verified was that the occasional input of AMD and the discharges of the treated water and $CaCO_3$ solution from the treatment station into Casinhas stream, increased heavy metal concentration in water and sediment, causing a great chemical impact in this stream. The impact of mine drainage to the stream could be avoided if the AMD inputs were completely prevented and if the AMD treatment was efficient, avoiding the presence of heavy metals in treated water and its release into the stream. These may be achieved by increasing the depth of the deriving channel, and by using NaOH (more effective than $CaCO_3$) to neutralize pH of AMD and to precipitate heavy metals of AMD (http://www.wvu.edu/~agexten/landrec/chemtrt.htm). Other types of AMD treatment, such as the passive treatment system (DeNicola and Stapleton 2002), could also be used.

When correctly designed, passive treatments have been shown to be efficient in improving chemical water quality (DeNicola and Stapleton 1999; Milavec 2000). The chemical impact caused by mine residue from open air deposits that reach the Zezere river, although lower than the chemical impact caused by AMD in Casinhas stream, is significant. The chemical impact caused by mine residue open air deposits could be eliminated or diminished by their environmental rehabilitation as was observed in Jales (Chapter 4). The results also suggest that the chemical impact of AMD from Panasqueira mine in Zezere river may be significant due to high concentrations of heavy metals in water in site 3, located in Casinhas stream near the Zezere river (Fig. 1). That, however, was not evaluated because it was not possible to find an accessible site in the Zezere river downstream and near the mouth of Casinhas stream.

Biological responses

The high levels of metal contamination in Casinhas stream caused negative effects on *C. riparius* larvae. During the *in situ* bioassays, there was a total inhibition of larval development and growth in the most contaminated sites (sites 4, 5 and 6) located in Casinhas stream, near the mine galleries entrance, the AMD and the treatment station (the major sources of heavy metals to the stream). There was also an inhibition in larval growth and a total inhibition in larval development in site 3, a high contaminated site located near the mouth of Casinhas stream (Fig. 1 and 2). Since growth and development of chironomids larvae are ecological important endpoints (Liber et al. 1996; Sibley et al. 1997a) and *C. riparius* larvae is an ecological important species in freshwater ecosystems (e.g. Vos 2001), the results suggest that Cabecinhas stream is an ecological impaired ecosystem due to metal contamination, i.e. the chemical impact, caused by AMD. The results also indicate that the treatment station is not efficient in improving the water quality because no significant differences in biological responses of *C. riparius* larvae (larval growth and development inhibition) were found between the site located downstream (site 4) and the sites located upstream (sites 5 and 6) the AMD treatment station.

At less extent, the low metal contamination found in Zezere river at site 2 also affected *C. riparius* larvae. During the laboratory bioassay, growth of larvae in treatment with water and sediment from site 2 was inhibited, but larval development was not affected. This suggest that even the low chemical impact caused by the mine residues from the open air deposit, in contact with the river at site 2 may be causing ecological impairment in river, at least in this site.

The fact that growth was less inhibited in site 3 than in sites 5 and 6, which had higher heavy metal concentrations in sediment but had lower heavy metal concentrations in water than site 3, shows that larval growth is more affected by heavy metals in the sediment than in the water of rivers. Furthermore, Linear regression analyses and stepwise multiple regressions indicated that

growth was highly negatively related to Zn, Cr, Fe, Cu concentrations in sediment, but was not related with heavy metals in water. In addition, although no significant differences where observed in larval development between sites 3, 5 and 6, linear regression analyses and stepwise multiple regressions indicated that development was highly negatively related to Cr concentrations in sediment but was not related with heavy metals in water. These findings show that in metal contaminated rivers *C. riparius* larvae are mostly affected by sediment contamination, as was observed in chapter 4, which is explained by the fact that the heavy metals enter *C. riparius* larvae mostly associated to sediment particles (chapter 4), indicating that *C. riparius* is a sensitive and responsive species to be used in sediment quality assessment in metal contaminated rivers.

