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ABSTRACT

The imperative need of water for life supporting activities (e.g., for agriculture and human consumption) has led to the construction of artificial lakes or dams worldwide. Nevertheless, unsustainable use and management of these water bodies are posing serious environmental and human concerns linked with intensive modern agricultural practices, and urbanization in their catchment areas. Thus, the evaluations of the water quality status of these reservoirs are essential to identify their main problems and adapt water management strategies to regional requirements for sustainable use. The present study was conducted to: (1) identify the ecological receptors at most risk in the Alqueva reservoir – a previously known freshwater reservoir contaminated mainly with pesticides, using a battery of laboratory and more ecologically relevant *in situ* assays, and (2) to set a cost-effective tool-box (i.e., a battery of short-term, easy and accurate assays) to allow rapid and integrative measures of environmental quality. The assay battery integrated a wide range of species that are representative of different taxonomic (algae, crustaceans, and insects) and functional groups (primary producers, primary and secondary consumers, benthic and epibenthic decomposers) as well as of different environmental compartments (water column, sediment-overlying water, sediment) in the study catchment. Overall, the surrogate organism used to evaluate ecological receptors at most risk did not show harmful effect on their biological responses, indicating no toxicity evidence at the Alqueva reservoir, except for a slight decrease in some laboratory responses at one of the five selected sites, which was not corroborated by the *in situ* responses. Both the *in situ* as well as the laboratory assay results revealed that the aquatic communities in Alqueva reservoir are generally safe. This fact was associated to changes in agricultural practices in the catchment of the

reservoir driven by the need to adopt environmentally friendly techniques which might have intercepted contaminant reaching to the reservoir.

Keywords: Large reservoir, Ecological receptors, Laboratory and *in situ assays*, Tool-box

RESUMO

A necessidade de água para atividades essenciais à vida,como por exemplo na agricultura e no consumo humano, levou à construção de lagos artificiais e barragens por todo o mundo. Contudo, o uso e o gestãonão sustentável destes corpos de água tornou-se um grande problema devido essencialmente àsintensas práticas agrícolas modernas e à urbanização das áreas de captação. Assim sendo, as avaliações de qualidade da água destes reservatórios são essenciais para identificar os seus principais problemas e adaptar estratégias de gestão da água aos requisitos regionais para uso sustentável. O presente estudo teve os seguintes objetivos: (1) identificar os recetores ecológicos em risco, usando uma bateria de ensaios em laboratório e os ensaios mais relevantes *in situ*, no reservatório do Alqueva, anteriormente conhecido como contaminado por pesticidas, e (2) estabelecer um conjunto de ferramentas com uma boa relação custo-benefício (i.e., um conjunto de testes rápidos, fáceis e precisos), que permita a rápida realização de medidas integrativas de qualidade ambiental. A bateria de ensaios integrou um grande número de espécies que são representativas de diferentes grupos taxonômicos (algas, crustáceos e insetos) e funcionais (produtores primários, consumidores primários e secundários, decompositores bênticos e epibênticos), assim como de diferentes compartimentos ambientais (coluna de água, água subjacente ao sedimento e sedimento) nos locais em estudo. De uma forma geral, os organismos utilizados para avaliar os recetores ecológicos em maior risco não demonstraram efeitos nocivos nas suas respostas biológicas, não indicando evidências de toxicidade no reservatório do Alqueva, excepto para uma ligeira diminuição em algumas respostas em laboratório para um dos cinco locais selecionados, o que não foi confirmado pelas respostas *in situ.* Tanto os resultados *in* situ como os resultados em laboratório

revelaram que as comunidades aquáticas presentes no reservatório do Alqueva estão seguras. Este facto foi associado às mudanças das práticas agrícolas nos limites do reservatório, induzidas pela necessidade de se adoptar técnicas ambientalmente favoráveis, que podem ter impedido os contaminantes de alcançarem o reservatório.

Palavras-chave: Grande reservatório, Receptores ecológicos, Ensaios de laboratório e em ensaios *in situ,* Conjunto de ferramentas

Chapter 1 Introduction

Multiple anthropogenic pressures pose an increasing threat to aquatic ecosystems ever nowadays than the past. Particularly, freshwater ecosystems have experienced multiple and intensive threats and their ability to provide sustainable ecosystem services has substantially decreased (Gurnell *et al*., 2009). In addition to the threats posed by habitat loss, invasive species, and different types of toxicants, such ecosystems are furthermore affected by climatic change phenomena (Coops *et al.,* 2003; Millennium Ecosystem Assessment, 2005). Linked with this, in most aquatic ecosystems, biota is exposed to multiple stressors and not to a single stressor alone, i.e., to a combination of chemical, biological and physical stressors (Liess *et al*., 2008; Statzner and Beche, 2010). In ecology, the importance of understanding and predicting the effects of multiple pressures on biological communities and ecosystems is well recognized (Rohr *et al.,* 2008). However, it still remains a central challenge to develop rapid, sensitive, low-cost, and ecologically relevant tools for precise risk estimation and environmental regulation.

In many aquatic habitats, organisms are chronically exposed to a huge variety of toxicants, often at low concentrations (Maltby *et al*., 2002; Mills *et al*., 2006; Relyea & Hoverman, 2006). Moreover, the consequence of subtle effects might be enhanced by interactions with other abiotic (e.g., pH, temperature) and biotic (e.g., pathogens, predators) stressors (Hanazato, 2001; Edginton *et al*., 2004; Relyea, 2004; Wenger, 2010), causing an increased sensitivity of the organisms to the other stressors or vice versa (Boone & Semlitsch, 2001). Such interactions of multiple stressors may not lead to direct and clear alterations of health and ecological status of the ecosystems, but may cause slight changes on individual traits, e.g., behaviour, immune status, feeding, growth or reproduction (Rose, 2000; Relyea & Hoverman, 2006).

Importantly, the combination of effects arising from exposure to multiple stressors might vary from the sum of their individual effects alone (Rose, 2000). It has been observed that variations in the abiotic environment can make insecticides more lethal to animals (Zaga *et al*., 1998; Boone & Semlitsch, 2001; Boone & James, 2003), depending especially, on whether the individuals are at the edges of their normal physiological range or whether populations are at the edges of their ecological potency (Van Straalen, 2003). Thus, it is important in ecological risk assessment approaches to evaluate the effects of multiple stressors and their interactions in a holistic manner for accurate and precise risk evaluation and characterization than focusing on single stress exposure scenarios (Allan *et al*., 2006; Roman *et al*., 2007; Wharfe *et al.,* 2007*)*

Traditionally, the characterization of ecological risk due to contaminants or recovery in aquatic systems has been conducted by the evaluation of the aquatic organisms and/or the environmental factors that have direct and indirect effects on biota (USEPA 1992; Werner *et al.,* 2007). Usually, it involves mainly the collection of water or sediment samples from potentially contaminated sites and then conducting chemical analysis to compare the concentrations to various benchmarks in the risk estimation process (Chapman, 1990; Stoddard *et al*., 2003; Rosen *et al.,* 2012). Though this approach is well established and accepted by many environmental regulators, its ecological relevance is restricted as it ignores the concept of bioavailability and stressors interaction effects. Moreover, it lacks accurate linkages among chemical exposure and biological responses needed to establish cause-effect relationship in risk (Liber *et al.,* 2007; Rosen *et al.,* 2009).

