Universidade de Aveiro Departamento de Biologia
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Joana Raquel de Aguiar Medeiros Correia

Toxicidade de extratos de eucalipto e pinheiro em peixes e anfíbios

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos, Investigadora de Pós-Doutoramento do Departamento de Biologia e CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro e co-orientação do Doutor Kieran Andrew Monaghan, Investigador auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro e do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave eucalipto; pinheiro; anfíbios; peixes; embriotoxicidade; LC50; biomarcadores.

resumo

A desmatação de florestas naturais, aliada à crescente extensão de florestas plantadas, de monoculturas, como o eucalipto e o pinheiro, leva à perda de biodiversidade e tem outras consequências que são particularmente nefastas em ribeiros intermitentes, prejudicando os organismos aquáticos, como peixes e anfíbios.

O presente estudo teve como principais objetivos: (i) avaliar as características físico-químicas de extratos aquosos de folhas de eucalipto e pinheiro produzidos em laboratório, e de amostras de água recolhidas em poças de rios intermitentes localizados em florestas de eucalipto, pinheiro e caducas nativas; (ii) avaliar a toxicidade dos extratos de eucalipto e pinheiro em três espécies-modelo, peixe-zebra (*Danio rerio)*, rã-verde (*Pelophylax perezi*) e rã-de-unhas-africana (*Xenopus laevis*), e comparar a sensibilidade das espécies usadas aos diferentes extratos, tendo em conta a mortalidade e as malformações; (iii) avaliar os efeitos sub-letais dos extratos aquosos de eucalipto e pinheiro no peixe-zebra, com recurso aos biomarcadores vitelogenina (vtg), catalase (CAT), glutationa S-transferase (GST) e colinesterase (ChE).

No geral, os extratos de folhas de eucalipto e pinheiro reduziram oxigénio e pH, e aumentaram fenóis, terpenos, lípidos e condutividade. O extrato de eucalipto mostrou ser mais tóxico do que o de pinheiro, para *P. perezi* e *D. rerio*, enquanto para *X. laevis* a toxicidade dos extractos é semelhante. A sensibilidade dos embriões aos extratos de eucalipto foi: *D. rerio* > *X. laevis* ≈ *P perezi*; e para o de pinheiro: *X. laevis* > *D. rerio* ≈ *P. perezi*. As malformações mais observadas foram cauda torcida e edemas. Os extratos aquosos de eucalipto e pinheiro induziram efeitos teratogénicos nos embriões do *X. laevis.*

A actividade da CAT diminuiu em ambas as exposições (eucalipto e pinheiro), enquanto a GST diminuiu quando os organismos foram expostos ao extrato de eucalipto, mas aumentou quando expostos ao extrato de pinheiro. Não se verificaram diferenças significativas na vtg e na ChE com nenhum dos extratos. Os resultados obtidos sugerem que os extratos de folhas de eucalipto e pinheiro têm efeitos, sobre o peixe-zebra, ao nível do stress oxidativo, mas não parecem ter acção anti-colinérgica ou de disrupção endócrina.

Com base nos efeitos tóxicos causados a embriões de peixes e anfíbios, obtidos neste estudo, por exposição dos mesmos a concentrações de extratos aquosos de folhas de eucalipto e pinheiro, que poderão ser encontradas no contexto real, afirmam-se os efeitos deletérios que os lixiviados das florestas plantadas de eucalipto e pinheiro podem ter nos estágios de vida primários das espécies aquáticas.

keywords

eucalypt; pine; amphibian; fish; embryo toxicity; LC50; biomarkers.

abstract

Deforestation of natural forests allied to the increasing extent of monoculture plantation forests, such as eucalypt and pine, causes biodiversity loss and has other consequences that are particularly damaging in intermittent streams, harming aquatic organisms, like fishes and amphibians.

The present study aimed to: (i) assess the physico-chemical characteristics of laboratory aqueous extracts of eucalypt and pine leafs, and of intermittent stream summer pools draining eucalypt, pine or broadleaf forests; (ii) evaluate the toxicity of leaf extracts of pine and eucalypt in the embryos of three model species, the Zebrafish *Danio rerio*, the Perez's frog *Pelophylax*. perezi and the African clawed frog *Xenopus laevis*, and compare the sensitivity of the studied species to the different leaf extracts using malformations and mortality as main endpoints; (iii) assess the sub-lethal effects of eucalypt and pine leaf aqueous extracts in zebrafish embryos, using the biomarkers vitellogenin (vtg), catalase (CAT) glutathione S-transferase (GST) and cholinesterase (ChE) as main endpoints.

In general, leaf extracts were found to reduce water oxygen and pH, and increase dissolved phenolics, terpenes, lipids and conductivity. The eucalypt leaf extracts were more toxic than pine extracts for *P. perezi* and *D. rerio* embryos. Whereas for *X. laevis* the toxicity of these extracts seems to be similar. The sensitivity of the embryos to the eucalypt extracts was: *D. rerio* > *X. laevis* ≈ *P. perezi*; and to the pine extracts: *X. laevis* > *D. rerio* ≈ *P. perezi*. The malformations most commonly observed were tail curvation and edemas. We observed that exposure to eucalypt and pine leaf extracts induced teratogenicity in *X. laevis* embryos.

CAT activity decreased for the eucalypt and pine leaf extracts. GST activity decreased in organisms exposed to the eucalypt leaf extract and increased for the pine. There were no significant differences in vtg nor in ChE for any of the extracts tested. These results suggest that eucalypt and pine leaf aqueous extracts affect the zebrafish embryos causing oxidative stress, but do not seem to have anti-cholinergic action nor to cause endocrine disruption.

Based on the toxic effects to fish and amphibian embryos, obtained in this study, by exposing them to concentrations of eucalypt and pine leaf aqueous extracts, which can be found in the real context, the harmful effects that eucalypt and pine plantation forest leachates might have on the aquatic species early life stages are affirmed.

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1 Chapter One

General Introduction, Objectives and Relevance of the Study

1.1 General introduction

Natural forests have been gradually replaced by other types of forests, plantations, mainly monocultures, often composed of introduced species. Due to the economic advantages, characteristics of growth or development, indigenous species lose their space for exotic tree species, especially eucalyptus and pine (Jaiyeoba, 2001). Compared to short vegetation, planted forests, especially coniferous, can significantly increase the transfer of acidifying pollutants, from the air to the surface, and then into groundwater (Cannell, 1999).

Afforestation of pine and eucalypt is associated with changes in water quality, and can have detrimental impacts on aquatic species. The impact on stream communities could be due to the release of soluble organic and inorganic compounds by leaching (Canhoto and Laranjeira, 2007). Forest ecosystems are extremely important for conservation because they sustain biodiversity and provide numerous ecosystem services (Shvidenko *et al.*, 2005), however information about the biological effects of pine and eucapliyt leachates on aquatic organisms is still scarce. This is especially true for intermittent stream habitats in Mediterranean climates that support fish and amphibians with a high conservation value. In this context, in a parallel work performed by our research team under the project DOMINO EFFECT (Degradation of lotic ecosystems associated with plantation forestry: an evaluation of plantation forest food-web communities) we investigated amphibian biodiversity in streams of contrasting forest management and the relative importance of habitat characteristics for the amphibian communities of 29 Portuguese streams draining catchments of maritime pine, eucalypt and natural broadleaf forests (Costa et al. submitted, see Annex I). Variance partitioning revealed natural biogeographic descriptors (e.g. stream link magnitude, altitude) and management descriptors (e.g. forest type, Eucalypt/*Acacia sp.* riparian trees) both explained a significant proportion of variation in amphibian assemblages across sites. These comparatively diverse and abundant communities emphasize the ecological importance of amphibians in the patchy, stochastic habitats of both natural and plantation forest streams dominated by an Atlantic-Mediterranean climate.

In the present work a more ecotoxicological approach was performed, in order to understand the direct lethal and sub-lethal effects that eucalypt and pine leachates might have to aquatic biota, namely fish and amphibian early life stages such as embryos.

In this way, the Fish Embryo Toxicity (FET) test with the zebrafish (*Danio rerio*) and the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) with Perez's frog (*Pelophylax perezi*) and the african clawed frog (*Xenopus laevis*) were conducted on exposure of newly fertilized eggs and are expected to reflect toxic risk in fish and amphibian in general and are used as an alternative test method to the acute toxicity tests with juvenile and adult fish (ASTM, 1998; OECD, 2006).

Biomarkers reveal changes in the physiological state of the organisms due to exposure to pollutants, thus providing sensitive early-warning signs of environmental risk of pollution or stress conditions to the organisms (Moore *et al*., 2004; Gastaldi *et al*., 2007), before other sub-lethal effects become apparent, such as inhibition of growth or reproduction (Van Gestel *et al*., 2009). In this work different biomarkers were used to assess the effects of eucalypt and pine leaf extracts on fish and frog embryos, namely: vitelogenine (vtg), catalase (CAT), glutathione S-transferases (GST) and Colinesterase (ChE).

The vitellogenin is tied to the endocrine system, which is a complex mechanism that regulates and coordinates the communication between cells, comprising combinations between glands and hormones and is responsible for biological functions such as reproduction, embryonic development, growth and metabolism (Reis-Filho *et al*., 2006).

CAT and GST were analyzed because they are enzymes responsible for the detoxification of the body, acting at the level of oxidative stress, avoiding excessive accumulation of harmful substances in the cells, which can cause serious injuries. Maintaining the balance between the production of free radicals and antioxidant defenses is an essential condition for the normal functioning of the body (Cogo *et al*., 2009). ChEs have been divided in two types: acetylcholinesterase (AChE) and butyrylcholinesterase. They are a group of enzymes from the family of esterases, and their main function is the hydrolysation of carboxylic esters. An inhibition of the ChE thereby interferes with neurotransmission in cholinergic synapses and neuromuscular junctions (Küster, 2005).

1.1.1 Description of the species

Plant leaf extracts tested:

***Eucalyptus globulus* Labill.** (Tasmanian blue gum) is a species belonging to the group of hardwoods, originating in southwestern Australia, and considered a fast-growing species, reaching 80 m tall as an adult. It has evergreen leaves with high capacity to absorb water and nutrients, and has a great ability to adapt to the ecological conditions in different environments. *E. globulus* adapts to almost any soil, even the poorest, but is more productive in soils with plenty of water, non-compacted and low in calcium. Since the organic matter decomposes readily and the finest roots absorb the nutrients and avoid losses, the nutrient cycle is quite fast. *E. globulus* is the species most used in the paper industry, in temperate regions around the world (Acuña *et al*., 2012; Machado, 2012).

***Pinus pinaster* Aiton** (maritime pine) is a species of conifer native to Europe and the Mediterranean. It belongs to the group of conifers, with rapid growth and can reach up to 40 m tall in adult and 200 years of life. The leaves are persistent, hard and needle-shaped, which assimilate carbon and store nutrients and the root system is constituted by surface roots and deep roots. *P. pinaster* provides good resistance in poor soils and light textures. It’s a species vulnerable to forest fires, since the surrounding layer is massive in dead pine needles, and is highly flammable due to the presence of volatile resin, however, it also has characteristics that give some resistance to fire as a thick trunk and seed release by the cones right after fire (Machado, 2012).

Model species used to test the plant leaf extracts:

Pelophylax perezi Seoane, 1885 (Perez's Frog) occurs throughout the Iberian Peninsula and reaches its northern limit in southern France, although this is not yet well defined (García-París, 1997; Llorente and Arano, 1997 cited in Brito, 2010). It is very dependent on water bodies, occupying almost all habitats, like ponds, swamps, marshes, lakes, dams and streams (Almeida *et al.*, 2001 cited in Brito, 2010), which along with being the most resistant species and has more tolerant ecological requirements of all the amphibians present in the Iberian Peninsula (Llorente *et al.*, 2002 cited in Brito, 2010): it's conservation status is considered to be stable (Brito, 2010).

Xenopus laevis Wagner, 1827 (African clawed frog) it's a species of African origin belonging to the family Pipidae. It has been introduced to several other continents outside its natural distribution limits. It is the most studied amphibian worldwide and its use as a laboratory model is associated with a wide range of important scientific findings in the fields of embryology, molecular biology and physiology, existing in animal facilities in virtually all laboratories worldwide. It's a species heavily dependent on aquatic ecosystems and occupies a variety of habitats. It has a high reproductive and dispersion capacity as long as humidity is high. It's a predator of larvae and adults of other species, with strong documented declines of native amphibian's density in places where it was introduced (Lillo et al., 2008 cited in Brito, 2010), and thus represents a threat to their conservation (Brito, 2010).

Amphibians life cycle switches between earth and aquatic phases. The larval status occurs normally in the aquatic medium and, after metamorphosis, most of the species are terrestrial, although depending on the aquatic medium to reproduce. Despite this ecological limitation, as they have to keep close to wet areas, they have been able to colonize most of the terrestrial biotypes.

Amphibians have permeable skin, which allows for breathing, water regulation and physical as well as chemical protection. Reproduction is oviparous and the embryo develops primarily with the reserves of the egg. These are surrounded by a gelatinous capsule, which serves as protection against pathogens, predators, ultraviolet and mechanical wear, and also allows respiratory exchanges and the maintenance of water balance (Almeida *et al.*, 2001).

Danio rerio Hamilton, 1822 (Zebrafish) is a tropical freshwater fish, member of the family *Cyprinidae*, indigenous to South Asia, and is broadly distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan (Kishi *et al*., 2003).

The zebrafish is omnivorous, and its natural diet is composed of zooplankton, insects and phytoplankton (Kishi *et al*., 2003; Spence *et al*., 2008). It has a short generation time of 3–5 months and large clutch sizes of about 100–200 eggs per female. Their relatively small size makes them easier to manage in large numbers in the laboratory environment. Also, the greatest advantage of the zebrafish as a model system comes from its well-characterised genetic code, and the availability of well-characterised mutants. Zebrafish are also a tractable species for behavioural experiments, readily acclimatising to new environments, being constantly active and little disturbed by the presence of observers (Kishi *et al*., 2003; Spence *et al*., 2008).

1.2 General Objectives

The main goals of this study were to answer the following questions:

1. Do eucalypt and pine leaf aqueous extracts have effects on fish and frog embryos?

2. Which physico-chemical changes in water quality are associated with eucalypt and pine leaf-leachates?

3. Do biomarkers respond to eucalypt and pine leaf aqueous extracts in fish embryos?

1.3 Relevance of the study

In this study we evaluated the toxicity of two aqueous extracts that from pine and eucalypt leaves to assess how these plantation forests might pose a risk to aquatic biodiversity. This evaluation was achieved through toxicity tests with embryos of three different species, one fish species (*D. rerio*) and two amphibian species (*P. perezi* and *X. laevis*). In these tests we evaluated lethal parameters and sub-lethal parameters like malformation and biochemical alterations, which give us information on adverse physiological effects, which could be detected before any alteration at higher levels of biological organization. In addition to the toxicity tests, physico-chemical parameters were also evaluated, using the aqueous extracts prepared in the laboratory and field collected samples, which bridge laboratory and reality, and give us guidance about which compounds may be granting these plantations and their toxicity.