Although the inhibition of larval development seems to be related to high metal contamination in sediment, it could be also a consequence of the inhibition of larval body length increase (growth). Hooper et al. (2003) suggested that it may be necessary a minimum body length increase to stimulate changes in development. In our study, although there was only a week or no correlation between larval head capsule width and body length within instars, the developmental stage of larvae (the percentage of larvae in fourth instar) was highly correlated with larval body length. Similar results were also observed in a previous study performed in artificial streams with the insecticide lambda-cyhalothrin (chapter 2). These findings suggest that effects on larval development can be a consequence of, and thus can indicate, the level of toxicity effect of a contaminant to the larval growth.

Conclusions

Acid drainage from Panasqueira mine have been causing a high chemical impact in Casinhas stream and the AMD treatment station has not been efficient enough to eliminate or significantly diminish this impact. Growth and development of *C. riparius* larvae were highly inhibited by high metal contamination in the Casinhas stream in the sites located upstream and downstream the AMD treatment station. This indicates that chemical impact in Casinhas stream caused by AMD resulted in an ecological impairment of this ecosystem and the treatment station was not efficient to improve water quality in the stream.

The low metal contamination caused by an open air mine residue deposit in Zezere river have caused an impairment in the growth of *C. riparius* larvae, indicating that even the low chemical impact caused by the mine residue deposit may cause an ecological impact in the river.

These findings suggest that the bioassay with *C. riparius* larvae using growth and development as endpoint can be used as bioindicator of water quality in metal contaminated rivers and to assess ecological effects of metal contamination in rivers. Thus, it can be used to assess

ecological impacts caused by mining activities and to evaluate the efficiency of remediation methods in decreasing the impact of mining activities in the water quality.

Acknowledgements

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Appendix 1. Physical and chemical parameters of water observed in reference and metal contaminated sites during *in situ* bioassays. Temp = temperature; Cond = conductivity; DO = dissolved oxygen. n.d. = not determined.

	Temp ºC		pH		DO (mg L^{-1})		Cond ($\mu s\ cm^{-1}$)		Nitrate (mg L^{-1})		Phosphate (mg L^{-1})	
Sites	Min	Max	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6
REF	15.0	25.5	6.8	6.9	8.9	8.5	65	71	0.9	1.0	0.29	0.26
2	n.d.	n.d.	6.1	n.d.	8.5	n.d.	360	n.d.	1.1	n.d.	0.14	n.d.
3	14.5	26.0	5.9	6.0	9.5	8.5	1003	1220	4.7	5.9	0.03	0.09
4	14.0	26.0	5.6	9.1	9.6	8.8	1024	1256	1.7	2.2	0.03	0.02
5	14.0	26.0	5.2	5.3	9.5	8.9	388	397	1.6	1.2	0.08	0.01
6	11.0	24.0	5.2	5.1	9.8	9.2	348	376	1.6	1.2	0.12	0.15

Appendix 2. Metal concentrations in reference and metal contaminated sites on day 0 and day 6 of *in situ* bioassays. n.d. = not detected.

	As		Zn		Cd		Pb		Ni		Mn		Cr		Fe		Cu		Ca	
Sites	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6
Water (mg L^{-1})																				
REF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	0.05	n.d.	n.d.	0.37	0.52	n.d.	n.d.	13.0	13.1
3	0.05	0.05	7.64	9.29	0.07	0.10	n.d.	n.d.	0.35	0.45	7.90	10.7	n.d.	n.d.	0.94	0.97	1.02	0.80	109	103
4	0.50	0.40	5.94	3.10	0.05	0.02	n.d.	n.d.	0.30	0.13	8.42	3.59	n.d.	n.d.	0.99	0.47	0.56	0.24	110	154
5	0.04	0.03	2.23	2.41	0.02	0.02	n.d.	n.d.	0.09	0.10	1.73	1.79	n.d.	n.d.	0.26	0.05	0.33	0.36	26.5	28.4
6	0.04	0.05	2.06	1.94	0.02	0.01	n.d.	n.d.	0.08	0.08	1.65	1.57	n.d.	n.d.	0.05	0.54	0.29	0.28	24.3	25.6
Sediment (mg Kg^{-1})																				
REF	0.06	0.05	0.15	0.13	n.d.	n.d.	0.03	0.05	0.01	0.02	0.34	0.50	0.02	0.03	29.1	43.6	0.08	0.01	2.38	2.31
3	28.8	25.8	18.1	17.9	0.13	0.10	0.55	0.43	0.66	0.76	6.49	6.87	0.34	0.32	482	417	15.8	16.2	43.3	14.0
4	125	150	21.5	22.5	0.27	0.29	1.57	1.68	0.39	0.39	4.34	4.31	0.29	0.30	673	718	29.9	22.9	25.7	26.5
5	138	110	17.2	12.8	0.22	0.16	1.43	1.14	0.36	0.28	4.14	3.60	0.26	0.24	644	529	24.7	16.8	22.3	26.2
6	142	135	22.7	22.2	0.28	0.27	1.66	1.60	0.60	0.62	4.91	5.04	0.31	0.33	554	551	27.4	24.5	20.4	24.4
Detection limit (mg L^{-1})	0.025		0.01		0.005		0.025		0.01		0.001		0.01		0.01		0.01		0.5	