Currently, the increased awareness on the inadequacy of the classical ecological risk assessment methods for contaminated aquatic ecosystems has led to the use of the

ecotoxicological line of evidence which promotes a more integrative approach in the context of bioavailability (Allan *et al.,* 2006). Such approaches are important especially when the stressors are unknown and exist in a mixture. In this regard, the European Water Framework Directive (WFD; Directive 2000/60/CE) suggests a holistic approach to catchment management and requires not only chemical and ecological quality objectives to be set but also that assessment methods should be developed to fulfil the goals for effective monitoring of the aquatic communities (Pollard & Huxham, 1998; European Community, 2000). Thus, it is believed that the integration of ecotoxicological approaches, mostly toxicity tests/bioassays with different levels of complexity, may contribute to monitor and manage waters failing to achieve good ecological status in a more multidisciplinary and holistic perspective as needed (ICES, 2004; Allan *et al*., 2006). Though bioassays cannot identify the constituent toxicants causing an effect alone, the triad approach integrating the chemical, ecotoxicological and ecological lines of evidence is considered critical (Schlekat *et al.,* 2002). Therefore, the integration of bioassays in the WFD is advantageous to effectively monitor aquatic systems in Europe. This integrative strategy contributes to reduce uncertainties in risk assessments (Burton *et al.,* 2002), so that it is possible to identify the ecological receptors at risk and establish comprehensive cause-effect relationship in addressing toxicant effects (Schlekat *et al.,* 2002; Wharfe *et al.,* 2007). The use of such an ecotoxicological line of evidence for pollution monitoring and control in regulatory frameworks is becoming increasingly important as it provides more accurate and precise risk estimations for environmental quality.

The elemental assumption of the ecotoxicological approach is that toxicants impact molecular, cellular, and physiological processes and therefore have the potential to

adversely affect the health of individual organisms (Baird *et al.,* 2007), which might propagate and impair ecosystem function through effects on populations (Loreau *et al.,* 2002). As sub-organismal level estimations (biomarker) are among the most sensitive and earliest detectable responses signalling exposure and/or effects, they may provide useful early warning and screening tools for monitoring environmental impacts (Baird *et al.,* 2007). However, their choice for regulatory purpose depends on factors such as ecological relevance and the monitoring objective as some endpoints might lack a mechanistic linkage to the ecosystem function and processes for extrapolating from lower to higher levels of ecological organization (Crane *et al.,* 2007; Baird *et al.,* 2007).

Conventional ecotoxicity assays to address the impacts of toxicants involve the collection, transportation and laboratory storage of samples, followed by measurements of their toxicity under controlled laboratory conditions using standard protocols (Crane *et al.,* 2007). Generally, such bioassays offer a rapid measurement of the potential environmental harm posed by contaminants using standard test species (Rand, 1995; Wharfe *et al*., 2007). In addition, such laboratory assays provide a causal linkage between contaminants and their biological effects, complementing the chemical characterization and the monitoring of indigenous biota, within the triad approach (Chapman, 1996; Burton *et al*., 2002). Laboratory assays when compared to measuring effect at higher levels of biological organization are more sensitive and least money/time consuming (Connell *et al.,* 1999; Liber *et al.,* 2007). Furthermore, the outputs from single-species toxicity assays are essential for other research areas such as ecotoxicological modelling (Vann der Brink *et al.,* 2002), trait-based risk assessment (Liess *et al.,* 2008) or assessment of mixture toxicity (Altenburger *et al.,* 2004).

However, laboratory toxicity testing using single-species does not always generate ecologically relevant information for the area of concern (Giesy and Hoke, 1989), mainly because field situations may not be accurately simulated in the laboratory, and sample collection, storage or handling can affect sample toxicity (Crane *et al*., 2007; Baird *et al.,* 2007). It is also important to consider that under natural systems organisms are generally exposed to a combination of multiple stressors which is difficult to simulate in the laboratory (Rand, 1995). In addition, assays based on grab sampling may not include exposure to volatile or time-varying stressors as such stressors will be lost during sample transport. Furthermore, the observed effects in the laboratory cannot be reliably extrapolated to real contamination scenarios (Connell *et al.*, 1999; Burton *et al.*, 2005; Liber *et al.*, 2007). This is particularly relevant in sediment toxicity testing as it is highly affected by artefacts related to sample collection and transport (Castro *et al.,* 2003). Thus, this suggests laboratory assays should ideally be used as part of integrative toxicity assessments, in combination with other more complex and realistic ecotoxicological tools, as well as physico-chemical parameters and biological monitoring surveys (Culp *et al.,* 2000).

In this respect, microcosms or mesocosms systems simulating natural communities are regarded as essential tools to validate extrapolations from single-species laboratory assay estimates (Coors *et al.,* 2006). Such complex assays involve conducting multiple-species toxicity tests while simulating more realistic exposure scenarios, where the direct and indirect effects of stressors can be assessed. However, conducting mesocosms tests to assess toxicant impacts requires skilled labour and high financial costs, which obstruct their routine use. Due to this, *in situ* assays are used as an alternative to partially validate

extrapolations from laboratory assay estimates in a cost-effective way, and are being increasingly used as part of integrative assessment studies to increase the ecological realism of environmental decisions for specific impacted systems (Moreira-Santos *et al.,* 2004; Burton *et al.,* 2005). Normally such *in situ* assays offer the possibility of assessing contaminant effects under fluctuating natural environmental conditions integrating site-specific physical, chemical and biological processes by exposing test species at the impacted system with a minimal manipulation (Crane *et al.,* 2007; Baird *et al.,* 2007). *In situ* assays integrate effects from multiple stressors and incorporate natural factors that may modulate toxicity (e.g. water current, light, sediment disturbance) (Burton *et al.,* 2005; Crane *et al.,* 2007). Furthermore, by locating organisms in their own environment, the range of organisms used in *in situ* toxicity testing can be extended to include indigenous species for risk assessment frameworks (Baird *et al.,* 2007).

Although *in situ* assays have many advantages in risk assessments, long-term *in situ* exposures present some drawbacks such as the rise of study costs and the emergence of effects associated with potential confounding factors (environmental factors or/and contaminants) (Adams, 2003). In addition, most *in situ* assessments are based, either directly or indirectly, on the concept of comparing current conditions to natural conditions in the absence of disturbance or alteration (i.e., comparison to a pristine, unpolluted, or anthropogenically undisturbed state) (Reynoldson *et al*., 1997). However, nowadays obtaining a pristine site to compare is almost impossible due to the extensive anthropogenic impacts on aquatic ecosystems. As a consequence, to overcome this problem most researchers use the concept of a reference condition approach, where the reference condition is defined by a set of reference sites representing undisturbed or the best available

conditions of a region though they are not still efficient (Reynoldson *et al.,* 1997). Beside this difficulty in establishing reference conditions, *in situ* bioassay has some other limitations as many variables are difficult to control, for example the stress induced to the test organisms due to their transport to the field and caging (DeWitt *et al.,* 1999), difficulty of deployments of chambers in deep or fast-moving systems (Burton *et al.,* 2005). Taking these drawbacks into account, it is more possible to precisely estimate risk by integrating *in situ* assays with other risk assessment tools. Moreover, their adaptation to a monitoring program can minimize costs and improve risk estimation accuracy.

It is widely accepted that single-species bioassays will never provide a full picture of the quality of the environment, as toxicity is both species-specific and chemical-specific; hence, the use of a battery of tests is advocated (Baird *et al.,* 2007). Such approach contributes to solve the problem of over or underestimates of actual risks of chemical stressors in the aquatic environment. Thus, in planning a battery of bioassays the choice of the test species is an important factor as it may highly influence the ecological relevance of the toxicity results, both in field and in laboratory approaches (Baird *et al*., 2007). In most cases, to overcome this problem, the potential ecological receptors under the study area are selected as a surrogate for the evaluation of the effects of toxicants (Van Straalen, 1993). These receptors should reflect important ecosystem components and be a representative of key ecosystem functions and major trophic levels at the study area (Fisher *et al.,* 2003; Baird *et al.,* 2007), as well as sensitive to a broad range of stressors (Baird *et al*., 2007).