This study investigates the effects of two plant extracts, complex mixtures of plant derivated compounds, in three model species, using parameters at different levels of biological organization It is intended to contribute to the knowledge of the impact that eucalypt and pine monocultures might have on aquatic species.

1.4 Organization of the thesis

Chapter 1 - General Introduction, Objectives and Relevance of the study

Introductory chapter with a short approach to the general context around the main theme of the work, description of the species involved, presentation of the general objectives and relevance of the study.

Chapter 2 - Sensitivity of zebrafish (*Danio rerio*) and frog embryos (*Pelophylax perezi* and *Xenopus laevis*) to eucalypt and pine leaf aqueous extracts (scientific paper format)

This chapter presents a study evaluating the sensitivity of the embryos of the different aquatic model species to eucalypt and pine leaf aqueous extracts assessed in terms of mortality and sub-lethal effects such as malformations. The effects obtained are discussed in relation to the physico-chemical parameters of leaf extracts.

Chapter 3 - Effects of eucalypt and pine leaf aqueous extracts on biomarkers of zebrafish (*Danio rerio*) embryos (scientific paper format)

Reports the findings of a the study of sub-lethal effects of eucalypt and pine leaf aqueous extracts, in *D. rerio*, through analysis of the biomarkers vtg, CAT, GST and ChE.

Chapter 4 – Concluding Remarks

Contains the main conclusions of the study and some ideas for the future research.

Annex I

Scientific paper submitted for publication in Biological Conservation. Joana Correia is co-author of this work. Her main input was in the field work and in the survey and identification of amphibian species.

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2 Chapter Two

Sensitivity of zebrafish (*Danio rerio*) and frog embryos (*Pelophylax perezi* and *Xenopus laevis*) to eucalypt and pine leaf aqueous extracts

2.1 Abstract

Deforestation of natural forests allied to the increasing extent of monoculture plantation forests causes biodiversity loss. Afforestation of pine and eucalypt is associated with alterations in stream hydrology, including increasing conductivity and acidity. These effects can be particularly emphatic in intermittent streams where leaf litter accumulating in isolated pools of standing water that represent important nursery habitat for fish and amphibians. Therefore, in the present study, the toxicity of eucalypt and pine leaf aqueous extracts was evaluated using embryo toxicity testing in the perez’s frog (*Pelophylax perezi*), african clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*). The endpoints monitored included mortality and malformations in embryo development. A gradient of leaf aqueous extract concentrations was tested, ranging from 1.6 to 32, 0.16 to 4.8 and 0.64 to 20.48 g L^{-1} of eucalypt leaf extract with *P. perezi*, *X. laevis* and *D. rerio*, respectively and from 1.6 to 32, 0.16 to 6.4 and 1.6 to 32 g L^{-1} of pine leaf extract with *P. perezi*, *X. laevis* and *D. rerio*. In general, leaf extracts were found to reduce water oxygen and pH, and increase dissolved phenolics, terpenes, lipids and conductivity. The eucalypt leaf extracts were more toxic than pine extracts for *P. perezi* and *D. rerio* embryos, whereas for *X. laevis* the toxicity of these extracts appeared to be similar. The sensitivity of the embryos to the eucalypt extracts was: *D. rerio* > *X. laevis* ≈ *P. perezi* and for pine extracts: *X. laevis* > *D. rerio* ≈ *P. perezi*. The malformations most commonly observed were tail curvation and edemas. We observed that exposure to eucalypt and pine leaf extracts induced teratogenicity in *X. laevis* embryos.

Overall, the toxic effects on fish and amphibian embryos obtained in the present study by the exposure to environmentally realistic concentrations of eucalypt and pine leaf extracts suggest that leaf leachates associated with eucalypt and pine plantation forest can have a negative effect on the early life stages of ecological contrasting aquatic fauna.

Keywords: afforestation; amphibian; fish; embryo toxicity

2.2 Introduction

Fundamentally caused by human activity, deforestation is a major cause of biodiversity loss (Wilson *et al*., 1998; Davis *et al*., 1995; Brook *et al*., 2003; Laurance, 2007). Between 2000 and 2010, the world lost per year, approximately 13 million hectares of forests that were converted to other land-uses or lost due to natural causes, compared to 16 million hectares during the 1990s. In addition, the total area of planted forest was estimated to be 264 million hectares on 2010, corresponding to 6.6 percent of the forest area (FAO, 2010).

Afforestation of pine and eucalypt is associated with changes in water quality, including an increase of phenolic compounds, conductivity and stream acidity and a reduction in base cations, nitrogen (N) and dissolved oxygen (Canhoto and Laranjeira, 2007; Farley *et al*., 2008). The impact on stream communities could be due to the release of soluble organic and inorganic compounds by leaching (Tukey, 1970 cited in Canhoto and Laranjeira, 2007). The influence of catchment and riparian vegetation is inversely proportional to stream size (Hynes, 1975; Vannote *et al*. 1980), so adverse effects on aquatic systems are mainly observed in low order streams running through these plantations, which are reduced to summer pools with stained water due to leaf leachates, but scarcely studied. Knowledge about the effects of leaf extracts of pine and eucalypt on the development of fish and amphibians embryos arises as extremely relevant towards the understanding of the impact of eucalypt and pine afforestation, particularly in intermittent streams.

Amphibian populations have been identified to be at major risk on a global scale, in part because of their high sensitivity to environmental contamination which makes them good indicators of ecological quality (Stuart *et al*., 2004; Gascon *et al*., 2005; Hopkins, 2007). Their thin permeable skin and jelly-coat egg membranes may function as a barrier to certain compounds, but absorbs moisture and many compounds and pollutants dissolved in water (Wells, 2007). The Perez's frog *Pelophylax perezi* has a biphasic life cycle, with an aquatic larval stage (tadpoles) and a terrestrial adult stage, which gives it an important role in the food webs of aquatic and terrestrial systems (Duellan and Trueb, 1994; Wilbur, 1987; Pough, 2007). The african clawed frog *Xenopus laevis* is a well-established model in developmental biology and toxicology (Pettersson *et al*., 2006).

Danio rerio is a small tropical freshwater fish which can be easily managed in large numbers in the laboratory (Kishi *et al*., 2003) and is one of the most widely used model fishes in the field of ecotoxicology, because of their physiological characteristics and the available background knowledge (Scholz *et al*., 2008). The zebrafish have short generation times of approximately three to five months and develop from a fertilised egg to an adult outside the female in a transparent egg, as well as the embryos themselves during their first days of life (Wixon, 2000).

Both amphibian and zebrafish embryonic development has been well characterized (Gosner, 1960; Nieuwkoop and Faber, 1975; Kimmel *et al*., 1995).

For the study of acute toxicity in amphibians, the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is currently used (ASTM, 1998). The FETAX assay is a 96-hour exposure standardized test, with *Xenopus laevis* embryos, developed to determine the teratogenic and toxicity potential of chemicals and complex mixtures (ASTM, 1998; Kapfer *et al*., 2007; Martini *et al*., 2012).

To determine acute toxicity of chemicals on embryonic stages of fish, the Organization for Economic Co-operation and Development (OECD) recommended a Fish Embryo Toxicity (FET) test with the zebrafish (*Danio rerio*) as an alternative test method to the acute toxicity tests with juvenile and adult fish (OECD, 2006).

The present study aimed to: (i) assess the physico-chemical characteristics of laboratory aqueous extracts of eucalypt and pine leafs, and of intermittent stream summer pools draining eucalypt, pine or broadleaf forests and (ii) evaluate the toxicity of leaf extracts of pine and eucalypt in the embryos of three model species, the zebrafish and the Perez's and the african clawed frogs and compare the sensitivity of the studied species to the different leaf extracts using malformations and mortality as main endpoints.

2.3 Material and Methods

2.3.1 Tested species

P. perezi eggs were collected from a freshwater pond (+40.596364N; -8.695690W) near Aveiro, Portugal, on May 2011, at Gosner developmental stage 8-9 (Gosner, 1960) and used in the assays at stage 9-11 (Gosner, 1960).

Eggs masses of *X. laevis* were obtained from individuals maintained at Watchfrog (Paris, France), by in vitro fertilization and incubated at 16°C until the blastula stage 8-9 (Nieuwkoop and Faber, 1975). The blastula selected were then incubated with leaf aqueous extracts and placed at 24°C.

D. rerio eggs were obtained from a culture established at the Department of Biology, University of Aveiro, which is maintained in carbon-filtered water at 27.0 ± 1 ºC; under a 16:8h (light:dark) photoperiod cycle; conductivity is kept at 550 ± 50 µS; dissolved oxygen at 95% saturation and pH at 7.5 ± 0.5. The eggs were collected approximately 30 minutes after mating, washed and selected. During cleavage the unfertilized eggs or with abnormalities were discarded (Kimmel *et al*., 1995).

2.3.2 Preparation of leaf aqueous extracts for FET and FETAX

The eucalypt and pine leaves were collected immediately after leaf fall. Leaf aqueous extracts were prepared on the same day as collection by immersing 32 g of eucalypt or pine leaves in 1 L of FETAX medium (*Pelophylax perezi* and *Xenopus laevis*) or FET medium (*Danio rerio*) and maintained for 48 hours (Canhoto and Laranjeira, 2007) in controlled conditions (photoperiod and temperature similar to those of the subsequent animal exposure experiments). Leaf extracts were collected, filtered, divided in aliquots and stored at -20º C until use. Before being used, the aliquots were thaw overnight at 4°C. The different leaf extract concentrations were prepared by diluting the initial leaf extracts with the appropriate medium, FETAX or FET medium, for amphibians or zebrafish embryo testing, respectively.

2.3.3 Chemical analysis of leaf extracts and water from intermittent streams

A total of thirteen 4th order intermittent streams located in the north and center of Portugal were selected for water physico-chemical analysis. Based on upstream catchment landuse seven were classified as monocultures of eucalypt (*Eucalyptus globulus* Labill.), four as maritime pine (*Pinus pinaster* Ait.) and two as broadleaf forest. Sampling was performed twice at each location during the summer of 2011 and 2012. Water physico-chemical parameters (temperature, dissolved oxygen, pH and conductivity) were analyzed *in situ* and a 500 ml water sample was colected for terpenes and lipids measurements. In order to simulate leaf leaching in intermittent streams of eucalypt and pine monocultures, eucalypt and pine leaf extracts were prepared in distilled water in the same conditions as explained above for FETAX and FET testing. The physico-chemical parameters were also measured, the extract filtered, divided in aliquots and stored at -20º C until chemical analysis. Before being used, the aliquots were allowed to thaw overnight at 4°C. Phenols, terpenes and lipids (Volatile Fatty Acids) were analyzed by HPLC, Headspace-GCMS and GCMS, respectively.

2.3.4 Exposure conditions

Pelophylax perezi

All procedures followed the guidelines defined by ASTM (1998) draft guideline FETAX test. The field collected eggs masses were dejellied with 2% cistein in FETAX solution (pH 8.1) to individualize eggs but keeping egg jelly coats undamaged. A total of 585 eggs were distributed in Petri dishes (55mm of diameter): 3 replicates, with 15 eggs each per 10 mL of test solution, were performed for the six concentrations (1.6, 3.2, 6.4, 12.8, 25.6, 32 g L^{-1}) of eucalypt or pine leaf extracts tested, and for the control (FETAX medium) as well. Eggs were then maintained under controlled conditions, with a 14:10h (light:dark) photoperiod and temperature of 23 ± 1ºC, during the exposure test period of 96 hours. Mortality, temperature, dissolved oxygen, pH and conductivity were checked once a day, dead individuals and excess organic waste (*e.g.* egg jelly) were removed to prevent oxygen depletion and excessive bacterial growth, and test solutions were renewed. After 96 h of exposure, malformations were identified.

Xenopus laevis

Similarly to *P. perezi*, the assays with *X. laevis* followed FETAX guidelines (ASTM, 1998) as explained above. A total of 440 eggs of *X. laevis* were exposed in 6-well microplates: 2 replicates, 10 eggs per 1-well microplate for each test concentration: 0.16, 0.32, 0.64, 1.28, 1.6, 1.92, 2.24, 2.56, 3.2, 4.8 g L^{-1} for eucalypt leaf extracts, and 0.16, 0.32, 0.64, 1.28, 1.6, 1.92, 2.24, 2.56, 3.2, 6.4 g L^{-1} for pine leaf extracts; 4 replicates of 10 eggs each in the case of control. After 96 h of exposure, malformations were identified.

Danio rerio

The *D. rerio* assays were performed based on OECD draft guideline FET test (OECD, 2006) and on the embryo test described by Fraysse (2006), with a 16h:8h (light:dark) photoperiod and temperature of 26 ± 1ºC. The bioassays were conducted during 96 hours, with the following concentrations: 0.64, 1.28, 2.56, 5.12, 10.24, 20.48 g L^{-1} for eucalypt leaf extracts, 1.6, 3.2, 6.4, 12.8, 29.26, 32 g L^{-1} for pine leaf extracts, and a control constituted by the FET medium. Using 24-wells microplates 396 eggs were placed one per well with 2 mL of test solution; 30 eggs per treatment (3 replicates, 10 eggs per replicate) and 36 eggs for control (representing 3 replicates with 12 eggs per replicate). Every 24 h, the medium was renewed, the mortality checked and physico-chemical parameters (temperature, dissolved oxygen, pH and conductivity) measured. After 96 h of treatment, malformations were identified.

2.3.5 Data analysis

Cumulative mortality was updated every 24 h, the mortality rates were calculated as the mean percentage of dead embryos every 24 h and the total mortality after 96 h.

For each sample with observable malformations at 96 h, probit analysis was employed to calculate effect concentrations in 50% of the cases (EC_{50}) with 95% confidence intervals (CI). The same analysis was used to calculate lethal concentrations in 50% (LC_{50}) and 10% (LC_{10}) of the cases, and their 95% CI, at 96 h period of exposure.

All data was checked for normality and homogeneity of variances (Zar, 1996) with Ryan-Joiner and Bartlett's tests, respectively.

Significant differences between the control group and concentrations for all assays, regarding mortality and malformations, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC), were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test, choosing a level of significance of 0.05 for rejecting the null hypothesis. All statistical analysis was performed using Minitab® 16.1.1, Inc 2010.