Appendix 3. Metal concentrations in reference (Ref) and low metal contaminated (Low) treatments on day 0 and day 6 of laboratory bioassays. n.d. = not detected.

	As		Zn		Cd		Pb		Ni		Mn		Cr		Fe		Cu		Ca	
Treatments	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6	day 0	day 6
Water (mg L^{-1})																				
Ref (REF site)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	0.07	n.d.	n.d.	0.58	0.41	n.d.	n.d.	13.4	15.2
Low (site 2)	0.10	0.11	0.22	0.36	n.d.	n.d.	n.d.	n.d.	0.021	0.023	0.95	0.82	n.d.	n.d.	2.88	8.03	0.25	0.33	23.0	26.7
Sediment (mg Kg^{-1})																				
Ref (REF site)	0.60	0.60	0.13	0.10	n.d.	n.d.	0.06	0.03	0.02	0.01	0.50	0.35	0.03	0.02	51.0	43.6	0.01	0.01	2.31	1.50
Low (site 2)	3.02	1.05	0.524	0.497	0.005	0.004	0.064	0.057	0.03	0.03	0.77	0.61	0.04	0.05	89.1	71.7	0.46	0.50	3.51	3.04

General discussion

The results from this study comprised several different aspects regarding *in situ* bioassays with C*hironomus riparius* larvae. Since the detailed discussion of the results was already done within each chapter, I will now focus on the major results, their implications and relevance for the biological assessment of water quality and for biomonitoring contamination in rivers.

In situ bioassays

The efficiency and usefulness of a single species bioassay as bioindicator of water quality and to biomonitor contamination in aquatic ecosystems depends of the type of contaminants that illicit a response, which depends of species and endpoint used, and the relevance of response, that is, the consequences for the individual, population and ecosystem.

In this study was found that *C. riparius* larvae were negatively affected by metal and insecticide contamination, were not affected by herbicides and were tolerant to organic contamination in streams, indicating that *C. riparius* is not a suitable species to assess herbicide and organic contamination. Tolerance of chironomids to organic contamination was observed by several authors (Gower and Buckland 1978; Pinder and Farr 1987; Arnwine et al. 2003).

It was also observed that endpoints of the *in situ* bioassay with *C. riparius* larvae, developed in this study, showed different sensitivity to different types and levels of contamination. Survival was less sensitive to contamination than the other endpoints used (chapters 1 and 2), which was expected because sublethal responses generally precede and are manifested at lower exposure concentrations than lethal responses (Gerhardt 1996), and thus, lethal endpoints are less suitable than sublethal endpoints to assess water quality and to biomonitor contamination in streams. On contrary to survival, growth was the most sensitive endpoint to metal and insecticide (lambda-cyhalothrin, endosulfan) contamination (all chapters). High sensitivity of growth of *C. riparius* larvae to metal and insecticide contamination was also observed by other authors (e.g. Timmermmans et al. 1992; Scott and Kaushik 1998; Gills et al. 2002; Milani et al. 2003).