Beside the consideration of the choice of the test species, the selection of the test endpoint remains equally important for both *in situ* and laboratory assays in risk characterization. Many bioassays measure biological effects such as mortality,

physiological (e.g., feeding, metabolism) and/or biochemical (e.g., enzyme activity) responses. Linking these endpoints to change at the population level is essential for the development of predictive indicators of the environmental impact of chemical stressors. However, not all the endpoints have the same predictive ability, as their responses cannot always be extrapolated to ecological functions (Forrow & Maltby, 2000; Crane *et al.,* 2006; Baird *et al.,* 2007; Krell *et al*., 2011). For instance, most often survival does not provide enough information on contamination effects since chemicals are usually discharged at concentrations that induce ecologically relevant effects before killing the organisms (Gerhardt, 1996; Maltby *et al*., 2002; Mills *et al*., 2006). Conversely, endpoints for sublethal effects (e.g. growth, feeding, metabolism, enzymatic activities, and reproduction) are preferred as it is a component of individual fitness which can be mechanistically linked to ecological functions and processes (Taylor *et al*., 1998; Krell *et al*., 2011; Agostinho *et al*., 2012).

1.2 Study Objectives

Many water legislations, including the European WFD, suggest a multidisciplinary and holistic approach to water management and require the establishment of chemical, hydromorphological and biological quality objectives as well as the implementation of assessment methods to fulfil the goals of effective monitoring of all quality elements (Pollard & Huxham, 1998). Hence, there are clear opportunities for the use of biological effects methods – ecotoxicological bioassays – as tools to contribute to establish cause-effect relationships in ecological quality assessments of water bodies, specifically to ascertain the causes of a deteriorating ecological status in a water body (ICES, 2004; Wharfe *et al*., 2007). In this regard, the main goal of the present study was to evaluate the

water quality at the big man-made Alqueva reservoir (South Portugal) based on an ecotoxicological line of evidence. Specifically the present study was conducted to: (1) identify the ecological receptors and functions at most risk in the Alqueva reservoir – a previously known freshwater reservoir contaminated mainly with pesticides – using a battery of laboratory and more ecologically relevant *in situ* assays, and (2) to set a cost-effective tool-box (i.e., a battery of short-term, easy and accurate assays) to allow rapid and integrative measures of environmental quality to complement the existing assessment and monitor tools for freshwater reservoirs in the study area as well as other regions.

To achieve these objectives while minimizing the likelihood of missing ecotoxicological effects, either because of differences in species sensitivity to the contaminants occurring in the study area, or because of differences in exposure routes through life-cycle traits, a broad battery of bioassays was performed. The assay battery integrated a wide range of species that are representative of different taxonomic (algae, crustaceans, and insects) and functional groups (primary producers, primary and secondary consumers, benthic and epibenthic decomposers) as well as of different environmental compartments (water column, sediment-overlying water, sediment) in the study catchments. Moreover, as many sub-lethal effects occur at more realistically discharged concentrations of contaminants than lethality, the proposed bioassays have included measurements of various sub-lethal endpoints that can be translatable into effects on the health of populations, communities and ecosystems (Schafer *et al.,* 2011). Also, in parallel to laboratory assays, to reduce assessment uncertainties, more ecologically relevant *in situ* assays were conducted with locally occurring species. Furthermore, both the species and the responses measured were selected taking into account the ecosystem under study and

the range of potential contaminants discharged to the catchment, to assess accurately the ecological receptors at most risk without missing biological effects. Finally, the study was conducted during spring season (in May), considered as the worst water quality case scenario, when contaminant concentrations in the system are expected to be highest as the result of the increased evaporation due to the high temperatures and no occurrence of rainfall dilution, and of the regime of pesticide application in the region (despite the limited occurrence of runoff events at this time of the year). At the end of the present study, following the evaluation of both laboratory and *in situ* assays, a set of easy-to-use assays showing a good correlation with toxicity and with the more demanding (in time and effort) ecologically relevant ones should preferably be recommended to be included in a tool-box which will be used in assessment and monitoring programs by the water quality managers in the areas.

Chapter 2 Materials and Methods

2.1. Characterisation of the study area and site selection

The imperative need of water for life supporting activities (e.g., for agriculture and human consumption) has led to the construction of artificial lakes or dams worldwide (Abramovitz, 1996; Bird, 2001). Nevertheless, unsustainable use and management of these water bodies are posing serious environmental and human concerns, as a result of excessive nutrient and contaminants loading mainly due to agricultural practices (Carpenter *et al.*, 1998).

The Alqueva reservoir is one of the results of a massive dam construction for multipurpose use, located at the Guadiana River basin in the Alentejo region, Southeast of Portugal (Figure 1). This reservoir is the largest artificial lake in the Iberian Peninsula with a sophisticated system of irrigation covering about 1 100 km^2 of land (World Wide Fund for Nature, 1995). The population density in the region ranges between 2 to 25 inhabitants/km^2, with a total of approximately 500 000 residents (Palma *et al*., 2010a and a reference therein). The purpose of this reservoir was to be a strategic water reserve for the reinforcement of water supply to the populations, agricultural activities and hydroelectric power generation (World Wide Fund for Nature, 1995)

The hydrological feature of the Alqueva reservoir is characterized by a Mediterranean climate, with dry and hot summers and mild and wet winters with concentrated rains (Morales, 1993). The annual average temperature ranges from 24 to 28°C in hot months (July/August) and 8 and 11°C in cold months (December/January). The average annual precipitation ranges from 450 to 550 mm. The region is affected by intense dry periods without precipitation since almost 80% of the precipitation occurs from October to April (Morales, 1993). Seasonal and temporal fluctuations in rainfall coupled with the

influence of the Mediterranean climate turns the flow of the tributary rivers to the reservoir greatly variable (Chícharo *et. al.,* 2006), causing strong seasonal and spatial variability of the reservoir water quality (Palma *et al.,* 2010b). In addition to this natural variation of influx in the basin, extensive urban and agricultural development in the catchment area, mainly since the 1950s, has progressively reduced the amount of freshwater, thereby contributing to a decrease in the available quantity (Dias *et al*., 2004), which might have a strong impact on the reservoir ecosystem in general.