2.4 Results

2.4.1 Chemical analysis of intermittent streams and leaf extracts

The values of phenols, terpenes, lipids, pH, conductivity, dissolved oxygen and temperature in the 100% leaf extracts and the extreme values achieved for the same parameters in the field water samples are presented in Table 1. In general, terpenes, lipids, conductivity and temperature are higher in the 100% leaf extracts than the field water samples, and pH and dissolved oxygen are lower. The same applies comparing 100% eucalypt and pine leaf extracts, respectively. Between the field water samples, the eucalypt samples follow the same trend described above, while the parameters for pine samples are similar to broadleaf.

Table 1 – Physico-chemical characterization of eucalypt and pine leaf extracts after 48 h of incubation of 32 g of leaves in 1 L of distilled water and of field water samples collected in intermittent streams.

Sample		**Phenols (mg L^{-1})**	**Terpenes (ųg L^{-1})**	**Lipids (ųg L^{-1})**	**pH**	**Conductivity (ųs/cm)**	**D.O. (mg L^{-1})**	**Temp (ºC)**
100% leaf extract	Eucalypt	0.31	38.00	1653.00	3.90	617.75	2.96	23.08
	Pine	0.87	11.00	480.00	4.81	482.75	1.67	22.85
Field water streams	Eucalypt	n.a.	12.50	77.00	5.68	156.10	1.31	14.00
	Pine	n.a.	0.00	14.00	5.63	49.90	6.32	14.50
	Broadleaf	n.a.	5.90	8.10	6.14	68.50	5.88	13.10

n.a. = values not available

The greatest variations in the physico-chemical parameters belong to the variable dissolved oxygen which reaches the lowest value of 0.2 g L^{-1} in the *P. perezi* test with the pine leaf aqueous extract, with the equivalent maximum value of 10.1 g L^{-1} which represents a 98% decrease in dissolved oxygen comparative to the control group. In other variables, differences when compared to the control group are less abrupt, maximum values of 2% for temperature (24.1ºC in the *P. perezi* test with pine leaf extract; 26.0ºC in the control) and 45% to the pH (7.7 in the *P. perezi* test with pine leaf extract; 4.2 in the control). Conductivity is the only variable that increased (68%) comparative to control group, particularly in the *D. rerio* tests, with the minimum value of 999 ųs/cm in the *P. perezi* test with the pine leaf extract (Table 2).

Table 2 – Minimum and maximum values of physico-chemical parameters during the 96 h exposure of *P. perezi* and *D. rerio* to eucalypt or pine leaf aqueous extracts and control solutions.

Organisms	**Leaf extracts**	**Concentration (g L^{-1})**	**pH**	**Conductivity (ųs/cm)**	**D.O. (mg L^{-1})**	**Temp (ºC)**
P. perezi		0.00	6.0 – 7.7	654 – 863	7.1 – 10.1	24.2 – 26.0
	Eucalypt	32.00	4.8 – 6.2	855 – 894	0.6 – 1.8	24.9 – 25.4
	Pine	32.00	4.2 – 6.7	677 – 881	0.2 – 0.7	24.1 – 25.1
D. rerio		0.00	7.3 – 7.7	1171 – 1211	14.3 – 14.9	22.6 – 24.5
	Eucalypt	20.48	4.3 – 4.5	1576 – 1677	2.3 – 3.6	23.7 – 25.0
		0.00	7.8 – 8.1	999 – 1145	15.4 – 16.5	22.4 – 25.2
	Pine	32.00	5.9 – 6.2	1143 – 1465	1.1 – 12.4	24.1 – 25.1

2.4.2 Lethal and sublethal effects

At the end of the assays, no mortality was registered in the control group of *P. perezi* test and was lower than 10% in the *X. laevis* and *D. rerio* tests (Table 3), as required to validate the tests.

Mortality has significantly increased in *P. perezi* embryos exposed to the three highest concentrations of eucalypt (F=1980.83; DF=6; P<0.001) and pine (F=761.75; DF=6; P<0.001) leaf aqueous extracts; and in *D. rerio* embryos exposed to the three highest concentrations of eucalypt leaf extract (F=326.07; DF=6; P<0.001) and the two highest concentrations of pine leaf extract (F=22.00; DF=6; P<0.001). Eucalypt and pine leaf extracts did not significantly affect the occurrence of mortality in *X. laevis* embryos (F=1.97; DF=10; P=0.126 and F=2.17; DF=10; P=0.096, respectively.

Table 3 – Percentage of cumulative mortality in *Pelophylax perezi*, *Xenopus laevis* and *Danio rerio* larvae at the end of the exposure period (96 h), on eucalypt and pine leaf aqueous extracts.

Cumulative mortality (%) – 96 h										
P. perezi			***X. laevis***				***D. rerio***			
C	**Euc**	**Pin**	**C**	**Euc**	**C**	**Pin**	**C**	**Euc**	**C**	**Pin**
0.00	0.0	0.0	**0.00**	2.5	**0.00**	5.0	**0.00**	2.8	**0.00**	8.3
1.60	2.2	6.7	**0.16**	10.0	**0.16**	0.0	**0.64**	0.0	**1.60**	16.7
3.20	0.0	0.0	**0.32**	0.0	**0.32**	0.0	**1.28**	10.0	**3.20**	10.0
6.40	2.2	2.2	**0.64**	0.0	**0.64**	0.0	**2.56**	6.7	**6.40**	23.3
12.80	100.0*	46.7*	**1.28**	5.0	**1.28**	10.0	**5.12**	100.0*	**12.80**	33.3
25.60	100.0*	100.0*	**1.60**	5.0	**1.60**	10.0	**10.24**	100.0*	**29.26**	100.0*
32.00	100.0*	100.0*	**1.92**	10.0	**1.92**	10.0	**20.48**	100.0*	**32.00**	100.0*
-	-	-	**2.24**	10.0	**2.24**	25.0	-	-	-	-
-	-	-	**2.56**	10.0	**2.56**	10.0	-	-	-	-
-	-	-	**3.20**	5.0	**3.20**	25.0	-	-	-	-
-	-	-	**4.80**	25.0	**6.40**	60.0	-	-	-	-

C = Extract concentration (g L^{-1}); Euc = Eucalypt leaf extract; Pin = Pine leaf extract; *$p \leq 0.05$

In the control group of *X. laevis* assay with eucalypt extract 20.51% of malformations were observed. Malformations were lower than 10% in the control groups of the *D. rerio* and the *X. laevis* assays with pine leaf extracts, and were not observed any malformations in the control of the remaining bioassays (Table 4).

After 96 h of exposure, eucalypt leaf extracts significantly increased malformations in *P. perezi* embryos exposed to the 6.4 g L^{-1} concentration (F=14.25; DF=4; P<0.001) and in *X. laevis* embryos exposed to the the three highest concentrations (F=11.44; DF=10; P<0.001). Eucalypt leaf extracts did not significantly affected the occurrence of malformations in *D. rerio* embryos (F=1.00; DF=4; P=0.452), in the same way that pine leaf extracts did not significantly affect the occurrence of malformations in *P. perezi* embryos (F=0.50; DF=4; P=0.737), in *D. rerio* embryos (F=0.50; DF=4; P=0.737) and in *X. laevis* embryos (F=2.58; DF=10; P=0.056) (Table 4).

Table 4 – Percentage of malformations in surviving *Pelophylax perezi*, *Xenopus laevis* and *Danio rerio* larvae at the end of the exposure period (96 h), on eucalypt and pine leaf aqueous extracts.

Malformations (%) – 96 h										
P. perezi			***X. laevis***				***D. rerio***			
C	**Euc**	**Pine**	**C**	**Euc**	**C**	**Pine**	**C**	**Euc**	**C**	**Pine**
0.00	0.0	0.0	**0.00**	20.51	**0.00**	7.89	**0.00**	0.00	**0.00**	3.03
1.60	4.6	2.4	**0.16**	6.0	**0.16**	0.0	**0.64**	3.33	**1.60**	4.00
3.20	0.0	2.2	**0.32**	5.0	**0.32**	5.0	**1.28**	0.00	**3.20**	3.85
6.40	25.0*	2.3	**0.64**	5.0	**0.64**	20.0	**2.56**	0.00	**6.40**	0.00
12.80	-	0.0	**1.28**	5.0	**1.28**	61.0	**5.12**	-	**12.80**	0.00
25.60	-	-	**1.60**	42.0	**1.60**	11.0	**10.24**	-	**29.26**	-
32.00	-	-	**1.92**	33.0	**1.92**	28.0	**20.48**	-	**32.00**	-
-	-	-	**2.24**	56.0*	**2.24**	67.0	**-**	-	-	-
-	-	-	**2.56**	17.0	**2.56**	28.0	**-**	-	-	-
-	-	-	**3.20**	53.0*	**3.20**	67.0	**-**	-	-	-
-	-	-	**4.80**	87.0*	**6.40**	75.0	**-**	-	-	-

C = Extract concentration (g L^{-1}); Euc = Eucalypt leaf extract; Pin = Pine leaf extract; *p≤0.05

Surviving zebrafish and frog embryos showed a variety of malformations in both leaf extracts, most common observed were: tail curvation, tail shortening, cardiac edema and abdominal swelling (Figure 1).

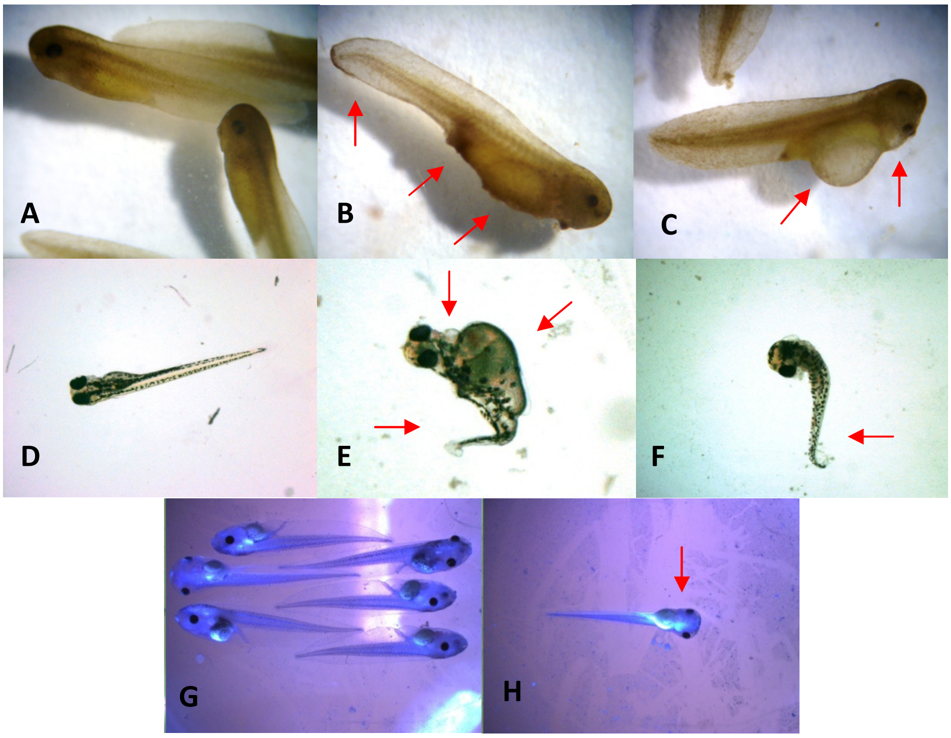

Figure 1 – Surviving organisms of *Pelophylax perezi* and *Danio rerio* after 96 h exposed to leaf extracts. A) *P. perezi* larvae from control treatment (FETAX medium) without malformations; B) *P. perezi* larvae exposed to 1.6 g L^{-1} eucalypt leaf extract with tail and body deformations; C) *P. perezi* larvae exposed to 3.2 g L^{-1} pine leaf extract with abdominal and cardiac edemas; D) *D. rerio* larvae from control treatment (FET medium) without malformations; E) *D. rerio* larvae exposed to 0.64 g L^{-1} eucalypt leaf extract with cardiac edema, tail and body deformations; F) *D. rerio* larvae exposed to 3.2 g L^{-1} pine leaf extract with tail curvation; G) *X. laevis* larvae from control treatment (FETAX medium) without malformations; H) *X. laevis* larvae exposed to pine leaf extract with microcephaly.

The values of LC$_{50}$, LC$_{10}$, and EC$_{10}$, EC$_{50}$ for malformations and their respective confidence limits are presented in Table 5. The eucalypt leaf extracts were more toxic than pine extracts for *P. perezi* (LC$_{50}$ 96 h = 9.0 and 13.3 g L^{-1}, respectively) and *D. rerio* embryos (LC$_{50}$ 96 h = 3.3 and 12.8 g L^{-1}, respectively), as the LC$_{50}$ values for both species were significantly lower when exposed to the eucalypt extracts (see Table 3). Whereas for *X. laevis* the toxicity of these extracts seems to be similar, since there are no significant differences between the LC$_{50}$ of the eucalypt and pine leaf extracts (LC$_{50}$ 96 h = 8.2 and 5.4 g L^{-1}, respectively).

For eucalypt leaf extract, the *D. rerio* present a significantly lower LC$_{50}$ than the two other species. They present the following order of sensitivity to the eucalypt extracts: *D. rerio* > *X. laevis* ≈ *P. perezi*. For pine leaf extract, the *X. laevis* present a significantly lower LC$_{50}$

than the two other species. The sensitivity of the embryos to the pine extracts is: *X. laevis* > *D. rerio* ≈ *P. perezi*.

While the EC_{50} for malformations in *X. laevis* presented similar values for both leaf extracts (EC_{50} 96 h = 3.0 g L^{-1}), the EC_{50} of eucalypt leaf extract are significantly higher for *P. perezi* than for *X. laevis* (LC_{50} 96 h = 8.8 (7.2-13.0) and 3.0 (2.5-3.8) g L^{-1}, respectively).

We observed that exposure to eucalypt and pine leaf extracts induced teratogenicity in *X. laevis* embryos (IT = 2.73 and 1.80, respectively), and did not induce teratogenicity in *P. perezi* (IT = 1.02). It was not possible to calculate the IT in the remaining bioassays (Table 5).

Table 5 – Values of lethal concentration in 10% (LC_{10}) and 50% (LC_{50}) of the cases and effect concentration for malformations in 10% (EC_{10}) and 50% of the cases (EC_{50}), with the respective 95% confidence limits, and teratogenic index (IT), after a 96 h period of exposure of embryos of *Pelophylax perezi*, *Xenopus laevis* and *Danio rerio* to eucalypt and pine leaf extracts.