Larval growth was highly affected by metal contamination in streams. It was slightly but significantly inhibited in low contaminated streams, inhibited in high contaminated streams and completely inhibited in very high metal contaminated streams, highly discriminating different levels and effects of metal contamination (chapters 1, 4 and 5). Growth bioassay was also able to: (1) discriminate water quality in metal contaminated streams between seasons (chapter 1); (2)

detect a significant improvement in water quality in streams related to the improvement in the chemical quality of streams, during and after an environmental rehabilitation of open air mine residue deposit program (chapter 4); and, (3) verified that a mechanical treatment of acid mine drainage did not result in an improvement in water quality of the nearest stream (chapter 5). These results show that growth is a sensitive and reliable endpoint to assess and biomonitor metal contamination in streams.

Larval growth was also affected by endosulfan (an organophosphorus insecticide) contamination in channels after the insecticide spraying in rice fields; it was highly inhibited in a rice field channel (highest contaminated site) and was also inhibited by the insecticide contamination in a wetland channel (reference and low contaminated sites). This, however, was only possible to determinate during a laboratory bioassay, with water and sediment collected in sites, by comparison with a control treatment, because even the reference site had high concentration of the insecticide. Larval growth was also highly affected by lambda-cyhalothrin (a pyrethroid insecticide) when the bioassay developed for use *in situ* was conducted in artificial streams, since it was inhibited in low and highly inhibited in high dosed streams by the insecticide. These results show that growth is also a sensitive and reliable endpoint to assess insecticide contamination in rivers.

Since effects on larval growth affect reproductive and demographic parameters of chironomids, this is an ecologically relevant endpoint because is an important component of fitness and determinant of population health (Liber et al. 1996; Sibley et al. 1997a). Furthermore, chironomids larvae are of great interest in ecology of freshwater ecosystems because are the most wide and abundant macroinvertebrates (Péry et al. 2002; Ristola 2000, Vos 2001) and are important preys for fish and birds (van de Bund 1994; Prat and Rieradevall 1995; García-Berthou 1999). These imply that an effect on growth of chironomids larvae is likely to be disseminated throughout the freshwaters ecosystems due to its high abundance and interactions (predator/prey) with fish and aquatic birds. If nothing else a toxicity effect on larval growth may indicate that there is an ecological risk to the ecosystem.

These findings show that *in situ* bioassay with *C. riparius* larvae using larval growth as endpoint is a responsive, reliable and relevant tool to: (1) use as bioindicator of metal contamination and insecticide contamination; (2) biomonitor metal contamination; and, (3) assess ecological impact caused by heavy metals and insecticides in freshwaters ecosystems, or at least can be used as early warning of ecological impairment in those ecosystems.

Larval development was completely inhibited in high metal contaminated streams and in streams high dosed with lambda-cyhalothrin, but was not affected by lower levels of these types of contamination (chapter 1, 2, 4 and 5) or by endosulfan contamination (chapter 3) in streams.

Although development of chironomids larvae is a less sensitive endpoint than larval growth to metal and insecticide contamination, since it is an ecological relevant endpoint (Liber et al. 1996; Sibley et al. 1997a), it can be used as endpoint with growth of *in situ* bioassays to discriminate: (1) different types of contamination; (2) discriminate levels of contamination; and, (3) effects caused by different contaminants in freshwaters ecosystems, i.e. the ecological impact or risk caused by different contaminants to aquatic ecosystems.

Post-exposure feeding rate of larvae was inhibited in larvae exposed to lambda-cyhalothrin in high dosed streams (chapters 2) and was not affected by metal contamination. This indicates that post exposure feeding rate can be used to assess persistent effects of pyrethroid insecticides after their decay. Since *C. riparius* larva is a detritivore of the collector-gatherer type that plays an important role in detritus processing (a key process for ecosystem functions) on freshwaters ecosystems, the post exposure feeding rate of the bioassay may be used to assess the functional impairment of freshwater ecosystems, depending of functional redundancy, caused by pyrethroid insecticides after their decay.