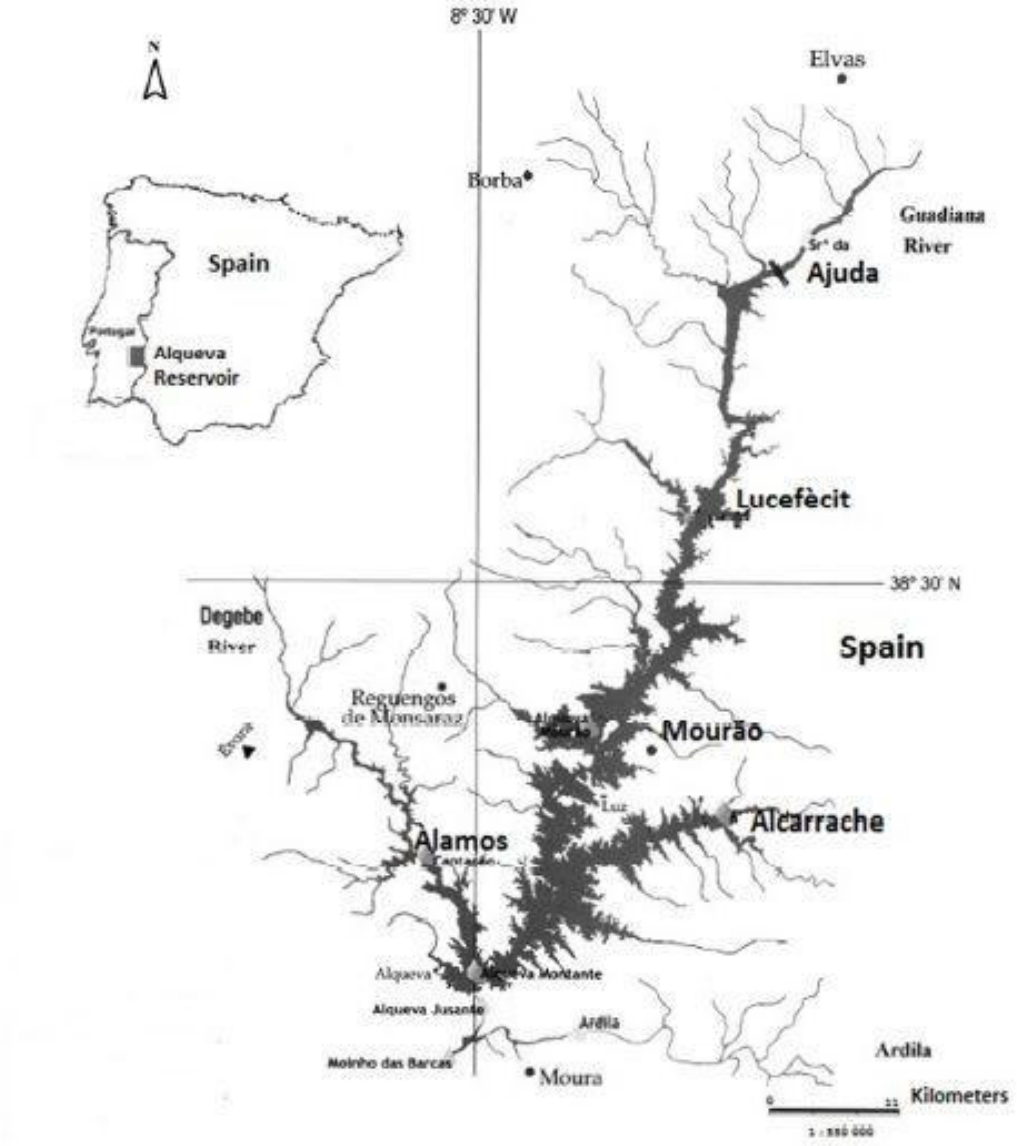

Figure 1. Alqueva reservoir catchment and location of the five study sites within the present study (Ajuda, Lecefécit, Mourão, Alcarrache, and Álamos) (source Palma *et al*., 2010a).

The major environmental concern related to the Alqueva reservoir is that it is subjected to different types of anthropogenic pressures causing serious water quality degradation. Both point source (municipal and industrial wastewater discharges and sewage

of pasture animals, namely mines and cattle breeding) and diffuse source (intense agricultural activity) contaminants constitute the main water quality violators in the reservoir (Instituto do Ambiente 2005). The reservoir catchment area is predominantly surrounded by agricultural fields, with only a small percentage of semi-natural areas, which makes agricultural contaminants one of the main environmental problems to this freshwater reservoir (Morais *et al*., 2007; Palma *et al.,* 2008; Palma *et al.,* 2010b; Rodriguez *et al.,* 2010). Moreover, the Guadiana River, the main tributary for the reservoir, receives the drain water from 3 000 km^2 of irrigated Spanish land and the residual water resulting from industries and from more than 1 650 000 inhabitants (Palma *et al.,* 2010b and a reference therein). This fact may be the main reason that the concentration of the sum of pesticides in the reservoir was above 0.5 ug/l in 2006 (Rodriguez *et al.,* 2010). Pesticide classes of phenylureas, triazines, chlroacetanilide, organophosphorous, organochlorine and thiocarbamates were the most frequently detected contaminants in the reservoir (Palma *et al.,* 2008; Palma *et al.,* 2009; Rodriguez *et al.,* 2010a). Furthermore, the Alqueva reservoir has been classified as "less than good status" based on the evaluation done by the European WFD and The River Basin Management Plan Implementation for Portugal using selected physicochemical parameter measurements and biological surveys (Silva *et al.,* 2011). In addition to this, it was observed that the anthropogenic impacts are not evenly distributed on this big man-made reservoir; the upstream section is highly impacted, thereby creating spatial water quality variability in the reservoir (Palma *et al.,* 2009; Palma *et al.,* 2010a).

The ecotoxicological characterization of this reservoir already showed a toxic effect on the growth of the microalgae *Pseudokircheneriella subcapitata* (Korshikov) which might be related with herbicides presence (Rodriguez *et al.,* 2010); even though the study

neglected the effect of nutrient variation across the sampling stations. Palma *et al.* (2010a) observed that the concentration of the insecticide chlorpyrifos in surface waters of the reservoir during 2006-2007 was in the same range as the EC_{50} (median effective concentration) values obtained for lethal toxicity levels with the crustacean species *Thamnocephalus platyurus* Packard and *Daphnia magna* Straus. Thus, the evaluation of the water quality status of this reservoir is essential to identify its main problems and adapt water management strategies for sustainable use.

2.2 Study design

In this work, the major goal was to identify ecological receptors at most risk in the Alqueva reservoir encompassing relevant short-term sub-lethal laboratory assays and more ecologically relevant *in situ* assays, to set a cost-effective (rapid, easy and sensitive) tool-box for assessment and monitoring programmes of contaminated lentic system in Portugal, particularly the Alqueva, and other regions. Considering the temporal variability of contaminant concentrations in the Alqueva reservoir (Palma *et al.,* 2009; Silva *et al.,* 2011), the present study was carried out during spring (in May 2013), taken as the best representation of a worst water quality scenario, when contaminant concentrations in the system are expected to be highest as the result of the increased evaporation due to the high temperatures and no occurrence of rainfall dilution, and of the regime of pesticide application in the region (despite the limited occurrence of runoff events at this time of the year).

In the present study, the following five representative sampling stations, distributed from upstream to downstream, were selected in an attempt to identify major ecological

receptors at risk in the reservoir: Ajuda – Aj (38°46'38''N, 07°10'11''W), Lucefecit – Lf (38°37'32''N, 07°17'20''W), Mourão – Mr (38°24'38''N, 07°24'56''W), Alcarrache – Ac (38°19'54''N, 07°19'53''W), and Álamos – Al (38°20'30''N, 07°34'35''W) (Figure 1). The differences in sediment allocations (deposition versus non-deposition areas), the worst-case sites of pesticide input and the intensity of known anthropogenic pressures were major factors considered when choosing the study sites. Moreover, all these sites were previously monitored to evaluate environmental risks, both by other researchers and government (EDIA; which is responsible for the management of the Alqueva reservoir) and were regard as strategic and representative of the reservoir (Palma *et al.,* 2009; Palma *et al.,* 2010a; Rodriguez *et al.,* 2010; Silva *et al.,* 2011).

To the extent possible, laboratory and *in situ* assays using the same species and based on measurements of the same responses were selected, in an attempt to more comprehensively interpret the responses obtained from the more ecologically relevant *in situ* assays while evaluating the role of laboratory assessments in ecological risk assessment studies. For the *in situ* assay a single location was selected at each study site. All water and sediment samples collected at the study sites were assayed in the laboratory at 100%, i.e., without applying a dilution series.

The battery of laboratory and *in situ* assays included species both originated from standard laboratory cultures and naturally occurring, being all indigenous in Portuguese habitats to minimize uncertainty, i.e., used as surrogates of the ecological receptors potentially at risk in the Alqueva reservoir. The assays were selected to cover a range of organisms representative of different taxonomic (algae, crustaceans, and insects) and functional groups (primary producers, primary and secondary consumers, epibenthic and

benthic decomposers) and environmental compartments (water column, sediment-overlying water and sediment). From a practical viewpoint, other criteria used to select the test species were availability in sufficient numbers, easily manipulated, and species for which data exist on the (autochthonous or related) species sensitivity to a broad range of stressors (environmental/chemical) so as to avoid under- or over-estimations of risk.