Organisms	**Leaf extracts**	**LC_{10}**	**LC_{50}**	**EC_{10}**	**EC_{50}**	**IT**
P. perezi	**Eucalypt**	6.4 (5.4 – 7.3)	9.0 (8.1 – 10.0)	4.6 (3.3 – 5.7)	8.8 (7.2 – 13.0)	1.02
	Pine	6.9 (5.2 – 8.3)	13.3 (11.9 – 15.2)	n.a.	n.a.	n.a.
X. laevis	**Eucalypt**	2.7 (1.6 – 4.9)	8.2(5.6 – 21.5)	1.0 (-0.8 – 0.6)	3.0 (2.5 – 3.8)	2.73
	Pine	1.8 (0.9 – 2.4)	5.4 (4.5 – 7.1)	n.a.	3.0 (2.4 – 3.9)	1.80
D. rerio	**Eucalypt**	1.8 (1.3 – 2.2)	3.3 (2.9 – 3.9)	n.a.	n.a.	n.a.
	Pine	1.6 (-1.4 – 3.8)	12.8 (10.7 – 15.5)	n.a.	n.a.	n.a.

n.a. = values not available

The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values for mortality and malformations are presented in Table 6. Concerning mortality the lowest NOEC and LOEC values calculated were for *X. laevis* embryos.

Table 6 – Values of no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for mortality and malformations, with the respective 95% confidence limits, after 96 h exposure of *Pelophylax perezi*, *Xenopus laevis* and *Danio rerio* embryos to eucalypt and pine leaf aqueous extracts.

Organisms	**Leaf extracts**	**Mortality**		**Malformations**	
		NOEC	LOEC	NOEC	LOEC
P. perezi	**Eucalypt**	6.40	12.80	n.a.	6.40
	Pine	6.40	12.80	n.a.	n.a.
X. laevis	**Eucalypt**	n.a.	n.a.	n.a.	2.24
	Pine	n.a.	n.a.	n.a.	n.a.
D. rerio	**Eucalypt**	2.56	5.12	n.a.	n.a.
	Pine	12.80	29.26	n.a.	n.a.

n.a. = values not available

2.5 Discussion

There is significant concern over the impacts of plant invasions on habitat quality for native fauna (Brown *et al*., 2006). Bioassays on plant extracts reported adverse effects on a few species of aquatic organisms (Maerz *et al*., 2005; Jancula *et al*., 2007; Morrongiello *et al*., 2011; Earl *et al*., 2012).

The results obtained in this study indicate that the leaf aqueous extracts presented toxic effects in the tested species, with a degree depending on the species and the extract itself. Eucalypt leaf extracts were more toxic than pine extracts for *P. perezi* and *D. rerio* embryos, whereas for *X. laevis* the toxicity of these extracts seems to be similar. The sensitivity of the embryos to the eucalypt extracts was: *D. rerio* > *X. laevis* ≈ *P. perezi*; and to the pine extracts: *X. laevis* > *D. rerio* ≈ *P. perezi*. We also observed that exposure to eucalypt (LC_{50}=8.2; EC_{50}=3.0; TI=2.73) and pine leaf extracts (LC_{50}=5.4; EC_{50}=3.0; TI=1.80) induced teratogenicity in *X. laevis* embryos.

However, the results concerning *X. laevis* should be interpreted with caution since the maximum mortality obtained was only 25% and LC_{50} was extrapolated, having therefore a high confidence interval associated. In addition, in the control the malformations exceeded 10%, which does not match the FETAX requirements.

The effects of pine tree extract in *Xenopus laevis* were also studied by Jones *et al*. (2009), and similar results were found (LC50=4.333; EC50=2.291; TI=1.89).

According to Earl *et al*. (2012), the non-native white pine extract was more acutely toxic than those from the other plants studied, and tadpoles of two chorus frog (*Pseudacris maculata* and *Pseudacris crucifer*) died within 3 days of exposure; when they examined only the effects of white pine extract, they observed reduced tadpoles survival (*Bufo americanus* and *Hyla versicolor*) 4 hours after being exposed.

Brown *et al*. (2006) explored the hypothesis that aqueous extracts of purple loosestrife leaves (*Lythrum salicaria*), a highly invasive plant in wetlands, could produce high levels of mortality, raising the possibility that soluble compounds in *L. salicaria* leaves affected the productivity or composition of food resources or acted directly on developing tadpoles (Brown *et al*., 2006 cited in Maerz *et al*., 2005).

Diverse studies in the Iberian Peninsula demonstrate that the replacement of the native deciduous vegetation by eucalypt monocultures changed 1) stream hydrology, 2) organic matter dynamics and 3) litter quality (Canhoto and Laranjeira., 2007). *Eucalyptus globulus* Labill. plantations cause negative effects on aquatic systems (Graça *et al*., 2002).

Acute and chronic eucalypt extract exposure affected the behavior and survival of a range of Australian native fish (Gehrke *et al*., 1993; McMaster and Bond, 2008 cited in Morrongiello *et al*., 2011) and on detritivores (Canhoto and Graça, 1996, 1999). *Eucalyptus calmaldulensis* leachates were responsible for high mortality rates on golden perch larva (Gehrke *et al*., 1993).

We observed that, in general, the eucalypt and pine leaf extracts were found to reduce water oxygen and pH, and increase dissolved phenolics, terpenes, lipids and conductivity. We hypothesize that these are the compounds that may be acting upon embryos. As generally recognized, following leaf immersion, a large array of solutes is released into the water resulting in a rapid loss of mass (Gessner *et al*., 1999 cited in Canhoto and Laranjeira, 2007). Plant phenolics include a large range of compounds such as simple phenols, phenolic acids, polyphenols including various brown oxidized products (widely called the tannins), and lignin (Ribereau-Gayon, 1972; Harborne, 1984; Robinson, 1991 cited in Chapuis-Lardy, 2002). According to Bernhard-Reversat *et al*., 2003, phenolic compounds reduce litter decomposition rate (Bignand and Schaefer, 1980; Palm and Rowland, 1997; Bernhard-Reversat, 1998) and tanning prevents the degradation of proteins and other compounds (Ribereau-Gayon, 1972; Palm and Sanchez, 1991) and affects the nitrogen nutrition of microbial decomposers (Aggangan *et al*., 1999). Trémolières (1988) and Canhoto and Laranjeira (2007) concluded that the toxicological effect of eucalypt leachates are explained by a mixture of various compounds, like phenols, oils and saponosides. These compounds can be toxic to amphibians (Maerz *et al*., 2005; Kerby *et al*., 2010), fish (Tremolieres, 1988; Steinberg *et al*., 2006; McMaster and Bond, 2008) and aquatic invertebrates (Canhoto and Laranjeira, 2007). Damage to gill tissues from exposure to tannins causes high and rapid mortality in fish (Temmink *et al*., 1989 cited in Maerz *et al*. 2005). As cited Canhoto and Laranjeira (2007), high phenolic and tannin contents are known to inhibit microbial activity (Bärlocher *et al*., 1995; Canhoto and Graça, 1999) and participate in the species specific biochemical oxygen depletion effect (Trémolières, 1988).

Chase and Ferullo (1957) studied the effect of autumn leaf fall on oxygen levels in lakes and streams, and also observed oxygen depletion (Trémolières, 1988). Inadequate dissolved oxygen in the aquatic environment is a well-established cause of fish morbidity and mortality (Choi *et al*., 2007). The reduction of dissolved oxygen in the water may have been accentuated by organic compounds oxidation, lipids and carbohydrates, which were abundant in the leachate media (Canhoto and Laranjeira, 2007). Some studies reported by Gehrke *et al*. (1993), Fisher *et al*. (2006) and McMaster and Bond (2008) indicate that dissolved organic carbon (DOC) compounds, such as the polyphenols and tannins, can negatively affect fish without involving hypoxia. The effects of DOC depend on the timing, duration and magnitude of DOC, and exposure will determine whether the effects are lethal or sub-lethal responses (Morrongiello *et al*., 2011).

The values obtained for the physico-chemical parameters of the laboratory eucalypt and pine leaf aqueous extracts are higher than those of the field water samples. The analyzed laboratory leaf aqueous extracts represent the maximum concentration (32 g L^{-1}) to which the organisms were exposed (100% leaf extracts) so, and given the LC50's values found, it wouldn't be expected to find such toxic effects on the sampled locals. However, Canhoto and Laranjeira (2007), that evaluated toxicity of such leachates to the shredder *Sericostoma vittatum* (Tricophetera Order), in a 2nd order stream, draining an eucalypt plantation, calculated that up to 32 g of leaf dry mass may accumulate per 1L of pond water, thus being possible to reach values for the physico-chemical parameters similar to those we obtained on the laboratory leaf aqueous extract and consequently higher toxicity. They estimated this value from the density of leaves in the riparian area and stream banks (894 leaves ± 246 (SE)), since the number of leaves inside the pools were difficult to quantify due to their degradation (Canhoto and Laranjeira, 2007). These differences might be related to the stream order, as we made the physico-chemical analysis in 4th order intermittent streams, and according to Speaker *et al*. (1984) the importance of all major retention mechanisms (debris dams, the ratio of wetted perimeter to cross-sectional area, pool area, the relative amount of stream margin, and the frequency of obstructions) tends to decrease as streams get larger.

Overall, the toxicity effects on fish and amphibian embryos obtained in the present study by the exposure to environmental realistic concentrations of eucalypt and pine leaf extracts indicate the deleterious effects that eucalypt and pine plantation forest leachates might have on the aquatic species early life stages.

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3 Chapter Three

Effects of eucalypt and pine leaf aqueous extracts on biomarkers of zebrafish (*Danio rerio*) embryos

3.1 Abstract

Deforestation is a major driver of biodiversity loss and a considerable loss global concern, however, the impact on forested streams is poorly known. Plantations forests are often associated with an increase in water consumption and changes in water quality and sustain a lower diversity of wildlife compared to natural forests. These alterations may be particularly damaging in intermittent streams where water in pool habitat has a long residence-time. The use of biochemical endpoints in ecotoxicology turned a complementary endpoint able to give information on toxicant effects at sublethal level. This work aims at assessing the sub-lethal effects of eucalypt and pine leaf extracts in zebrafish (*Danio rerio*) embryos, using biomarkers as main endpoints. The biomarkers selected for this study were vitellogenin-like proteins (vtg), catalase (CAT), glutathione *S*-transferase (GST) and cholinesterase (ChE). Zebrafish embryos were exposed to sublethal concentrations of eucalypt (0.14, 0.29, 0.58, 1.15, 2.30 g L^{-1}) and pine (0.11, 0.22, 0.45, 0.90, 1.79 g L^{-1}) leaf aqueous extracts and a control following the Fish Embryo Test (FET) draft guidelines. No significant differences were observed between treatments concerning embryo development effects. CAT activity decreased for the eucalypt and pine leaf extracts. GST activity decreased in organisms exposed to the eucalypt leaf extract and increased for the pine. No significant differences in the vtg content and ChE activity were found in exposed embryos. These results suggest no effect at endocrine level or anticholinergic action of these leaf extracts, whereas the alterations on CAT and GST activity suggest that the compounds present in the eucalypt and pine leaf aqueous extracts induce oxidative stress.

Keywords: afforestation; fish; embryo toxicity; biomarkers; sublethal endpoints

3.2 Introduction

Human exploitation, conversion of the ecosystems and losses of natural and semi natural forests, are the major causes of biodiversity loss, since they are causing decline in ecosystem condition (Brockerhoff *et al.*, 2008; Bullock *et al.*, 2011). Against this trend of deforestation, the area of plantations forest is increasing. As more than half of the known terrestrial plant and animal species live in forests (MEA, 2005), it is important to understand the potential conservation opportunities that plantation forests might provide.

Planted forest monocultures, such as eucalypt and pine forests, are high consumers of water, and intensify soil and water acidification directly by increasing base cation uptake from the soil and the production in the soil of organic acids from decomposing needles. They indirectly alter hydrology, as a result of soil drying and site disturbance, which leads to increased solute concentrations and the mineralization of organic sulfur and nitrogen with the production of mobile anions (Cannell, 1999; Canhoto and Laranjeira, 2007).

The quality of stream water from upland regions with a large proportion of forested catchments is generally high with low levels of nutrients and sediment. The adverse effects on the aquatic systems are mainly observed in low order streams running through these plantations, which are reduced to summer pools with stained water due to leaf leachates, but scarcely studied (Hopmans and Bren, 2007). The aquatic organisms living in isolated pools, are affected not only by the decreasing water quality (Molinero and Pozo, 2004), but also by the increased predation pressure by fish and predatory invertebrates (Pires *et al.*, 2000, Boulton, 2003; Acuña *et al.*, 2005; Power *et al.*, 2008; Walters 2011).

Fish are an important group in aquatic ecosystems, playing a key role in the ecological dynamics of communities via trophic interactions. They are often sensitive to environmental change and therefore represent useful indicators of water quality (Hued and Bistoni, 2005).

The small tropical freshwater fish *Danio rerio* was chosen as model species in this study because it can be easily managed in large numbers in the laboratory (Kishi *et al.*, 2003), is one of the most widely used model fishes in the field of ecotoxicology and because of its physiological characteristics and well-documented background knowledge (Braunbeck and Lammer, 2006; Schirmer, 2006).

The use of biological methods turned an alternative for evaluation of the aquatic contamination or as a complement to chemical methods, from integral perspectives for the monitoring process of water quality (Martins *et al*. 2007; Maggioni *et al*. 2012).

The use and development of biomarkers in environmental research has increased during the last two decades (Monferrán *et al*., 2011), probably due to the fact that biological analyses give additional information not detected by chemical methods: (1) biomarkers are able to reflect contamination effects on the living components of ecosystems (Contardo-Jara *et al*., 2009); (2) have the capacity to show acute as well as chronic toxicological impacts, thus showing the consequences of various substances whose concentrations vary over time and space (Soto-Galera *et al*., 1998). The biomarkers also provide important information concerning the mode of action of the compounds tested (Hallare *et al*., 2005; Hahn, 2002). Biomarkers have been defined in literature as "a biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also of toxic effect'" (Peakall and Walker, 1994, cited in Hallare *et al*., 2005).

Vitellogenin is a phosphoglycolipoprotein synthesized in the liver in response to estradiol stimulation and is transported via the blood to the gonads, where it is processed into lipovitellin and phosvitin - nutrient sources of the developing embryo (Versonnen *et al*., 2003). This protein is commonly used as a biomarker of endocrine disruption, namely of estrogenic compounds and is known to respond to the presence of phytoestrogens (Zhang *et al*., 2002) and phytosterols (Nakari, 2005). Catalase is an antioxidant enzyme widely used biomarker of oxidative stress, which converts hydrogen peroxide (H_2O_2) to oxygen and water (Oruc and Uner, 2000; Ribera *et al*., 2001; Osman and Van Noort, 2007). Glutathione-S-transferase is an enzyme involved in the detoxification of various xenobiotics and, exogenous and endogenous substances, by conjugation to reduced glutathione (GSH) with electrophilic functional groups (Hayes and Pulford, 1995; Hyne and Maher, 2003; and Hayes *et al*., 2005). Acetylcholine is a neurotransmitter, present in the central and peripheral nervous system, which plays an important role in signal transduction across synapses. ChE belongs to a class of serine hydrolases which catalyze the splitting of choline esters at different rates and are classified as acetyl- (AChE), propionyl- (PChE) and butyryl-cholinesterase (BChE) (Talesa *et al.*, 1995). This enzyme is tipically inhibited by organophosphate and carbamate pesticides but can also be altered by plant derived products such as phytoestrogens (Isoda *et al.*, 2002).