Although chironomids (mostly *Chironomus tentans* and *C. riparius*) are commonly used in bioassays to assess sediment contamination (Giesy et al. 1990; Hoke et al. 1993; Pellinen and Soimasuo 1993; Pery et al. 2003c), as chapters 2 and 3 showed, *C. riparius* larvae are highly affected by insecticide contamination in water, thus can be used to assess insecticide contamination in river water column exposures. On the other hand, chapter 4 showed that *C. riparius* larvae are more sensitive to metal sediment-associated contamination than to metals dissolved in the water, indicating that they are more suitable to assess metal contamination in the sediment than in the water. This is of the most importance because in metal contaminated rivers, especially in mine areas, contamination in rivers is mostly due to high concentration of metals in sediment, which resulted mostly of the neutralization of acid mine effluents, by natural processes or due to restoring measures that cause the precipitation, into sediment, of dissolved metals from the water column. Thus, the evaluation of rivers sediment contamination and sediment quality can predict, respectively, the contamination of river and river water quality.

Confounding factors

The chemical analysis can determine which chemicals are present and their concentrations in aquatic ecosystems. However, concentrations of contaminants is often a week predictor of toxicity caused by contamination to organisms in aquatic ecosystems, because several confounding factors, such as organic matter and oxygen levels, may significantly affect the bioavailability of contaminants to organisms (Nebeker et al. 1989; Krantzberg 1994; Halpern et al. 2002). This however depends of how organisms are exposed to contaminants, i.e. how contaminants are

bioavailable to organisms. Thus, to know how contaminants enter the organisms is of the most importance to understand how contaminants are bioavailable to organisms, and, consequently, to understand and predict the toxicity effects of contaminants to organisms.

In chapter 4 it was found that the most important route of heavy metals metal entrance in the *C. riparius* larvae is through ingested material associated to sediment particles. Therefore, larvae exposure to heavy metals and, thus, their bioavailability to larvae, depend primarily on ingestion of contaminated sediment particles, which are ingested along with organic matter (food). So, the organic content in sediment may affect the amount of sediment particles ingested not only directly, by changing the relative organic matter and sediment particles content in sediment (food source) and, thus, ingested, but also indirectly, because by changing the nutrient value of the food source this affects, consequently, the ingestion rate. Since, the ingestion of inorganic material along with organic matter reduce the nutritional value of the food source (Vos 2001), collectors-gatherers, such as *C. riparius* larvae, maximize ingestion rate in order to maximize food intake (Cummins and Klug 1979). It was also found that particle size of sediment may greatly affect the bioavailability and the toxicity of heavy metals in sediment to larvae, because the ingestion of sediment particle by larvae depends of the particle size. This happens because the particle size that larvae can ingest is limited and determined by the larval mentum width (Vos 2001).

In chapter 4 was found that the most important way of entrance of heavy metals in the *C. riparius* larvae is through ingested material associated to sediment particles. Therefore, larvae exposure to heavy metals and, thus, their bioavailability to larvae, depend primarily on ingestion of contaminated sediment particles, which are ingested along with organic matter (during feeding). Since organic matter content and the particle size of sediment affect the amount of sediment particles ingested by larvae (see Chapter 4), they may highly affect the bioavailability and, consequently, the toxicity of heavy metals in sediment to larvae. In chapter 4 was also found that, at lesser extent, heavy metals may enter *C. riparius* larvae by transport of dissolved ions across external membranes and/or adsorption on body surfaces. The larvae exposure to heavy metals in water and, thus, their bioavailability to larvae, depend of the contact between larvae and water. According to Halpern et al. (2002), apart their role as anti-predation shelter, the tubes that chironomids larvae build from detritus, algae and sediment particles for protection against predators, serve also as chemical shields, protecting the larvae from water contaminants. So, the bioavailability of heavy metals in water to larvae also depends of the degree of protection of shelter against contaminants, which depends, according to Halpern et al. (2002) of particle size of sediment. Thus the particle size of sediment can affect also the bioavailability and, consequently, the toxicity of heavy metals in water to larvae.