Ideally, *in situ* assessments are based either directly or indirectly on the concept of comparing conditions under study to natural conditions in the absence of disturbance or alteration (i.e., comparison to a pristine, unpolluted, or anthropogenically undisturbed state) (Reynoldson *et al*., 1997). Ordonez (2012) used the Beliche reservoir (Guadiana River basin) as a reference reservoir, as it is considered to have a minimum disturbance in its catchment (Silva *et al.,* 2011). However, as reported by Ordonez (2012), the differences in reservoir size, hydrological characteristics and water chemistry (mainly related with trace elements susceptible of influencing organism performance) suggested the Beliche reservoir not to be the best achievable target to compare to the Alqueva sites toxicity; accordingly Beliche was not used in the present study. To circumvent this problem in the present study, the ecotoxicity of the Alqueva sites in the laboratory assays was evaluated by comparing the performance of the standard test species in the Alqueva waters/sediments with the standard control within the same assay, even though confounding factors may occur as a response inhibition of the organisms in the natural samples compared to the control may not imply the existence of toxicity but rather that the water/sediment characteristics (e.g. trace elements, micronutrients, organic matter content) are different. For the species collected at a single reference site their performance in the Alqueva samples was compared to that of samples from the site of collection, to minimize potential stress induced as result

of using artificial medium/sediment on local organisms. As for the *in situ* assays, the ecotoxicity of the Alqueva sites was evaluated by deploying all organisms also at the single reference site, except for the microalgae *in situ* assay, which incorporates strategies to discriminate environmental differences across sites from toxic effects, and for the cladoceran assay due to logistic reasons.

The spring source of the Anços River (Central Portugal: 39º58'43.40"N, 8º34'23.30"W) was selected as the single reference site for collection of the epibenthic species; all other species used were from standard laboratory cultures or commercially available. The Anços River was previously classified as "minimally disturbed" based on classes of water quality for multiple uses by the National Water Agency (INAG, Portugal), species composition and abundance in the riparian corridor, channel morphology, and urbanization and industrial activities in the catchment area (Feio *et al*., 2010). It was also regarded as a "reference site" through predictive models for water quality assessments in compliance with the WFD using macroinvertebrate communities as biological indicators and various environmental explanatory variables (Feio *et al*., 2007).

All assays performed in the present study have largely been used in other ecotoxicity studies and most of them have already been standardized, and guidelines/standard operational procedures for recommended test methods have been published. The laboratory toxicity evaluation included: 72-hours growth of the green planktonic microalgae *P. subcapitata* (primary producer), 24-hours feeding and 21-days reproduction of the planktonic cladoceran *D. magna* (primary consumer) for the water column assays, 6-days growth of the epibenthic omnivorous ostracod *Heterocypris incongruens* (Ramdohr) (primary consumer), 96-h postexposure feeding of the epibenthic

grazer snail *Theodoxus fluviatilis* (Linnaeus) (primary consumer) and the epibenthic detritivorous amphipod *Echinogammarus meridionalis* (Pinkester) (decomposer) for the sediment-overlying water assays, and 96-h postexposure feeding and 10-days growth of the benthic midge deposit feeder *Chironomus riparius* Meigen (decomposer) for the benthic assays. The *in situ* toxicity evaluations included: 8-days growth of *P. subcapitata* and 96-hours postexposure feeding of the planktonic cladoceran *D. longispina* (Mueller) (primary consumer) for the water column assays, the 96-hours postexposure feeding of *T. fluviatilis* and *E. meridionalis* for the sediment-overlying water assays, and 96-hours postexposure feeding of *C. riparius* for the benthic assays.

2.3 Water and sediment sample collection and physico-chemical measurements

To conduct the laboratory assays, water and sediment samples were collected at the five Alqueva sites and at the Anços reference site during the performance of the *in situ* assays (overall a maximum 4 days difference). From each site, twice 5-L water samples were collected at a depth of 5 cm using acid washed polyethylene bottles; prior to use in laboratory assays the two samples from each site were pooled. Similarly, composite sediment samples (upper 10 cm) were collected with a scoop from each site and placed in black airtight plastic containers. All water and sediment samples were stored at 4ºC in darkness immediately upon arrival to the laboratory, and used within less than two months.

Physico-chemical parameters measured on site during *in situ* deployment and retrieval were dissolved oxygen (Wissenschaftlich Technische Werkstatten, OXI 92 oxygen meter, Weilheim, Germany) and temperature by deploying a data logger (HOBOware Pro, Bourne, MA, USA) along with the assay chambers. Measurements of pH (WTW 537 pH

meter) and conductivity (WTW 315i/SET conductivity meter) were taken immediately upon the samples arrival in the laboratory. Water physico-chemical measurements (pH, conductivity and dissolved oxygen) were also taken during the laboratory assays (see section for measurements specific for each assay).

The sediment samples collected from each station were used for water content, organic matter content (percent volatile solids), and particle size distribution analysis. Water content was determined by measuring the loss of sediment weight after oven drying at 60°C for 24 hours, and was expressed as the percentage of the initial wet weight of the sediment (Buchanan and Kain 1971). Organic matter content was determined by quantifying the loss of weight after ignition at 500°C for 24 hours, and was expressed as the percentage of the oven-dry weight of the sediment (Buchanan and Kain 1971). The sediment particle size distribution was analyzed by sieving it into eight fractions according to the Wentworth scale (Buchanan and Kain 1971): silt (< 63µm), very fine sand (63 – 125 µm), fine sand (125 – 250 µm), medium sand (250 – 500 µm), coarse sand (500 µm – 1 mm), very coarse sand (1 – 2 mm), very fine gravel (2 – 4 mm), and fine gravel (4 – 8 mm).

2.4. Test organisms: origin and laboratory maintenance

The green microalgae *P. subcapitata* was selected for the present study as it is a species easily maintained in the laboratory under reproducible culture conditions. Moreover, it is among the most widely used and recommended species for freshwater toxicity testing, for which standard guidelines have already been established (OECD, 1984) and are currently endorsed for regulatory purposes. The culture maintained at the University of Coimbra (Laboratory of Ecotoxicology) was established from a strain (Nr. WW 15-2521)

obtained from the Carolina Biological Supply Company (Burlington, NC, USA). Non-axenic stock cultures of the freshwater algae were maintained in 250-ml sterile glass Erlenmeyer flasks, with Woods Hole MBL growth medium (Stein, 1973) supplemented with vitamins (0.1 mg/L B1, 0.5 µg/L B12 and 0.5 µg/L biotin), at 19 – 21 °C, under continuous cool-white fluorescent illumination (100 µE/m2/s) . To start new cultures and also to obtain organisms for the assays, algae were harvested while still in the exponential growth phase (5–7 d old).

Cladoceran species are one of the most sensitive groups of organisms that play an important role in lentic ecosystem food chains (Hanazato, 2001). Moreover, due to their small size, short life cycle and high parthenogenetic reproduction rates, cladoceran species are commonly used to determine toxicity of chemicals and set environmental health standards (Hanazato, 1998). In the present study, the cladocerans *D. magna* (Clone A originated from IRCHA in France) and *D. longispina* (originated from a reference population in an aquatic systems in Southeast Portugal; Ribeiro and Lopes 2013) were obtained from stock cultures at the University of Coimbra (Laboratory of Ecotoxicology). The stock cultures were maintained at 19 – 21 °C, under a 14:10-hours light:dark photoperiod, in reconstituted hard water medium (ASTM, 2002), hereafter referred to as ASTM medium, supplemented with vitamins (7.5 µg/L B1, 1 µg/L B12, and 0.75 µg/L biotin) and Marinure extract (Glenside, Stirling, UK), which was renewed every other day, and fed daily with *P. subcapitata* (3×10^5 cells/ml). Cultures of *D. magna* were started with 25 organisms/L till the first brood is observed and then reduced to 15 organisms/L from there onwards, while for *D. longispina* cultures were started with 15 organisms/120 ml and reduced to 8 after the first brood observation. All organisms used in assays were from third

to fifth broods. Whereas feeding assays were started with 4-days old juveniles, the reproduction assays were started with less than 24-hours old neonates.