The present study aimed to assess the sub-lethal effects of eucalypt and pine leaf aqueous extracts in zebrafish embryos, using biomarkers as main endpoints. The biomarkers selected for this study were vitellogenin (vtg), catalase (CAT), glutathione *S*-transferase (GST) and cholinesterase (ChE).

3.3 Material and Methods

3.3.1 Preparation of leaf aqueous extracts

The eucalypt and pine leaves were collected immediately after leaf fall. Leaf aqueous extracts were prepared on the same day of collection by placing 32 g of eucalypt or pine leaves in 1 L of FET medium (*Danio rerio*) and maintained for 48 hours (Canhoto and Laranjeira, 2007) in controlled conditions (photoperiod and temperature similar to those of the subsequent animal exposure experiment). Leaf extracts were collected, filtered, divided in aliquots and stored at -20º C until use. Before being used, the aliquots were thaw overnight at 4°C. The different leaf extract concentrations were performed by diluting the initial leaf extract with FET medium..

3.3.2 Exposure design

D. rerio eggs were obtained from a culture established at the Department of Biology, University of Aveiro, which is maintained in carbon-filtered water at 27.0 ± 1 ºC; under a 16:8h (light:dark) photoperiod cycle; conductivity is kept at 550 ± 50 µS; dissolved oxygen at 95% saturation and pH at 7.5 ± 0.5. The eggs were collected approximately 30 minutes after mating, washed and selected. During cleavage the unfertilized eggs or with abnormalities were discarded (Kimmel *et al.*, 1995).

The assays were performed based on OECD draft guideline FET test (OECD, 2006) and on the embryo test described by Fraysse (2006). A total of 2760 eggs of *D. rerio* were exposed during 96-hours in 6-well microplates, 23 replicates, 10 eggs per 1-well microplate for each tested concentration: 0.14, 0.29, 0.58, 1.15, 2.30 g L^{-1} for eucalypt leaf extracts, and 0.11, 0.22, 0.45, 0.90, 1.79 g L^{-1} for pine leaf extracts. In addition a control constituted by the FET medium was performed in duplicate, for each test leaf extract. The gradient of concentrations chosen is below the LC_{10} calculated for each extract in zebrafish embryos (see previous Chapter: LC_{10} = 1.8 (1.3–2.2) and 1.6 (-1.4–3.8) for eucalypt and pine leaf extracts, respectively).

Every 24h, the medium was renewed, dead individuals removed and physico-chemical parameters (temperature, dissolved oxygen, pH and conductivity) measured.

3.3.3 Mortality and development effects

Mortality was checked daily and the total cumulative mortality was registered for each treatment at the end of 96h. Malformations were analyzed in surviving organisms, for each treatment at the end of the assays.

3.3.4 Biomarker analysis

At the end of 96h, all surviving embryos, whether hatched or unhatched, were frozen in liquid nitrogen, and stored at -80ºC prior to biomarker analyses. All samples were defrosted on ice on the day the enzymatic activities were determined. Assays were performed to analyze vtg-like protein levels and the activities of CAT, GST and ChE. All biomarkers were analyzed in the whole body of the organism.

The levels of vitellogenin-like proteins were determined by an indirect alkali-labile phosphate method (ALP). Groups of 10 organisms, 5 replicates for each treatment, were homogenized at 4ºC in 120 µL of a buffer solution (adjusted to pH 8): 125 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA and 1 mM dithiothreitol; and kept on ice. The homogenate was then centrifuged at 12.000 g for 20 min at 4°C. A subsample (5 µL) was removed from each tube for the determination of the total protein concentration using the Bradford method (1976) as described below.

To evaluate the activity of enzymes (CAT, GST and ChE), 10 replicates, with 5 organisms each, for each enzyme and each treatment, were homogenized on a potassium phosphate buffer (0.1 M, pH 7.4 for CAT and GST; 0.1 M, pH 7.2 for ChE) and kept on ice. The homogenate was then centrifuged at 4ºC and 3.000 g for 4 min and enzymatic activity was analyzed directly from the supernatant.

CAT activity was determined according to the method described by Claiborne (1985), based on decomposition of H_2O_2 into H_2O and O_2, at 240 nm.

GST was performed following the method described by Habig *et al.*, (1974) and adapted to microplate (Frasco and Guilhermino, 2002). The GST activity was analyzed by quantification of the product resulting from the conjugation of reduced 10 mM glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene (CDNB) as substrate and monitored by the increase of absorbance at 340 nm.

ChE activity was measured at 414 nm according to Ellman's method (Ellman *et al.*, 1961), adapted to microplate (Guilhermino *et al.*, 1996). Acetylthiocholine was used as substrate that was degraded by ChE into acetate and tiocholine.

The activity of enzymes CAT, GST and ChE was expressed as nmol of substrate hydrolyzed per minute per mg of protein. The protein content in the supernatant obtained from centrifuged samples was calculated in quadruplicate using the Bradford method (Bradford, 1976), adapted to microplate, at 595 nm, using bovine γ-globulin as standard.

3.3.5 Statistical analysis

Where assumptions of normality and homogeneity of variance were met, data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's or Dunn's tests test to compare the treatment means with respective controls. Whenever necessary, data were transformed. Where data did not meet the criteria of parametric analysis, nonparametric Kruskal-Wallis ranked test was employed. Data were considered significantly different at $p \leq 0.05$ (*). All data analyses were performed using SigmaPlot Version 11.0 (Systat Software Inc.).

3.4 Results

3.4.1 Physico-chemical parameters

The greatest variations in the physico-chemical parameters belong to the variable dissolved oxygen which reached the lowest value of 7.3 mg L^{-1} in the test with the pine leaf aqueous extract, with the equivalent maximum value of 11.4 mg L^{-1}; representing a 64% decrease in dissolved oxygen comparative to control. The other variables had similar minimum and maximum values between highest concentration and control solutions (Table 7).

Table 7 – Minimum and maximum values of physico-chemical parameters during the 96 h exposure of *D. rerio* to eucalypt or pine leaf extracts and the respective control solutions.

Leaf extracts	**Concentration (g L^{-1})**	**pH**	**Conductivity (ųs/cm)**	**D.O. (mg L^{-1})**	**Temp (ºC)**
Eucalipt	0.00	7.7 – 8.4	1112 – 1179	13.8 – 14.9	25.6 – 28.1
	2.30	7.1 – 7.2	1137 – 1193	7.3 – 11.4	24.3 – 28.1
Pine	0.00	7.7 – 8.3	1164 – 1180	14.5 – 14.8	25.1 – 25.4
	1.79	7.4 – 7.6	1230 – 1264	13.2 – 14.3	23.3 – 24.8

3.4.2 Mortality and development effects

At the end of the assays, mortality was lower than 10% in the control groups for eucalypt and pine leaf extracts (2.12% and 1.74%, respectively) validating the tests performed. As the range of concentrations chosen is below the LC$_{10}$'s, mortality lower than 10% was registered as well in the treatments with eucalypt and pine leaf extracts.

No significant differences were observed between control and treatments concerning embryo development effects (Eucalypt leaf extract - Kruskal-Wallis: H=7.879; P=0.163; Pine leaf extract - Kruskal-Wallis: H=6.912; P=0.227; Table 8). The maximum percentage of malformations was registered with intermediate concentrations of eucalypt and pine leaf extracts, 0.29 g L^{-1} and 0.22 g L^{-}1 respectively. The malformations observed included tail curvation, tail shortening, cardiac edema and abdominal swelling.

Table 8 – Percentage of cumulative mortality and malformations at 96h in surviving *D. rerio* embryos exposed to eucalypt and pine leaf aqueous extracts.

Eucalypt leaf extract			**Pine leaf extract**		
Concentration (g L^{-1})	**Cumulative mortality (%)**	**Malformations (%)**	**Concentration (g L^{-1})**	**Cumulative mortality (%)**	**Malformations (%)**
0.00	2.12	2.16	**0.00**	1.74	2.21
0.14	1.30	2.64	**0.11**	1.30	3.08
0.29	0.43	6.55	**0.22**	0.00	4.78
0.58	0.00	2.61	**0.45**	0.00	2.17
1.15	0.00	3.96	**0.90**	0.00	3.95
2.30	0.43	3.49	**1.79**	0.00	3.91

3.4.3 Biomarkers

The effects of eucalypt and pine leaf extracts on the biomarkers vtg, CAT, GST and ChE are presented in Figure 2. No significant difference in the vtg-like protein levels was found on embryos exposed to eucalypt (DF=5; F= 1,991; P= 0,118) and pine (DF=5; F= 1,127; P=0,374) leaf extracts treatments when compared to the respective controls. An inhibition of the CAT activity was observed in organisms exposed to the eucalypt leaf extract concentrations of 0.29, 0.58 and 1.15 g L^{-1} (Kruskal-Wallis: H=34,361; P=<0,001) and to the pine leaf extract concentrations of 0.22 and 1.79 g L^{-1} (Kruskal-Wallis: H=13,334; P=0,020). GST activity was inhibited at eucalypt leaf extract concentrations of 0.58, 1.15 and 2.30 g L^{-1} (Kruskal-Wallis: H=46,269; P =<0,001) and increased at 0.90 and 1.79 g L^{-1} (One-way ANOVA: DF=5; F=10,391; P=<0,001). ChE activity showed a statistically significant difference in the mean values among the treatment groups in zebrafish embryos exposed to eucalypt leaf extracts (One-way ANOVA: DF=5; F=2,416; P=0,049), however the Dunnett's post-hoc test performed revealed no significant difference between the control and the treatments. There was no significant difference observed for ChE activity in embryos exposed to pine leaf extracts (One-way ANOVA: DF=5; F=0,000; P=1,000).

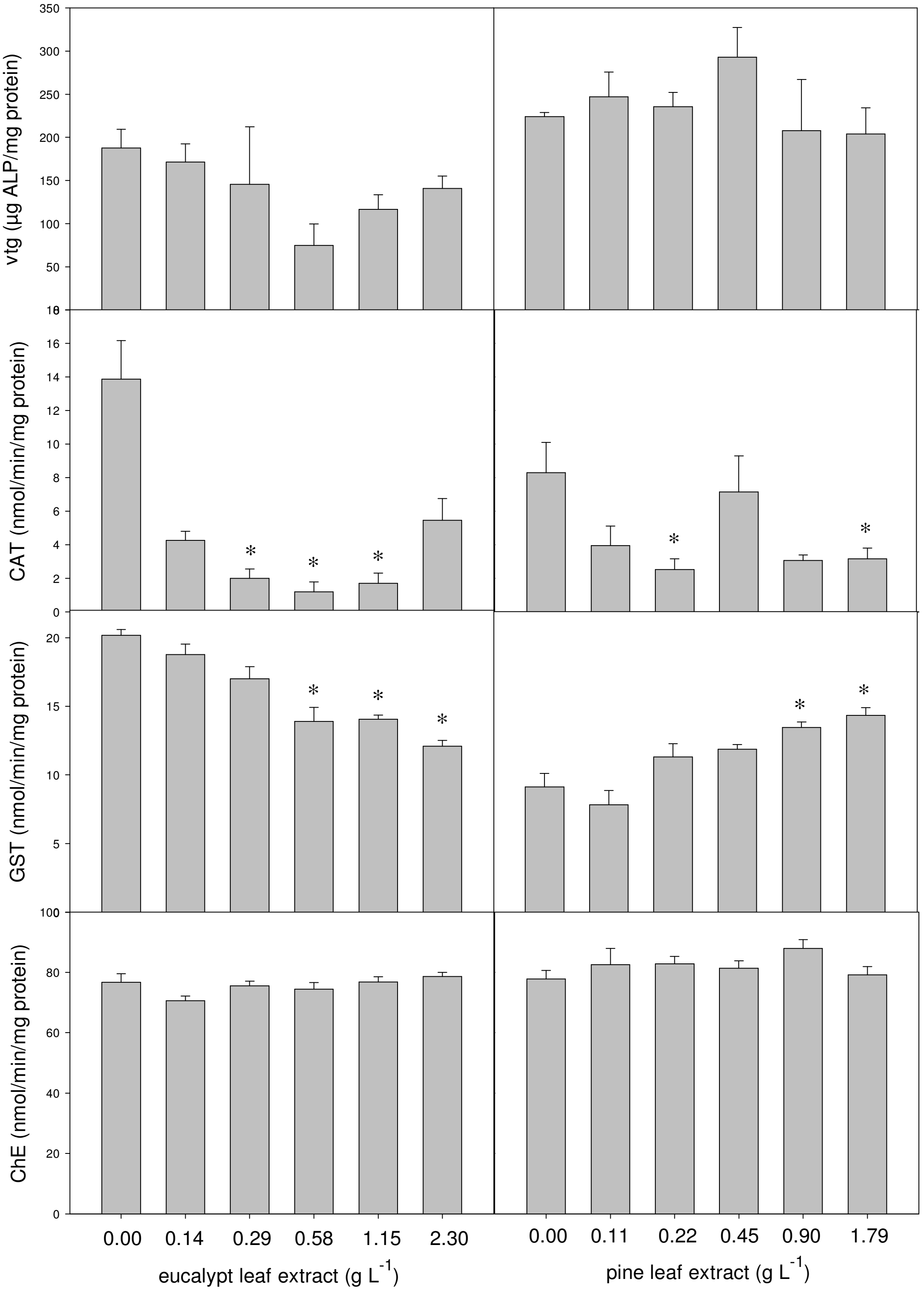

Figure 2 - Response of the biomarkers vtg (vitellogenin-like proteins), CAT (catalase), GST (glutathione S-transfaerase) and ChE (colinesterase) in zebrafish embryos after 96 h exposure to eucalypt and pine leaf aqueous extracts. Bars represent mean values with the respective standard error. Significant differences are presented for $p \leq 0.05$ (*).

3.5 Discussion

Fish, in general, have shown to be highly sensitive to the toxic effects of organic pollutants (Van der Oost *et al*., 2003). These effects can be evaluated resorting to biomarkers, which are biochemical and physiological indicators of environmental exposure to pollutants. They are used to detect sub lethal impacts on individual fish, thus providing an early warning for adverse effects before the onset of serious pathological damage (Mosse *et al*., 1996).

The results obtained in this study demonstrate that eucalypt and pine leaf aqueous extracts induce a response in some biomarkers of zebrafish embryos, mainly CAT and GST, whereas no significant malformations on embryo development were observed.