The low predictability of toxicity caused by contamination to organisms can be related to reasons other than alteration in bioavailability of contaminants. Some confounding factors, such as organic matter, can inhibit or enhance the toxicity effects of contaminants. Stuijfzand et al. (2000) observed that in organic enriched rivers, organic matter of sediment stimulated growth of *C. riparius* larvae at such an extent that effects of high toxicant concentrations of metals to larval growth were reduced or neutralized.

These findings imply that in studies with *C. riparius* larvae to assess: (1) metal contamination in rivers, (2) effects of metal contamination; or, (3) biological quality of sediments; the organic matter content and particle size of the sediment need to be taken into account because they can be confounding factors on that assessment.

In toxicity tests with caged *C. riparius* larvae, feeding is necessary because of high risk for false positives (e.g. reduced survival, growth, and reproduction due to feeding privation and not to toxicity) (Ristola 2000; Pery et al. 2003c). In chapter 4, an inhibition in development and growth of *C. riparius* larvae was observed in a low metal contaminated treatment with no added food, but larvae was not affected in a similar in which food was added. However, in toxicity tests with chironomids larvae the feeding quantity can have a substantial influence on the outcome of the bioassays (Sibley et al. 1997a; Ristola et al. 1999). So, feeding can also be a confounding factor in toxicity studies with caged chironomids larvae.

Advantages and disadvantages

In situ bioassays with enclosed specimens from single species, have several advantages over more traditional methods based on measures of benthic macroinvertebrate community structure (e.g. biotic indices) and laboratory toxicity tests (whole effluent toxicity tests, sediment toxicity tests).

Bioassays with single-species provide a more rapidly indication of alteration (improvement or deterioration) in water quality than biotic indices, because effects at the individual level will be manifested more rapidly (hours to days) than resulting changes in community structure (months to years) and therefore are particularly useful as early warning indicators (Maltby 1994; Maycock et al. 2003), and because they are more effective in discriminating different human impacts of running waters and sediments (Charvet et al. 2000; Ingersoll et al. 2001). However, *in situ* bioassays have also some disadvantages: (1) they need always a reference condition because results of impacted sites have to be compared with the results of a reference site, while biotic indices only need to be compared with a regional reference condition, i.e. a reference value determined for non-impacted sites in a region, and (2) are usually more expensive due to logistical procedures. They require at least two visits to each test location (at the beginning and at the end of the bioassay) and more

equipment is required (chambers and deployment material) in comparison with the simple techniques used to census macroinvertebrates.

In chapter 1, the *in situ* bioassay with *C. riparius* larvae, using growth as endpoint, showed several advantages over biotic indices BMWP' score and ASPT' as bioindicator of stress, such as: (1) do not only provided information about the biological quality of water but identified metal contamination and level of contamination; (2) assessed biological effects of metal contamination at individual level, which allowed the identification of causal mechanisms between environmental stressors and population or community level; (3) was more sensitive to low levels of metal contamination, which was also observed by Ingersoll et al. (2001) with *Hyalella azteca* bioassays, and (4) was able to detect changes in metal contamination between seasons. The structure of benthic macroinvertebrate community is very sensitive to several factors other than pollution (e.g. habitat alteration, water depth decrease, low flow rate) (Coimbra et al. 1996; Coimbra and Graça 1998; Ingersoll et al. 2001) and benthic macroinvertebrates can develop tolerance to pollution during the long term chemical exposure (McWilliam and Baird 2002b). These evidences may explain, respectively, why biotic indices missassigned water quality in some reference rivers and failed to detect low levels of metal contamination. However, the bioassay was not able to detect organic contamination while biotic indices were able to detect both types of contamination detected metal contamination but could also detect organic contamination.

In situ bioassays assess water quality and toxicity of contamination to organisms under more environmental realistic conditions than laboratory toxicity testing, which usually involves the use of bioassays performed with water and/or sediment collected *in situ* under static controlled conditions. However, laboratory toxicity testing can be performed under more realistic conditions, through the use of microcosms or mesocosms (e.g. artificial streams) (Boxall et al. 2002).