Regarding the ostracod *H. incongruens*, organisms to perform the assays were obtained by hatching cysts. The latter are commercially available together with all the materials needed to perform an assay in the form of a kit, the Ostracodtoxkit F (Creasel, 2001). For hatching, cysts were incubated in ASTM medium, though moderately hard (ASTM, 2002), at 25^{o}C under continuous cool-white fluorescent illumination (75 μE/m2/s), for 52 hours; after the first 48-hours pre-feeding of the freshly hatched organisms was carried out with *Spirulina* powder. This assay organism was selected as the test species is prevalently occurs in the freshwater system and plays an important role in ecosystem functioning.

The epibenthic organisms *T. fluviatilis* and *E. meridionalis* were selected because they are autochthonous available all year round in sufficient numbers and also play an important role in freshwater ecosystem functioning (Kirkegaard, 2006; Agostinho *et al*., 2012). Both species were collected at the spring of the Anços River. Snails were handpicked from stones of the river stream-bed, whereas a hand net with a mesh size of 1.5 mm was used to collect the amphipods after gently disturbing the sediment. Pre-copula pairs of *E. meridionalis* were separated on site and only young males were used for assays; females were excluded to prevent variability associated to the reproductive status. Upon collection organisms of both species were transported in thermally insulated boxes filled with local water to the laboratory or directly to the *in situ* assay sites (see below section 2.6). For the laboratory assays organisms were conditioned for transportation (30 minutes trip) in plastic containers (length × width × height of 21 × 15.4 × 9.4 cm). Laboratory cultures were

maintained in a temperature-controlled room at 19 – 21°C under a 14:10-hours light:dark photoperiod, in plastic containers (similar to those used for transportation) with aerated local water and stones (approximately 5-cm height), for a maximum of 48 hours prior to assay initiation. The mean (± standard deviation [SD]; *n* = 30) shell height and length of the snails used in the laboratory assay was 4.30 (± 0.19) and 5.40 (± 0.22) mm, respectively, whereas for the *in situ* assay was 3.94 (± 0.16) and 4.92 (± 0.21) mm; within this size range there is no effect of organism size on feeding rate (Correia *et al*. in press). The amphipods used had a mean (± SD; *n* = 20-23) wet weight of 7.79 (± 2.1) and 8.36 (± 1.4) mg in the laboratory and *in situ* assays, respectively. For wet weight estimations the water in excess was fist removed using absorbing paper and organisms were weighted to the nearest 0.1 mg in a microbalance (Kern ALS 120-4, Kern & Sohn, Balingen, Germany).

The benthic *C. riparius* was included in the assay battery as it belongs to the most widely distributed and frequently most abundant group of insects in freshwater environments (Pinder 1986). Besides, it is the most frequently used test organism in sediment ecotoxicological testing (OECD 2004). Organisms for testing were obtained from laboratory cultures at the University of Coimbra (Laboratory of Ecotoxicology). Cultures were held inside a 40 × 60 × 120 cm closed transparent acrylic box to freely allow adult swarming and copulation. Newly hatched larvae were reared in crystallizing dishes at a density of 30 larvae per 190 g of quartz sea sand (0.1 – 0.4 mm particle size; Merck, Darmstadt, Germany) filled with 300 ml of ASTM medium, until day 7. After 7 days, larvae were transferred to newly prepared crystallizing dishes and their density reduced to 15 until emergence. Larvae were fed on grounded fish flake food (Tetramin, Tetrawerk, Melle, Germany) at a quantity of 0.1 g/crystallizing dish (a suspension in 2 ml of distilled

water/0.1 g) every 2 days. Newly hatched larvae were used for the growth assays whereas 8-days old larvae were used for the postexposure feeding assays.

2.5 Laboratory assays

2.5.1 Water column assays

The 72-hours *P. subcapitata* growth assay was performed following the OECD (1984) and EC (1992) guidelines. To carry out this test, the water samples were vacuum-filtered (0.45 m) to remove locally occurring microalgae that might compete with the test organisms. The potential effect of nutrient limitation was discriminated from potential toxic effects by performing the assay with local waters supplemented with nutrients in the same amount as the control medium (EC 1992), which was the MBL culture medium diluted 2.5 times to be in accordance with recommended nutrient levels (OECD 1984). To perform the assay, 24-wells microplates (Coastar, Cambridge, MA) were used with each replicate (six for the standard control and three for each site water) consisting of 900 µl test solution plus 100 µl of algal inoculums (an initial cell concentration of 10^4 cell/ml was established for each wells. The border line wells of each microplate were filled with distilled water to minimize water evaporation during the test duration and each microplate had a control replicate to verify for homogeneous environmental conditions within the tested area. The assay was incubated under the same temperature and light conditions used for the stock cultures, while it was well mixed with a micropipette, to promote gas exchange and prevent cell sedimentation, every 12 hours. After the 72 hours exposure period, a well mixed 800 µl of each replicate of samples were preserved in 200 µl Lugol's solution. The final cell densities were counted from well-mixed aliquots of preserved samples under a microscope (x400 magnification) using a Neubauer chamber (American Optical Buffalo, NY, USA).

The assay endpoint was estimated as the daily specific growth rate, calculated from the initial and final cell densities (Nyholm & Kallqvist, 1989). The physico-chemical parameters (pH and conductivity) were measured in two replicates of fresh medium at the start of the assay.

The 24-hours *D. magna* feeding assay was conducted based on a methodology developed by McWilliam & Baird (2002). The control medium was ASTM medium not supplemented with vitamins or seaweed extract. For each tested water and control five replicates were setup in 175-ml glass vials filled with 120 ml of test solution plus food (3.5 $\times 10^5$ cells/ml of *P. subcapitata*) and five 4-days old organisms. To control for algal growth during the test period a blank treatment, consisting of ASTM medium without organisms, was also performed. Site waters were not vacuum filter to remove local microalgae as the observed quantities were minimal (below 10^3 cells/ml). The test was incubated at 19 – 21°C in darkness. After the 24 hours exposure period the test endpoint was estimated as the feeding rate (number of ingested cells/organism/24 hours), calculated from initial and final cell densities; algal counting was performed as described for the 72-hours *P. subcapitata* growth assay above explained. The physico-chemical parameters (pH, conductivity and dissolved oxygen) were measured in two replicates of fresh medium at the start of the assay.

The 21-days *D. magna* reproduction assay was carried out according to the OECD (1998) guideline. For each water sample and control (same medium used for stock culturing, see section 2.4), 10 replicates were set up in 60-ml glass vials filled with 50 ml of test water and one less than 24-hours old neonate. Together with the control medium water samples were also supplemented with seaweed extract to ensure direct comparison of reproduction

results with the control. Feeding regime, medium renewal frequency and incubation conditions were similar to those outlined for the stock cultures. During the assay duration, the number of living offspring produced per parent animal was recorded for each brood. After the 21-days exposure period, the assay endpoint was estimated as the total number of living offspring laid per live parent animal. The physico-chemical parameters (pH, conductivity and dissolved oxygen) were measured in two replicates of old and fresh medium at all medium renewal days.