No significant differences in the vtg-like protein levels were found on embryos exposed to eucalypt and pine leaf extracts treatments, when compared to the respective controls. Although a number of authors (Versonnen *et al*., 2003; Versonnen and Janssen, 2004; Chow *et al*., 2012; Chang *et al*., 2012) report significantly effects on the endocrine system of zebrafish, when exposed to stressors, Van der Oost *et al*. (2003) concluded that zebrafish can be considered a suitable, but not the most sensitive species for the measurement of VTG as biomarker for estrogenic exposure.

According to Domingues *et al*. (2011), ChE is a key enzyme in the neurotransmission processes (Olsen *et al.,* 2001), important in the neuronal and muscular development of zebrafish (Hanneman, 1992; Behra *et al*., 2002). Eucalypt and pine aqueous leaf extracts had no effects on the nervous system function, since ChE activity showed a statistically significant difference in the mean values among the treatment groups in zebrafish embryos exposed to eucalypt leaf extracts, however the Dunnett's post-hoc test performed revealed no significant difference between the control and the treatments, and there was no statistically significant difference observed for ChE activity in embryos exposed to pine leaf extracts.

CAT activity demonstrated the same response with a decrease in the presence of eucalypt and pine leaf extracts, while the activity of GST demonstrated the contrasting responses with a decrease in organisms exposed to the eucalypt leaf extract and an increase for the pine.

Catalase is the primary cellular enzymatic defense against H_2O_2 and it's critical for the process of scavenging free radicals (Dorval *et al*., 2003). We hypothesize that a decrease of CAT activity is likely to affect the cells capacity to defend themselves and respond to oxidative stress. Wu *et al*. (2011) also found a decrease on CAT activity, when zebrafish embryos are exposed to phenols, in which our leaf aqueous extracts were rich.

Glutathione-S-transferase (GST) are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites, and are involved in the detoxification of both reactive intermediates and oxygen radicals (Sherratt and Hayes, 2001; Van der Oost *et al*., 2003).

Many environmental pollutants, such as phenol, may cause oxidative stress in aquatic species, by inducing reactive oxygen species (ROS) production (Avilez *et al*., 2008).

We hypothesize that, according to Sayeed et al. (2003), GST activity increases to provide protection against the ROS damage, and CAT response could be due to the flux of superoxide radicals, which have been reported to inhibit CAT activity (Kono and Fridovich, 1982). This might be explained by the results found for the pine leaf extract, where there was an inhibition of CAT and an increase of GST. In the eucalyptus extract the less pronounced increase in concentration of phenols may provide the reason for the decrease of the GST.

Overall, eucalypt and pine leaf aqueous extracts seem to affect the zebrafish embryos by inducing oxidative stress. CAT and GST activities were significantly affected before other endpoints evaluated at higher levels of organization, namely embryo malformations. These enzymes might be considered in future studies as useful biomarkers to evaluate the effects of eucalypt and pine leaf leachates on fish early life stages.

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4 Chapter Four

Concluding Remarks

4.1 Concluding Remarks

The main objective of this thesis was to understand the consequences that the eucalypt and pine leaf extracts might have for the aquatic fauna.

The leaf extracts tested were from the species:

- *Eucalyptus globulus* Labill. (Tasmanian blue gum);
- *Pinus pinaster* Aiton (maritime pine).

The model species used to test leaf extracts toxicity were:

- *Pelophylax perezi* Seoane, 1885 (Perez's Frog);
- *Xenopus laevis* Wagner, 1827 (African clawed frog);
- *Danio rerio* Hamilton, 1822 (Zebrafish).

The eucalypt and pine leaf extracts were prepared in laboratory, being the analyzed effects mortality, at a lethal level, and embryo malformations in the three animal models as well as biomarkers responses, at a sub-lethal level.

The analysed biomarkers in zebrafish were:

- Vitellogenin (vtg);
- Catalase (CAT);
- Glutathione S-transferases (GST);
- Colinesterase (ChE).

Traditional embryo ecotoxicological bioassays (Chapter 2) allowed the determination of the toxicity of eucalypt and pine leaf aqueous extracts, in *P. perezi*, *X. laevis* and *D. rerio*. The endpoints monitored included mortality and malformations in embryo development.

In general, leaf extracts were found to reduce water oxygen and pH, and increase dissolved phenolics, terpenes, lipids and conductivity. Results indicated that eucalypt leaf extracts were more toxic than pine extracts for *P. perezi* and *D. rerio* embryos, whereas for *X. laevis* the toxicity of these extracts appeared to be similar. The sensitivity of the embryos to the eucalypt extracts was: *D. rerio* > *X. laevis* ≈ *P. perezi* and for pine extracts: *X. laevis* > *D. rerio* ≈ *P. perezi*. The malformations most commonly observed were tail curvation and edemas. We observed that exposure to eucalypt and pine leaf extracts induced teratogenicity in *X. laevis* embryos. The lower sensitivity of *P. perezi* to the extracts was expected, being a species which, although very dependent on water bodies, occupies almost all aquatic habitats (Almeida *et al*., 2001), and is the most resistant species with the most tolerant ecological requirements of all amphibians present in the Iberian Peninsula (Llorente *et al*., 2002), hence having the status “of less concern” at the conservation level (Brito, 2010).

Along with these assays, some physico-chemical parameters were also analyzed in the laboratory produced leaf aqueous extracts and compared with field collected samples (Chapter 2). We observed that, in general, the eucalypt and pine leaf extracts were found to reduce water oxygen and pH, and increase dissolved phenolics, terpenes, lipids and conductivity. We hypothesize that these physico-chemical alterations may be acting upon embryos causing the observed toxicity.

The toxicity effects on fish and amphibian embryos obtained in the Chapter 2, by the exposure to environmental realistic concentrations of eucalypt and pine leaf extracts indicate the deleterious effects that eucalypt and pine plantation forest leachates might have on the aquatic species early life stages.

In future research, more physico-chemical parameters or compounds (e.g. tannin, falvone, nitrogen) should be analyzed, in order to better understand the factors that grant toxicity to these plantations, which may not have been reviewed by us, and manipulate those possible, to test whether there are parameters which combined have other effects. It would also be relevant to produce in the laboratory broadleaf extracts, as it was done for eucalypt and pine, and make the same assessment of physico-chemical parameters, in order to compare with the values of the parameters measured in the samples collected in the field (Chapter 2), and whether the differences between them are similar or not to those that occur in the extracts of eucalypt and pine.

To better understand the toxicity mechanisms, the effects of leaf extracts were evaluated at a molecular level (Chapter 3). Zebrafish embryos were exposed to sublethal concentrations (below the LC_{10} calculated on Chapter 2) of eucalypt and pine leaf aqueous extracts and a control to assess the sub-lethal effects of eucalypt and pine leaf extracts in zebrafish (*Danio rerio*) embryos, using biomarkers (vtg, CAT, GST and ChE) as main endpoints.

Eucalypt and pine leaf aqueous extracts seem to affect the zebrafish embryos by inducing oxidative stress. CAT and GST activities were significantly affected before other endpoints evaluated at higher levels of organization, namely embryo malformations. These enzymes might be considered in future studies as useful biomarkers to evaluate the effects of eucalypt and pine leaf leachates on fish early life stages. In general, the measured biochemical responses, which responded to this kind of stress (CAT and GST) were sensitive, with observed effects even in low concentrations (below the LC10 calculated in Chapter 2). No significant differences in the vtg and ChE activity content were found, between the control and the treatments, on embryos exposed to eucalypt and pine extracts.

In future research, other biomarkers should be evaluated in order to know if there are more changes at the molecular level, to better understand the mechanisms involved in the effects caused by plantations of eucalyptus and pine, to allow the foreseeing of these consequences and intervene in the protection and / or recovery of aquatic ecosystems. Since the oxidative stress mechanism was affected, it would be relevant to explore other enzymes acting at this level, such as the glutathione peroxidase (GSH-Px), which catalyzes the reduction of hydrogen peroxide (H_2O_2) and organic peroxides to their corresponding alcohols (Shan *et al.*, 1990), the superoxide dismutase, a key enzyme used in combating oxidative damage caused by reactive oxygen species (Cogo *et al.*, 2009) and lipid peroxidation, which leads to changes in the structure and permeability of cell membranes (Mello-Filho *et al*. 1983).

In addition, to be entirely possible to compare the two approaches (Chapters 2 and 3) the same biomarkers should be analysed (vtg, CAT, GST and ChE) in the Perez's frog (*P. perezi*) and African clawed frog (*X. laevis*).

4.2 References

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5 Annex I

Amphibian assemblages of forested streams in an Atlantic Mediterranean climate: the importance of forest and stream management

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Abstract

Production forests are expanding in Mediterranean countries, yet knowledge of the effects for aquatic biodiversity remains scarce. We studied the amphibian communities of Portuguese streams draining catchments of maritime pine (n=9), eucalypt (n=12) and natural broadleaf forests (n=8). Eight different species were recorded with richness ranging from two to six across sites. Regression models indicated that characteristics of the channel, banks and riparian zone differed in their explanatory importance for individual species and were reflected in community organization. Cold water streams with low pH and gently sloping banks in natural broadleaf forests were dominated by *R. iberica* and to a lesser extent *C. lusitanica*, contrasting with the warmer waters and steeper banked sites in eucalypt forests where *P. perezi* was most abundant. Sites in maritime pine forests with a high frequency of loose-stone walls and riparian broadleaf trees where characterized by the newts, *L. boscai* and *T. marmoratus*. Variance partitioning revealed biogeographic descriptors and forest management explained significant proportions of community structure. Representing a key vector in the energy dynamics of aquatic-terrestrial systems with intrinsic conservation value, endorsed by the widespread occurrence of IUCN red-listed *C. lusitanica* and other endemic taxa, these amphibian communities represent an important component in the sustainable management of these forest ecosystems.

Keywords: Atlantic-Mediterranean, amphibians, conservation, forest management production forests, riparian habitat

1. Introduction

Planted forests account for approximately 264 million ha of landuse world-wide with coverage expanding at a rate of 5 million ha/yr in the last decade (FAO, 2010). In contrast, 13 million ha of natural and semi-natural forest cover disappear every year (FAO, 2010). While deforestation is a major cause of species loss (Brook et al., 2003; Dirzo and Raven, 2003; Laurance, 2007) afforestation presents opportunities for biodiversity conservation (Norton, 1998; Hartley, 2002; Brockerhoff et al., 2008). However, low structural complexity and the fragmentation of contrasting habitats of old growth forest result in comparatively low biodiversity in plantation forests (Magura et al., 2000; Raman, 2006). River corridors are a major nexus of biodiversity that can boost the conservation value of forest landscapes (Fausch et al., 2002; Robinson et al., 2002; Fukui et al., 2006; Olson et al., 2007; Strayer and Dudgeon, 2010; Stendera et al., 2012). However, their role in contributing to the biodiversity of plantation forests is often overlooked (Hartley, 2002; Brook et al., 2003; Quinn et al., 2004; Baker et al., 2006) and may limit management initiatives to promote the conservation value of forest streams (Olson and Burnett, 2009).

Conversion of natural forests to production forests changes the energy input, physical habitat structure, water quality and hydrologic regime of stream ecosystems (Gurnell et al., 1995; Canhoto and Laranjeira, 2007; van Dijk and Keenan, 2007; Farley et al., 2008). Plantation forests limited to early growth phases tend to have a high demand for water consumption (Hatton et al., 1998; Farley et al., 2005; Jackson et al., 2005) and are associated with reduced stream flows and altered discharge patterns (van Dijk and Keenan, 2007). The high stand densities and narrowly circumscribed growth-cycles followed by clear-cutting, present low structural diversity and a high level of disturbance to terrestrial and aquatic communities alike (Hartley, 2002; Bremer and Farley, 2010). Harvesting riparian trees alters the quantity and quality of solar radiation, leaf-litter, woody debris and invertebrate input to stream waters, transforming stream ecosystems (Hawkins et al. 1983; Quinn et al., 2004; Reid et al., 2010; Brooks et al., 2012). These changes can cause a feed-back effect for terrestrial riparian fauna (Nakano et al., 1999; Kato et al., 2003; Kawaguchi et al., 2003; Ballinger and Lake, 2006; Brooks et al., 2012).

Some of the largest increases of planted forests have occurred in Europe (FAO, 2010), with an increase in the Mediterranean region of 4.05 million ha in the last decade (FAO, 2013). Under a Mediterranean climate, rivers are characterized by highly variable hydrology with stochastic flooding and drying, causing dynamic change in river morphology, longitudinal connectivity and the spatio-temporal periodicity of associated pool habitats (Gasith and Resh, 1999). Throughout southern Europe, rivers have been highly modified by human intervention with dams, channelization, and abstraction contributing to potential stressors of catchment landuse (Allan and Castillo, 2007).

Planted forests for production often have short rotation life-cycles with harvesting of eucalypt and maritime pine plantations following periods of maximum growth that can be as little as 10-15 years (Kardell et al., 1986) and 15-50 years (Alegria, 2011), respectively. While previous studies have elucidated how plantation forests can change the productivity and diversity at the base of aquatic food-webs in Mediterranean streams (Abelho and Graça, 1996; Canhoto and Graça, 1996; López et al., 2001; Bärlocher and

Graça, 2002; Graça et al., 2002; Molinero and Pozo, 2004; Canhoto and Laranjeira, 2007), information on how these changes in the energy and habitat affect higher organisms remains scarce.

Amphibians depend on both aquatic and terrestrial habitats to complete their life-cycle, and can be particularly vulnerable to forestry management practices (Dupuis and Steventon, 1999; Wahbe et al., 2004; Stoddard and Hayes, 2005; Kluber et al., 2008; Kroll, 2009; Lowe, 2009; Semlitsch et al., 2009). The structural simplicity of monoculture forests with sparse understory vegetation and an absence of accumulated dead-wood can limit the availability of both food and habitat for foraging adults (deMaynadier and Hunter, 1995; Gómez and Anthony, 1996; Semlitsch et al., 2009). Logging activities and catchment erosion reduce interstitial space and result in the loss of refugia and breeding sites (Corn and Bury, 1989; Stoddard and Hayes, 2005; Lowe, 2009). With comparatively low mobility, the amphibians of isolated headwater stream habitats may be particular vulnerable to these short and long-term disturbance effects (deMaynadier and Hunter, 1995; Hawkes and Gegory, 2012).

Yet in stark contrast to the knowledge-based strategies for amphibian conservation in the forest streams of North America, the amphibian fauna of Europe have rarely been studied in relation to running waters and their relative importance in forested streams (e.g. Rodríguez-Prieto and Fernández-Juricic, 2005; De Bernardi et al., 2009; Ficetola et al., 2011).