In situ bioassays have some advantages over laboratory bioassays: (1) they allow stressor concentrations to fluctuate naturally (Tucker and Burton 1999); (2) can integrate physical, chemical and biological processes, which can not be reproduced in the laboratory (Chappie and Burton 1997), and thus, reveal ecotoxicological relevant information for the area in concern (Giesy and Hoke 1989); (3) eliminate laboratory-to-field extrapolations (Tucker and Burton 1999); and (4) eliminate sampling-related artefacts associated with laboratory testing (Chappie and Burton 1997). However, laboratory bioassays have also same advantages: (1) provide an effective way to discriminate effects caused by contaminants from effects caused by natural stressors (e.g. adverse physical and chemical conditions) (Tucker and Burton 1999); and (2) can be very valuable to assess low levels of contamination by comparison with a control treatment (no contaminated treatment), which may not be possible to assess *in situ* because in contaminated areas (e.g. agricultural areas, mining areas) it can be difficult to find a site with none or minor contamination

to compare with contaminated sites, as was observed in chapter 3.

Laboratory-to-field extrapolations can be biased due to sampling-related artefacts, such as handling or storage of samples, but that may depend on the type of contaminant and endpoint used. It was observed, in chapter 3, that toxicity of endosulfan insecticide to larval growth during a bioassay conducted in a rice field was detected during a laboratory bioassay with water and sediment from rice field conducted in same day, while toxicity of the insecticide to larval biomass *in situ* was not detected in laboratory exposure. Tucker and Burton (1999) also found higher toxicity to survival of *Chironomus tentans* larvae exposed to contaminated rivers surrounded by an agricultural area than to larvae exposed to water and sediment collected from that rivers in laboratory. In chapter 4, was found that the toxicity effects of heavy metals to larval growth and biomass observed during bioassays conducted in metal contaminated rivers were detected and were similar to the toxicity effects of heavy metals observed during laboratory bioassays with water and sediment from rivers conducted 24 and 36 hours after *in situ* bioassays, showing comparability of results for both endpoints between the two tests environments. These findings suggest that bioassays with *C. riparius* larvae conducted in laboratory can indicate with high reliability the *in situ* toxicity of metal contamination, if water and sediment collected from study sites are used shortly after its collection. However, laboratory-to-field extrapolations in toxicity tests with pesticides are dependent on the endpoint used. Nevertheless, laboratory testing should be complemented with *in situ* bioassays to achieve site-specific insecticide ecotoxicological information.

While bioassays (*in situ* and laboratory) and biotic indices can give information about the biological state of water and/or the biological impact of contaminants, only chemical analysis is able to determine the chemical quality of water and which contaminants are present and their concentrations. Therefore, chemical analysis is indispensable to accurately assess chemical quality of water. However, they are usually more expensive than bioassays or biotic indices and they only quantify the instantaneous water quality at the time of sampling. Furthermore, the chemical quality of water is usually a bad predictor of toxicity to organisms. In this scope, the use of biological monitoring can reduce the need for routine chemical monitoring, as was suggested by Chutter (1995).

Relevance

Acid drainages from mines and pesticide spray drift and runoff from agricultural fields are of the most important sources of contamination in aquatic ecosystems. Thus, assessing the water quality and the ecological impact of heavy metals and pesticides is essential for the management of surface waters and the protection of aquatic ecosystems in mine and agricultural areas. Ecological

assessment of water as been based mainly on measures of benthic macroinvertebrates community structure (e.g. species richness, composition, diversity and pollution tolerance) (Rosenberg and Resh 1993) or whole effluent toxicity tests (Maltby et al. 2000). However, the *in situ* bioassay with *C. riparius* larvae, developed in this study, using growth as endpoint, proved to be a more reliable and realistic tool to be used in water-quality assessment and to assess ecological impact of metal contamination in rivers ecosystems, in mining areas (chapters 1, 3 and 5). Although growth of the *in situ* bioassay with *C. riparius* larvae can be useful to detect and assess insecticide contamination, *C. riparius* larvae is not affected by other types of pesticides (e.g. herbicides) or by nutrients. So, to detect and assess effects of diffuse pollution in agricultural areas, resulting from pesticide spraying or fertilizer application, the bioassay with *C. riparius* larvae, should be complemented with bioassays using species sensitive to other pesticides and nutrient/organic contamination, such as larvae of stoneflies, which are very sensitive to organic contamination (Hilsenhoff 1987), or algae, which are highly affected by herbicides (Sáenz et al. 1977; Pereira et al. 2000; DeLorenzo et al. 2001).