2.5.2 Sediment-overlying water and sediment assays

A 6-days ostracod *H. incongruens* growth assay was conducted according to the Ostracodtoxkit F standard operating procedure for sediment toxicity (Creasel, 2001). All the necessary materials were provided in the commercial kit to perform the test. The standard control consisted of reference sediment included in the kit plus the same medium used for hatching the cysts (see section 2.4). The assay was conducted in 6-wells microplates with each replicate well consisting of approximately 1 ml of sediment plus 4 ml of test water inoculated with food (3.75×10^6 cells/ml of a green microalgae provided in the kit) and 10 less than 24-hours old organisms; 6 and 4 replicates were established for the control and tested water/sediments, respectively. After the 6-days exposure period, survival and final length (in µm) were recorded, being the assay endpoints.

The 96-hours snail *T. fluviatilis* postexposure feeding assay was performed according to the methodology developed by Correira *et al*. (in press). For each treatment four replicates were set up in 60-ml glass vials filled with a layer of approximately 5-cm of sediment plus water until the top and five snails. The vial was then covered with a 200-µm

mesh and completely immersed into a 250-ml polyethylene container, filled with the respective local water, to ensure constant exposure of the test organisms. In the same way, four replicates of control with sediment and water from the reference site were prepared. The set up was prepared 12 hours prior to the beginning of the test and left with continuous aeration to stabilize sediment and allow pore-water equilibrium; to add the test organism aeration was stopped for a few minutes. The water level was daily adjusted with distilled water and the organisms were not provided food. The assay was incubated under the same temperature and light conditions as the *D. magna* reproduction assay. After the 96-hours exposure period, postexposure feeding rates were immediately estimated according to Correia *et al.* (in press). Each organism was individually transferred to 1 ml ASTM medium hard water and 150 defrosted *nauplii* (< than 24-hours old) of *Artemia franciscana.* Then the organisms were allowed to feed at 19 – 21°C in darkness for 3 hours. The test endpoint, postexposure feeding rate, was estimated as number of consumed *nauplii*/organism/3 hours. The physico-chemical parameters were measured in three (dissolved oxygen) and two replicates (pH and conductivity) at the start (prior to organism transfer) and end of the assay.

The 96-hours amphipod *E. meridionalis* postexposure feeding assay was carried out based on a methodology developed by Agostinho *et al*. (2012). For each sample five replicates were setup in 175-ml glass vials filled with approximately 30-ml of sediment sample plus 100 ml of the respective site water and five male amphipods. As for *T. fluviatilis*, the control was prepared with sediment and water from the reference site and all vials were prepared 12 hours prior to the beginning of the assay and left with continuous aeration (to stabilize sediment and allow pore-water equilibrium), which was stopped for a

few minutes for introducing organisms. Also, water levels were daily adjusted with distilled water and organisms were not provided food. The assay was incubated under the same temperature and light conditions as the *D. magna* reproduction assay. After the 96-hours exposure period, postexposure feeding rates were immediately estimated according to Agostinho *et al*. (2012). Each amphipod was individually transferred to a 30-ml glass vial filled with 5-ml ASTM medium hard water and 100 defrosted *nauplii* (< than 24-hours old) of *A. franciscana*. The exposed organisms were allowed to feed at 19 – 21°C in darkness for 30 minutes. The test endpoint, postexposure feeding rate, was estimated as number of consumed *nauplii*/organism/30 minutes. The physico-chemical parameters were measured in three (dissolved oxygen) and two replicates (pH and conductivity) at the start (prior to organism transfer) and end of the assay.

The 96-hours *C. riparius* postexposure feeding assay was performed following the methodology developed by Soares *et al.* (2005). Before starting the assay, sediment samples were well homogenized and locally occurring midges were removed. For each sediment sample, three replicates were setup in 175-ml glass vials containing 40-ml of sediment and 100 ml of overlying local water. Similarly, three control replicates were prepared using sediment and water similar to those of stock cultures. The glass vials were prepared 12 hours prior to the beginning of the test and left with continuous aeration to stabilize sediment and allow pore-water equilibrium. In each treatment and control replicate, five larvae were introduced and aeration was stopped and restarted after 30 minutes to allow larvae to bury into the sediment. The test was incubated under the same conditions used for culturing and no food was provided during the testing period. After the 96-hour exposure period, each larva was individually transferred to a 60-ml glass vial filled with 30

ml ASTM medium hard water and fed on 100 defrosted *nauplii* (< than 24-hours old) of *A. franciscana* for 1 hour under dark condition at 19 – 21°C, to estimate postexposure feeding rates (Soars et al. 2005). The test endpoint, postexposure feeding rate, was estimated as number of consumed *nauplii*/larva/hour. The physico-chemical parameters (pH, conductivity and dissolved oxygen) were measured in two replicates of fresh medium at the start of the assay.

Correspondingly, the 10-days *C. riparius* growth assay was conducted according to the EC (1997) and OECD (2004) guidelines using first-instar larvae. Unless otherwise stated, assay procedures, incubation conditions and physico-chemical measurements were similar to those of the postexposure feeding assay. Four and three replicates, each with three larvae, were setup for the control and tested local samples, respectively. The water levels were daily adjusted with distilled water and a feeding regime consisting of 1 and 1.5 mg of a suspension of ground Tetramin per larva per day up to day 2 and from day 3 onwards, respectively, was applied. After the 10-days exposure period, the larvae were retrieved from each replicate and killed with 90% Ethanol to individually estimate the assay endpoint growth as the body dry weight (in mg); larvae were dried at 60°C for 72 hours.

2.6 *In situ* assays

2.6.1 Water column assays

The 8-days *P. subcapitata* growth assay was carried out following a methodology developed by Moreira-Santos *et al.* (2004). The immobilization of algal cells in beads of calcium alginate was carried as follows. A 1.3% (weight/volume) sterile solution (autoclaved 15 min at 120^{o}C) of sodium alginate (A-7128; Sigma Chemical, Steinheim,

Germany) was mixed with an inoculum of algal cells (less than 1 ml) to prepare an alginate-cell suspension with approximately 10^5 cells/ml. The beads were then formed by forcing this mixture through a syringe (fitted with a needle) into a 2% (w/v) aqueous solution of $CaCl_2$. After being washed with distilled water, beads were stored in diluted MBL medium (20 times) in the dark at 4°C for four days until the assay initiated.

The *in situ* chamber used included a chamber for bead exposure (CBE) and an outer chamber consisting of a 1.1-L white-translucent plastic box with two side windows and nearly all the lid made of a 200-mm nylon mesh. The CBE was composed of two acrylic plates, each with four holes covered on one side by a 64-mm nylon mesh. Four cavities for placing the beads were formed by joining the two plates with the mesh to the outer sides, providing four sub-replicates within each replicate to reduce assay variability (see Moreira-Santos *et al.* (2004) for detail chamber design). To discriminate the potential effect of temperature, light and nutrient levels across sites from those due to potential toxicity, the *in situ* assay included the deployment of two treatments, each with three replicates, at each study site. One consisted of control (closed) chambers filled with MBL medium (2.5 times diluted) to determine to what extent differences in temperature and light conditions across sites influence on *P. subcapitata* growth. The other comprised open chambers exposing the algae to the local water, but provided with 60 g fertilizer particles (Blaukorn, 0008, Aventis CropScience, Lisboa, Portugal) as an additional nutrient source (LW+N). A previous study by Moreira-Santos *et al.* (2004) showed that addition of nutrients to local water (LW+N) allows algae to growth without nutrient limitations as in the MBL control. Thus, any growth inhibition could in the LW+N infer toxicity effect compared to the MBL control. Before used as supplement, the fertilizer particles were thoughtfully mixed with distilled water (500 ml) for 45 seconds with the help of a metal spatula, and washed through a 1-mm

nylon screen, to remove their blue coating, and dried (4 hours at 40^{o}C) to prevent their immediate dissolution.