In mainland Portugal forests represent 35.4% of land cover (3.15 million ha), with maritime pine (*Pinus pinaster* Ait.) and eucalypt (*Eucalyptus globulus* Labill.) plantations accounting for 23% and 26% of forest cover, respectively (ICNF, 2013). The resultant hydrophobic soils are associated with increased runoff and reduced stream discharge during dry periods (Abelho and Graça, 1996; Keizer et al., 2005). In eucalypt plantations the recipient aquatic ecosystems reveal characteristic changes in the community structure of aquatic fungi and invertebrates characterized by an overall reduction in diversity and abundance (Bärlocher and Graça, 2002; Larrañaga et al., 2009) may ramify through higher trophic levels.

According to Gardner et al., 2007, studies attempting to define individual trait responses, amphibian community patterns and assessing the conservation value of each species, are still a small fraction of the worldwide research reporting the consequences of habitat change for amphibians. With 7 endemic species and 17 species in total, the comparatively diverse Portuguese amphibian fauna has been little studied (e.g. Soares and Brito, 2007; Malkmus 2004) and their ecological importance in river ecosystems remains largely unknown. To address this major knowledge gap we surveyed the amphibian communities of low-order forested streams in northern and central Portugal collecting data on the river habitat and riparian and catchment landuse with the aim of: (i) describing amphibian biodiversity in streams of contrasting forest management: eucalypt, maritime pine and broadleaf; (ii) elucidating the relative importance of habitat characteristics at the reach and landscape scale as determinants of species distributions and community structure.

2. Methods

2.1 Site selection

Candidate sites were selected using Geographic Information System (GIS) topographic maps (scale 1:25000; ArcGIS software, ESRI inc., 1999). Sites were identified by dominant landuse, stream order and an absence of major (unrelated) anthropogenic disturbance (e.g. urban and industrial development). Site selection was limited to 4th order streams where upstream landuse was classified as monocultures of eucalypt (*Eucalyptus globulus* Labill.), maritime pine (*Pinus pinaster* Ait.) and broadleaf forest. Ecological surveys were conducted at twenty-nine sites in central and northern Portugal representing tributaries within the major river basins of the Minho, Lima, Ave, Douro, Vouga and Mondego (Figure 1).

2.2 Amphibian sampling

Each site was visited six times in surveys conducted in consecutive seasons representing autumn (October-November), spring (March-May) and summer (July-August) between 2010 and 2013. Adult amphibians were sampled by an active visual search performed in aquatic and terrestrial habitats. River channels were surveyed over a longitudinal reach of 60 meters for a period of 45 minutes by three operators using dip-nets and a battery-powered electrofishing back-pack. Steep valley form and time-constraints limited terrestrial surveys to the river corridor. Terrestrial habitat was sampled by a visual survey conducted along four transects, perpendicular to the stream (each 3m wide, 10 m length). Visual surveys were accompanied by an exhaustive, active search (lifting stones and woody debris, sifting leaf-litter, vegetation, etc.) completed within three sub-sample units per transect each representing 6 m^2, located 0-2 m, 4-6 m and 8-10 m from the water's edge. This provided visual terrestrial surveys covering a total area of 120 m^2 including an exhaustive active search over a sub-sampled area of 72 m^2 at each site, on each occasion.

2.3 Habitat description

Habitat characteristics considered to be potentially important to amphibians were recorded by a combination of three surveys protocols, each based on the classification of habitat features at spot-checks that were repeated at fixed intervals along a defined transect. In aquatic habitats details of the channel, bank and riparian features were documented over 200 m using a version of River Habitat Survey modified for use in upland Portuguese streams (RHS; Environment Agency, 1997; Monaghan and Soares, 2008). Detailed information on channel dimensions, flow regime, in-stream and out of stream cover in aquatic habitats was based on the classification system applied in the HABSCORE survey (Wyatt et al., 1995). Transects of terrestrial habitat were characterized by an amphibian-based habitat survey including a description of canopy shade, ground cover, vegetation complexity, topography and quantitative estimates of available refugia. As upland Portuguese river corridors are characterized by a high frequency of loose-stone walls used to reinforce riverbanks and erect terraces on adjacent terrain, these features were incorporated into surveys by documenting the presence/absence of walls and characterizing the degree of associated wall refugia (extent and size of interstitial space). Water quality (temperature, dissolved oxygen, pH, conductivity and flow) were assessed on-site using portable meters. Water samples were also collected at each site visit and analysed for nutrients and key ions. Following the two summertime surveys water samples were also analyzed for lipids and terpenes.

2.4 Data analysis

Habitat structure described by RHS and HABSCORE surveys was separated into channel and bank characteristics and subjected to Principal Component Analysis (PCA) conducted on the correlation matrix to provide a reduced summary of explanatory variables. PCA was also used to summarize gradients in terrestrial amphibian habitats and water chemistry. Site scores for each habitat descriptor were based on the site centroid for respective axes. Because of the unknown probability of recapturing an amphibian on a sequential site visit, data for respective streams were standardized over the entire study period and expressed as catch per unit effort (CPUE; representing the average capture rate per visit) to provide comprehensive data on species richness and standardized estimates of abundance across streams and thus enable a robust descriptive analysis of general patterns in biodiversity. Prior to analysis data were examined and, where appropriate, transformed to provide the best approximation to a normal distribution.

To assess the importance of local environmental conditions as a determinant of the distribution and abundance of species we conducted multiple regression analysis considering variables from habitat PCA's (channel, banks and water chemistry), on-site measurements of water quality (pH, temperature, conductivity) and riparian zone (aquatic and terrestrial habitat). Because the potential importance of biogeography was unknown, geographic co-ordinates (Northings, Eastings) and altitude were also included in the analysis. Fully saturated models were fitted to ecological data, eliminating non-significant terms ($P<0.05$) to develop a minimum adequate model (Crawley, 2007). Model development included testing the effects of two-way interactions between variables and quadratic terms for geographic co-ordinates and altitude. Where the ratio of explanatory variables to (amphibian) data points were low, the selection of variables for inclusion was randomized and model elaboration was repeated to ensure that all potentially important variables and interactions were considered.

At the community level, the relationship between environmental variables and ecological trends were described by constrained ordination. Because overall diversity was low, we employed RDA which approximates to a linear gradient that provides a more appropriate representation of ecological change when species turnover is low (Lepš and Šmilauer, 2003) To avoid the problem of double-zeros associated with Euclidian distance, amphibian data were subjected to Hellinger transformation prior to analysis (Borcard et al. 2011). Interpretation with respect to environmental variables at contrasting spatial scales was achieved by considering the importance of local habitat characteristics and landscape variables in respective analyses. At the local site-scale ordination was constrained by the same environmental variables applied in individual regression models. At the landscape scale ordination was constrained by natural biogeographic descriptors (geographic co-ordinates, stream link magnitude, slope, altitude, distance from source, discharge and valley form) and descriptors associated with management intervention (catchment landuse, riparian landuse, channel modifications, bank modifications, terraced-walls, wall-refugia and the presence/absence of irrigation channels). For ordination analysis at both the local and landscape scale the parsimonious selection of explanatory variables was based on evidence from models for individual species and via examination of variables' “variance inflation factors” (Lepš and Šmilauer, 2003). For ordination at the landscape scale, variance partitioning based on the calculation of adjusted R^2 values (Peres-Neto et al., 2006) was employed to assess the relative importance of natural and management descriptors, testing their respective statistical significance by partial RDA. Tests of significance for ordination axes were achieved by permutation tests estimated from 1000 permutations. All statistical analysis was performed using R 2.10.1; ordinations were conducted within the vegan package.

3. Results

3.1 River habitat

Streams where characterized by cool circum-neutral waters and low conductivity with mean temperatures ranging from 10.2 to 16.8 ^{o}C, pH from 5.9 to 7.9 and conductivity from 19.8 to 190.4 μS cm^{-1}. The primary axes of PCA on water chemistry represented 49% of the overall variation, describing a gradient from waters with comparatively high concentrations of sulphate, chloride and key metals ions contrasting with waters that tended to be higher in aluminum, terpenes, lipids and dissolved organic carbon (Table 1).

Dividing the 47,190 data points from habitat surveys into the three categories followed by ordination resolved approximately one-third of the overall variation in each case (Table 1). The primary axis of channel habitat (15 %) described a generalized change from eroding channels with large substrate and interstitial space to depositing environments with sand and silt substrates and accumulated leaves and woody debris. The second channel axis (14%) ranged from step-pool habitats with bedrock and in-stream vegetation to shallow riffle streams with cobble, pebble substrate (Table 1). Eighteen percent of variation was resolved by the first axis for bank habitat, describing a transition from stable and eroding cliffs and steep profiles to banks with gentle profiles and complex vegetation structure. The second axis (14%) characterized a change from low banks with a vertical profile to lower gradient slopes with high bank tops (Table 1). The primary axis for terrestrial habitat captured 19% of the variation ranging from riparian zones with heavy canopy shade and simple understory vegetation with abundant organic litter to habitats of steeper terrain with more abundant growth of herbs and shrubs; the secondary axis (15%) presented a transition differentiated by broadleaf trees and terraced walls with spacious interstices to a dominance of eucalypt trees and increasingly complex understory vegetation (Table 1).

3.2 Amphibian assemblages

Adult amphibians were present at all sites; in total 1799 individuals representing eight species were captured with mean CPUE ranging from 1.5 to 27.0 amphibians per site visit. Overall richness ranged from two to six across sites (average ± sd: 3.79 ± 1.08). The Iberian frog (*Rana iberica*) was present at all sites and the most widespread species, followed by Boca's newt (*Lissontriton boscai*) > golden-striped salamander (*Chioglossa lusitanica*) > Perez's frog (*Pelophylax perezi*) > European toad (*Bufo bufo*) > marbled newt (*Triturus marmoratus*) > fire salamander (*Salamandra salamandra*) > midwife toad (*Alytes obstetricians*). The overall rank abundance of species broadly corresponded to species' site incidence (Table 2). Mean species richness was significantly higher in eucalypt plantations (4.6 ±. 0.42) compared to broadleaf (2.88 ± 0.31) and maritime pine plantations (3.6 ± 0.43; $F_{27, 3}$ =5.829; P=0.0036) however, these landuse patterns included concomitant differences in other key environmental variables (Table 3).

Regression models revealed channel, bank and terrestrial habitat characteristics as key determinants of species distribution and abundance although the importance of habitat characteristics differed between species (Table 4). The occurrence of *R. iberica* and *B. bufo* increased with increasing substrate size and stream flow. *C. lusitanica* was commonly found in streams with small stony substrate and shallow riffle habitats (Table 4). *R. iberica* and *C. lusitanica* both revealed affinities towards low gradient, sloping banks, while *L. boscai* was more common where walls and bedrock or earth formed stable or eroding cliffs. Although not related to habitat characteristics of the channel or banks *P. perezi* tended to be comparatively more abundant at sites that lacked canopy shade and had well developed herbaceous and shrub vegetation; these sites were generally characterized by higher pH and water temperature (Table 4). Insufficient data and irreconcilable problems with statistical assumptions precluded the elaboration of regression models for the other species recorded.

The first two axes of RDA explained 41% of the variation and a significant portion of ecological variability. The first axis (30%; F=13.3, P = 0.001) defined broadleaf forest sites where *R. iberica* and to a lesser extent *C. lusitanica* where abundant and stream habitats were characterized by cold waters and low pH with banks that gently sloped towards high bank tops. At the other extreme, sites where characterized by eucalypt forests with steeper banks and warmer water where *P. perezi* was more common. The secondary axis (F=4.98, P=0.070) distinguished sites of maritime pine forests with a high frequency of broadleaf trees in riparian zones and increased refugia associated with terraced walls and stream channels with stony substrate where *L. boscai* and *T. marmoratus* tended to be more abundant (Figure 2).

At the landscape scale variance partitioning revealed that natural biogeographic descriptors (stream link magnitude, altitude, slope, geographic co-ordinates) and forest management descriptors (forest type, modified banks, loose-stone walls, Eucalypt/*Acacia sp.* riparian trees) explained almost equal proportions of ecological variation, each accounting for about 20% of overall variation (Figure 3). Partial RDA revealed that both management and catchment variables accounted for a significant proportion of the structure in ecological data (Figure 3). A slightly smaller fraction (16%) represented shared explanatory value between biogeographic and management variables, while 44% of the ecological variation remaining unexplained.

4. Discussion

While the role of amphibians is often overlooked in European lotic systems (e.g. (Rodríguez-Prieto and Fernández-Juricic, 2005; De Bernardi et al., 2009; Ficetola et al., 2009, 2011) the abundant and diverse populations in these streams demonstrate their important contribution to the ecological function and conservation value of these forest ecosystems. Their seasonally punctuated life-cycles are well-adapted to the pronounced periodicity of aquatic habitats in climates of Mediterranean influence (Beja and Alcazar, 2003; Ficetola and De Bernardi, 2004; Ficetola et al., 2009, Malkmus 2004), facilitating the exploitation of stochastic, ephemeral resources that represent unsustainable habitats for potential aquatic predators (Magalhães et al., 2002; Mesquita et al., 2006). In consequence these invertebrate-vertebrate consumers are a key component of ecosystem processes with larvae exploiting aquatic productivity and adults consuming a wide-range of terrestrial and aquatic invertebrates (Davic and Welsh Jr., 2004; Lowe et al., 2005; Ficetola et al., 2009; Joseph et al., 2011). In turn, amphibians themselves often contribute to the diet of larger predatory animals including a wide range of reptiles, mammals, and birds (Fasola et al., 1993; Beja, 1996; Clavero et al., 2005; Lanszki et al., 2007; Santos et al., 2007; Novais et al., 2010). By hosting the reproductive cycles of populous amphibian communities, stream ecosystems represent the focal point of a functional pulse that drives the transfer of nutrients and energy throughout terrestrial food webs and helps sustain catchment biodiversity (Davic and Welsh Jr., 2004; Baxter et al., 2005).

The amphibian assemblages of these streams represented high intrinsic conservation value. Of particular note was the widespread occurrence of the International Union for Conservation of Nature (IUCN) red-listed golden-striped salamander (*C. lusitanica*), present in both natural and plantation forests. While an endemic limited to a specific sub-region this species abundance opposed to its known rareness, indicating its potential as an indicator of ecosystem dynamics. These comparatively remote hill streams also provide important habitat for the endemic marbled newt (*T. marmoratus*) and a strong-hold for the Iberian frog (*R. iberica*). Although the dominance of exotic trees at the catchment scale appeared not to influence the site incidence of these species, it provided an important explanatory factor in overall community structure. The frequency of natural, broadleaf trees in the riparian zone was explicitly associated with the relative abundance of *R. iberica* and occurrence of *T. marmoratus* and *L. boscai*, suggesting that the presence of riparian buffer strips had an important effect on the biodiversity we observed.