To achieve a sustained development of mining industry and agriculture is essential to take into account environmental issues (Allan 1995; Helliwell and Stevens 2000). In mining areas, environmental restoration measures have been attempting to reduce the impact of metal contamination in the environment, principally by acid mine drainage treatments (passive treatment system and mechanical treatment with chemicals), to restore aquatic ecosystems (DeNicola and Stapleton, chapter 5), and by impermeabilization and forestation of open air mine residue deposits (chapter 4 and 5). In agricultural areas, the improvement of aquatic environment could be achieved by using less harmful pesticides for environment.

During bioassays with *C. riparius* using growth and development as endpoints it was observed that *C. riparius* larvae were more affected by lower concentrations of the pyrethroid insecticide (lambda-cyhalothrin) than by higher concentrations of the organochlorine insecticide (endosulfan) (chapter 2 and 3). This indicates that the pyrethroid is more effective than the organochlorine insecticide and since it is lower persistent in the environment (chapter 2 and 3), the ecological risk of the pyrethroid insecticide is probably lower than the ecological risk of the organochlorine insecticide to aquatic ecosystems. Helliwell and Stevens (2000) have also found that alphacypermethrin, other pyrethroid insecticide was more effective and less harmful for the environment than organophosphorus insecticides (e.g. chlorpyriphos), which are highly toxic for a broad range of organisms. Therefore, the use of pyrethroid insecticides in agricultural treatments as an alternative to the use of organochlorine or organophosphorus insecticide can decrease the impact of insecticides in aquatic ecosystems. The ecological risk of different pesticides to aquatic ecosystems can be assessed by laboratory, mesocosms or microcosms toxicity tests. The impact of

pesticides in aquatic ecosystems could be also reduced by adopting measures to manage the resistance of organisms to pesticides, to allow for longer effects of these substances (Plapp and Wang 1983). The resistance of insects to insecticides is common in agricultural regions and is usually the result of their long exposure to insecticides, which increases costs for the farmers and pesticide industry and increases impact in environment (Plapp and Wang, 1983).

Even more important than assessing water quality, is to biomonitor water quality and assess the biological recovery of contaminated aquatic ecosystems that are subject of restoration measures and, thus, evaluate the efficiency of these measures. DeNicola and Stapleton (1999) observed that a passive treatment system of acid mine drainage in Slippery Rock Creek (USA) showed to be efficient in improving chemical quality of water but recovery of benthic community was not observed during the four years monitoring. This happened because a high heavy metal concentration on sediment still remained or could also be due to the fact that alterations in water quality may only induce changes in community structure at long term (months to years), as mentioned above. The *in situ* bioassay with *C. riparius* larvae using growth as endpoint proved to be a sensitive, reliable, relevant and fast tool to biomonitoring water quality and to indicate biological recovery of metal contaminated rivers (Chapter 4). The bioassay allowed to evaluate the efficiency of an environmental rehabilitation program of open air residue deposit of Jales mine and also allowed to verify the inefficiency of the mechanical treatment of acid mine drainage in the Panasqueira tungsten mine. The bioassay with *C. riparius* larvae can also be used, at least potentially, to biomonitoring insecticide contamination in aquatic ecosystems of agricultural areas, since larval growth was highly affected by insecticide contamination in streams and could discriminate levels of insecticide contamination.

Conclusions

At the end of this thesis, I became confident that *in situ* bioassays with *C. riparius* larvae proved to be a reliable and fast tool for the assessment of water quality and the impact of heavy metals and insecticides in river ecosystems. Thus, *in situ* bioassays with *C. riparius* larvae can form an integral part of monitoring schemes designed for the management of water quality in rivers.

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