Despite revealing comparatively rich assemblages by European standards, this study probably underestimated overall diversity (i.e. richness and abundance of species). While the standardized surveys facilitated a robust comparison between sites, the contrasting ecology of respective species coupled with potential differences in sampling efficiency in different habitats is expected to have biased capture rates. These species differ in their diel behavior (Malkmus, 2004). Visual surveys inevitably favored the capture of larger, more animated animals (e.g. *R. iberica and P. perezi*) whereas the smaller, more cryptic newts and salamanders were overwhelmingly captured by active searches in aquatic and terrestrial habitats. Sighting of fire salamander (*S. salamandra*) in the catchment of numerous study sites where they were otherwise not captured (*pers. ob*. MJC) suggests that these wide-ranging foragers (Semlitsch and Bodie, 2003; Ficetola et al., 2009) were often missed by our standardized surveys that focused on aquatic habitats and the immediate riparian zone. Although a complementary suite of survey methods would provide more accurate population estimates (Heyer et al. 1994), these comparative data demonstrate that this type of low cost, day-time surveys provide sufficient information to relate amphibian assemblages to environmental conditions and provide a relative indication of habitat quality.

Regression models indicated how species' populations respond to local environment conditions. Optimal cover and egg-laying habitat for the golden-striped salamander (*C. lusitanica*) appears to explain their strong association with shallow stony channels and rocky, creviced banks (Sequeira et al., 2001). As newts attach eggs to submerged plant material or wrap them in leaves, the presence of steep banks and overhanging herbaceous vegetation presumably facilitated the direct input of these resources into stream channels and suggests the particular importance of riparian buffer strips of deciduous trees and herbaceous plants in maritime pine forests. The anuran's of these forested streams were characterized by generalist species with *B. bufo* and *P. perezi* proportionally more common in the more productive, exposed sites while the endemic *R. iberica*, was most abundant in the fast-flowing, rocky mountain streams of deciduous forest that offered more extensive refugia and reproductive habitat (Malkmus 2004).

Strong gradients in climate and relief make biogeographic descriptors useful predictors of amphibian distributions throughout the Iberian Peninsula (Sequeira et al., 2001; Teixeira et al., 2001; Soares and Brito, 2007). Altitude integrates a range of ecologically deterministic variables including temperature, precipitation, slope and vegetation type and has been distinguished as a key correlate of amphibian distribution in many regions (Corn and Bury, 1989; Dupuis and Steventon, 1999; Stoddard and Hayes, 2005; Lowe et al., 2006; Olson et al., 2007; Kluber et al., 2008). Slope and stream link magnitude represent proxies for key characteristics of stream habitat such as discharge and flow regime, substrate size and water quality (Jeffers, 1998) that amphibians respond to at the local site-scale (Wilkins and Peterson, 2000; Stoddard and Hayes, 2005). Management intervention aimed at controlling discharge, reducing erosion and exploiting land for different purposes can override the ecological importance of these natural landscape predictors as species respond to the changes in their local environment (e.g. Power et al., 1996; Boothroyd et al., 2004; Parris and Lindenmayer, 2004; Jackson et al., 2005; Monaghan et al., 2008). However, despite the significant contribution of management parameters in statistical models, understanding how these auto correlated variables with complex interactions influence amphibian communities is difficult. Differences in landuse at the riparian and catchment level were accompanied by differences in other deterministic environmental variables. The influence of walled banks and riparian terraces are context dependent and related to their qualitative character, in particular the availability of interstitial space that can be exploited as refugia by amphibians and other animals (*pers. ob*. KAM). It therefore seems likely that the traditional use of loose-stones provide superior (managed) habitat compared to tightly packed blocks or the increasing use of concrete in the maintenance of this widespread and ancient practice (Ferreira et al. 1996).

5. Conclusions

The demonstrative need to begin monitoring and protecting amphibian biodiversity in the forest ecosystems of southern Europe is facilitated by experience in North America (e.g. Welsh Jr. et al., 1997; Quinn et al., 2007; Kroll, 2009). Indicating how amphibian assemblages are defined by a nested hierarchy of deterministic parameters at the landscape and local scale, these data support the need for an integrated spatio-temporal understanding of amphibian habitats (Lowe and Bolger, 2002; De Bernardi et al., 2009; Ficetola et al., 2011). While this study indicates the close coupling of these aquatic-terrestrial ecotones and the critical importance of riparian buffer strips, effective conservation strategies must be grounded in a more comprehensive knowledge of spatial dynamics that link breeding, foraging and overwintering habitats and the dispersal to new sites (Olson et al., 2007). Considering the relatively recent and widespread increase in intensive production forestry in southern Europe, the lack of historical data, potential time-lags in ecological responses and the difficulties of distinguishing temporally distinct ecological signals (deMaynadier and Hunter, 1995; Kroll, 2009), detailed spatio-temporal studies designed to provide a more comprehensive understanding of the population dynamics of these forest amphibians is fundamentally important to their conservation.

Similar scenarios of landscape pressures compounding site-scale impacts also threaten the spatially discrete metapopulations of amphibian in lentic habitats of southern Europe (Beja and Alcazar, 2003; Ficetola and De Bernardi, 2004). By linking fragmented habitats and providing a dynamic continuum for aquatic and terrestrial organisms, the conservation of river corridors may facilitate better ecological resilience in the face of global change. Representing intrinsically important habitats for amphibian biodiversity, the conservation management of these streams should represent an integral part of any forestry management that is striving for credibility as a sustainable practice.

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Chemistry		Channel				Banks				Terrestrial			
Chem1 (49%)		Ch1 (15%)		Ch2 (14%)		Bk1 (18%)		Bk2 (14%)		Ter1 (19%)		Ter2 (15%)	
SO4	1.161	Boulder	2.104	Bedrock	2.034	Features (L)	1.178	Profile (R)	1.274	Shading	1.939	Broadleaf Trees	1.653
Cl	1.138	Cobble	1.867	Flow	1.117	Profile (L)	1.129	Profile (L)	1.079	Bryophytes	1.397	Wall Refugia	1.555
Mg	1.138	Cover-rocks	1.631	Cover-veg.	1.090	Profile (R)	1.017	Veg. structure 1m (R)	0.981	Organic Litter	1.292	Wallfreq	1.457
Na	1.071	Width	1.443	Vegetation	0.972	Features (R)	1.016	Veg. structure face (R)	0.569	Veg Structural	1.130	Herbs	0.820
N	1.033	Flow	1.1757	Depth	0.950	Subst-Size (L)	0.978	Veg. structure face (L)	0.479	Broadleaf Trees	1.129	Bryophytes	0.747
K	0.979	Pebble	1.1542	Features	0.732	Subst-Size (R)	0.901	Veg. structure 1m (L)	0.411	Woody Debris	0.703	Profile	0.570
NO3	0.861	Features	1.115	Width	0.609	Height (R)	-0.948	Features (L)	0.051	Terraced Walls	0.391	Irrigation channel	0.415
Ca	0.838	Vegetation	1.038	Sand	0.493	Height (L)	-0.949	Features (R)	-0.122	Wall Refugia	0.362	Boulder Cobble	0.110
Lipids	0.456	Cover-veg	0.853	Boulder	-0.194	Veg. structure 1m (R)	-1.174	Sub. Size (R)	-1.180	Boulder Cobble	0.250	Shrubs	-0.560
DOC	0.340	Depth	0.403	Woody debris	-0.206	Veg. structure 1m (L)	-1.573	Sub. Size (L)	-1.393	Irrigation Channel	0.217	Shading	-0.676
Terpenes	0.213	Tree roots	0.008	Cover-rocks	-0.623	Veg. structure face (R)	-1.735	Height (R)	-1.903	Eucalypt/ *Acacia sp.*	-1.054	Organic Litter	-0.967
Al	-0.019	Woody debris	-0.352	Gravel	-0.988	Veg. structure face (L)	-1.927	Height (L)	-1.978	Profile	-1.148	Woody Debris	-1.021
		Sand	-0.397	Leaf litter	-1.106					Shrubs	-1.433	Veg Structural	-1.239
		Leaf litter	-0.629	Cobble	-1.376					Herbs	-1.562	Eucalypt/ *Acacia sp.*	-1.564
		Silt	-0.882	Pebble	-1.825								

Table 1. Summary of the major gradients in habitat characteristics for 29 forested stream sites in northern and central Portugal describing water chemistry, stream channel, stream banks and terrestrial riparian habitat according to PCA axes. Percentages represent the proportion of total variation associated with the respective axes, numerical values represent the axis weighting for corresponding variables. (R) and (L) represent Right and Left bank, respectively.

Species	Common name	Total abundance (%)	Site occurrence
			Number of sites (%)
Caudates			
Chioglossa lusitanica	Golden-striped salamander	133 (7.4)	20 (69)
Lissontriton boscai	Bosca's newt	210 (11.7)	26 (90)
Triturus marmoratus	Marbled newt	9 (0.5)	5 (17)
Salamandra salamandra	Fire salamander	4 (0.2)	4 (14)
Anurans			
Rana iberica	Iberian frog	1193 (66.3)	29 (100)
Pelophylax perezi	Marsh frog	216 (12.0)	13 (45)
Bufo bufo	Common toad	33 (1.8)	12 (41)
Alytes obstetricians	Midwife toad	1 (0.1)	1 (3)

Table 2. Summary statistics for the adult amphibians captured from 29 forested stream sites in northern and central Portugal.

Environmental Variable	Broadleaf mean	Maritime pine Plantation	Eucalypt Plantation
Conductivity	42.0 (21.2)	33.9 (11.6)	91.7 (55.0)
Stream link magnitude	16.5 (14.8)	47.6 (59.2)	85.9 (63.8)
Slope	0.13 (0.07)	0.12 (0.09)	0.04 (0.02)
Altitude	539 (213)	358 (222)	140 (102)
Channel character (Ch2)	0.048 (0.27)	0.18(0.20)	-0.24 (0.08)
Bank character (Bk2)	-0.12 (0.24)	0.012 (0.19)	0.11 (0.05)
Riparian trees – Eucalypt/*Acacia sp.*	0.25 (0.61)	10.86 (7.22)	9.71 (5.17)
Riparian trees - Broadleaf	23.25 (1.44)	12.14 (7.66)	12.69 (6.69)

Table 3. Mean value (standard deviation) for respective environmental variables with respect to land use. Only variables that were significantly different (P <0.05) are presented.

Species	Intercept	Ch1	Ch2	Bk1	Bk2	Ter1	Ter2	Conductivity (μS cm^{-1})	Latitude (Eastings)	Riparian trees Broadleaf	pH	Temperature (^{o}C)
R. iberica	2.641	1.339			-1.690					0.047		
C. lusitanica	1.099		-2.094		-2.102							
L. boscai	-4.794			2.046				0.798	7.405×10^{-6}			
B. bufo	-0.839	1.556				-0.886		0.348				
P.perez	4.267						-1.243				1.254	0.374

Table 4. Summary of regression models describing amphibian species abundance across 29 forested stream sites in forests of maritime pine, eucalypt and broadleaf trees (all terms are significant at $P<0.05$). Ch1 and Ch2 represent channel characteristics defined by PCA; Bk1 and Bk2 represent bank characteristics defined by PCA; Ter1 and Ter2 represent terrestrial riparian characteristics defined by PCA (see Results 3.1 for variable descriptions).

Figure 1. Map indicating the location of the 29 forested stream sites in northern and central Portugal.

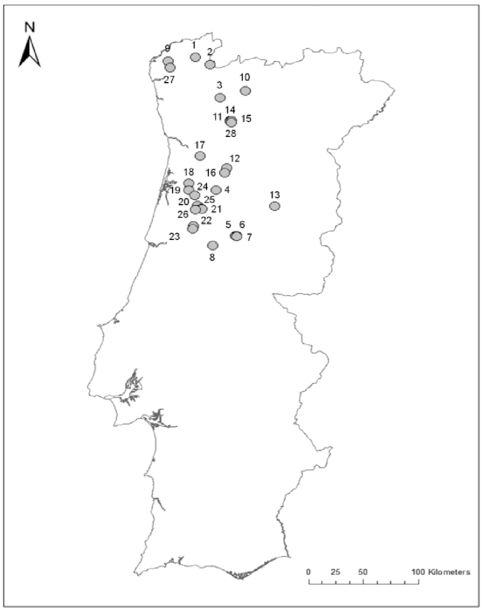

Figure 2. RDA ordination of amphibian assemblages from the 29 forested stream sites in northern and central Portugal in relation to landuse and key descriptors of the local habitat (see Results 3.1 for habitat description). Amphibian species are Riber – *R. iberica*; Tbosca – *L. boscai*; Clusit – *C. lusitanica*; Pperez – *P. perezi*; Bbufo – *B. bufo*; Tmarm – *T. marmoratus;* Ssalam – *S. salamandra*; Aobst – *A. obstetricians.*

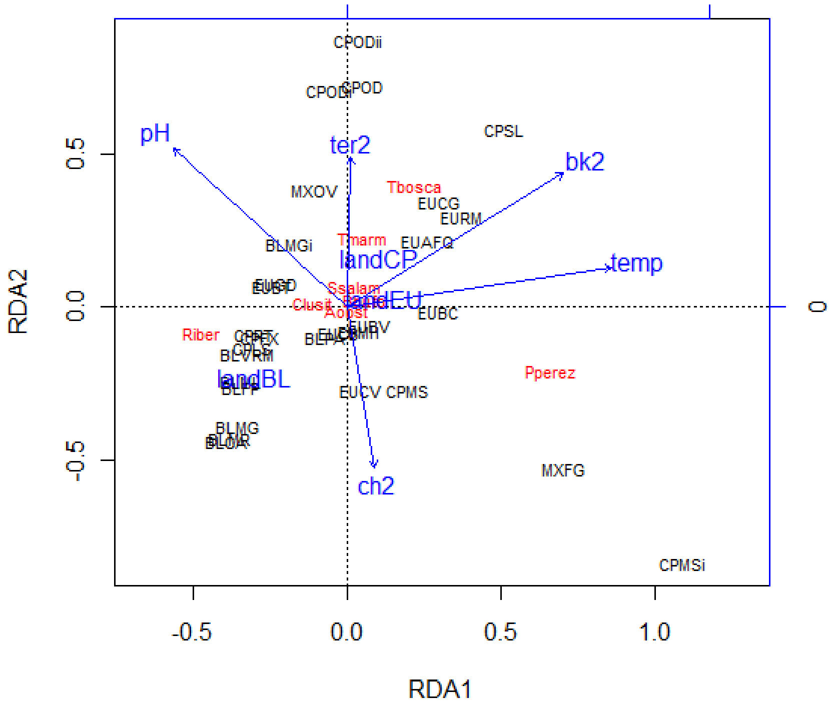

Figure 3. Variance partitioning distinguishing the respective influence of natural catchment variables (stream link magnitude, altitude, slope, geographic co-ordinates) and forest management variables representing intervention in stream habitat and landuse (forest type, modified banks, loose-stone walls, Eucalypt/*Acacia sp.* riparian trees). Fractions represent the proportion of total ecological variation explained by each component; F and P values are based on statistical tests of partial RDA