At the day of deployment, three times 10 beads were preserved in Lugol's solution to estimate the initial cell density and beads were transported to the site already in the CBE with the respective site water (50 µm filtered). After the 8-days exposure period, each group of beads was collected, preserved in Lugol's solution, and transported to the laboratory for microscopic count of cell density. The assay endpoint was estimated as the daily specific growth rate, calculated from the initial and final cell densities as for the laboratory microalgae assay.

The 96-hours *D. longispina* postexposure feeding assay was done by adapting a methodology developed by McWilliam & Baird (2002), using three replicate chambers per site, each with 12 4-days old organisms. Assay chambers were similar to the meshed outer chambers used for the *in situ* microalgae assay, without the CBE and nutrients. At the deployment date, the organisms were transported to the site in 50-ml polyethylene vials filled with culture ASTM medium; one vial/replicate chamber. After a 4-days exposure period, the assay organisms were retrieved and transported to the laboratory in the same vials used for transportation at deployment filled with local water (4-6 hours transport). Upon arrival to the laboratory, immediately the postexposure feeding quantification was carried out according to the procedures described above for the laboratory feeding assay, except that feeding duration was four hours.

2.6.2 Sediment-overlying water and sediment assays

The proposed short-term *in situ* assays for sediment-overlying water (96-hours postexposure feeding of *T. fluviatilis* and *E. meridionalis*) and sediment (96-hours postexposure feeding of *C. riparius*) were conducted simultaneously by exposing all three the test species in the same chambers for the first time up to our knowledge, aiming to increase the ecological relevance of the assays, while decreasing costs (in materials and money) for field assays. The *C. riparius, T. fluviatilis*, and *E. meridionalis* in situ assays were performed by adapting methodologies developed by Soares *et al.* (2005), Correira *et al.* (2012) and Agostinho *et al*. (2012), respectively. Each assay chamber, previously developed by Moreira *et al.* (2005) for *in situ* assays, consisted of a 20-cm long and 5-cm inner diameter acrylic tube with open ends with 0.5-cm wall thickness, being the bottom edge slimmed for 1-cm to facilitate penetration into the sediment. The exchange of overlying water (at the water-column level) and pore-water (at the sediment level) between the interior and exterior of the chamber was guaranteed by two opposite rectangular windows (4 x 10-cm) covered with a 200-μm nylon mesh. To cover the top end of the assay chamber during exposure, a nylon mesh (15 x 15-cm), held by an elastic band, was used. To deploy chambers, they were carefully pushed into the sediment to a depth of about 10 cm, so that the lateral windows allowed both the flow of the overlying water at the water-column level and of pore-water at the sediment level.

At each study site, including the reference site Anços, three replicate chambers were deployed, each with 10 snails, six amphipods and six chironomids; for feeding rate quantifications only eight, five and five organisms were used but such difference was to ease retrieval while respecting natural population densities. All organisms were transported

to the study sites in plastic vials equal to those used for *D. longispina* transportation, one vial/species/chamber. Plastic vials were filled either with reference water for snails and amphipods, which were collected at the day of deployment, or with ASTM medium used for chironomids cultures. At deployment chambers were carefully pushed into the sediment to a depth of about 10-cm and disturbed sediment was allowed to settle for few minutes. Following this, in a procedural manner; *T. fluviatilis* were gently directly inserted into the chamber, which was immediately closed with a mesh, held by a 1-cm wide elastic band, equipped with a plastic tube to introduce the amphipods and chironomids with the help of a plastic funnel. Assay chambers were retrieved carefully after a 96-h exposure period and to avoid organism losses with the sediment during retrieval, the bottom end of each chamber was closed by hand and the chamber contents were directly transferred to a white tray where assay organisms were retrieved and transported to the laboratory following exactly the same procedures as at deployment, but using local water. Upon arrival to the laboratory (4-6 hours later), the postexposure feeding rate determinations were immediately started following exactly the same procedures described for the laboratory assays.

2.7 Data Analysis

For the *in situ* assays, the mean percentage of dead organisms recovered from each chamber after the exposure period was calculated to assess whether any organism escaped during exposure or chamber retrieval. For all assays, except the microalgae in situ assay, the measured endpoints were explored for significant differences across treatments using one-way analysis of variance (ANOVA) or nested ANOVA followed by the Dunnett's multiple comparison test to verify the existence of significant differences between the assay standard controls/reference and the tested samples. In the cladoceran feeding/postexposure

feeding assays, a paired Student's *t*-test was first performed to compare algal cell densities in the control without organisms at the start and end of the feeding period to confirm that no algal growth took place. Also, due to the highly unbalanced nested design (due to low recovery/some mortality) in the amphipod and chironomids *in situ* assays, a one-way ANOVA, instead of a one-way nested ANOVA were used. As for the microalgae *in situ* assay, a one-way ANOVA (not nested ANOVA because a single specific growth rate was estimated per chamber, instead of the four possible sub-replicates) was first performed to confirm that minor temperature and light differences across sites did not lead to differences in growth, and only after a Student's *t*-test was performed to compare the control and LW+N treatments within each site. Prior to all analysis of variance, the assumptions of normality (Shapiro–Wilk's test) and homoscedasticity (Barlett's test) were checked. All analyses were performed using the software Statistica 7.0 (StatSoft, Aurora, CO, USA), and the level of significance was set at 0.05.

Chapter 3 Results

3.1 Sediment characteristics

Characteristics of all collected sediments from each site, in terms of water and organic matter contents and particle size distribution (using the Wenthworth classification), are summarized in Table I. The reference sediment covered a wide range of particle size composition from fine gravel to silt. Compared to the other sites, it was mainly composed of coarse sand and medium sand accounting for 35 and 36%, respectively. Overall, Alqueva sediments were comparable to each other, with water content varying from 18 to 34% and organic matter from 4 to 9%, except Ajuda which had the highest percentage of silt (31%) and the lowest percentage of fine gravel and coarse sand.

Table I. Water and organic matter contents and particle size distribution (following the Wenthworth classification) (all in %) of the sediments (reference site at the Anços River and five sites at the Alqueva reservoir) used for the laboratory and *in situ* assays.

Wentwoth Class	**Patricle size (µm)**	**Site**					
		Reference	**Álamos**	**Alcarrach**	**Mourão**	**Lucefécit**	**Ajuda**
Fine gravel	4 - 8 mm	0.17	0.00	0.00	0.00	0.00	0.00
Very fine gravel	2 - 4 mm	1.61	59.50	68.09	53.54	42.13	16.70
Very coarse sand	1 - 2 mm	17.53	12.03	13.02	13.44	15.02	9.21
Coarse sand	500 µm - 1 mm	35.80	8.52	6.21	9.52	12.90	7.02
Medium sand	250 µm - 500 µm	36.04	3.36	0.44	4.69	5.02	3.93
Fine sand	125 µm - 250 µm	8.37	5.13	2.33	7.51	7.35	12.69
Very fine sand	63 µm - 125 µm	0.34	5.54	5.06	6.97	10.65	18.97
Silt	<63 µm	0.14	5.92	4.85	4.33	6.93	31.47
Organic matter content		0.26	5.56	8.72	4.42	4.63	6.37
Water content		18.01	28.87	25.90	16.88	17.83	33.71

3.2 Laboratory assays

3.2.1 Water column assays

All laboratory assays fulfilled the validity criteria for control performance required in the respective guidelines/standard operational procedures. The overall range of pH, conductivity and dissolved oxygen measurements taken in the water column during each assay are summarized in Table II. The pH and conductivity values were similar among sites and assays, ranging from 7.30 to 8.34 and from 307 to 717 µS/cm, respectively. Dissolved oxygen levels were more variable, ranging from 3 (exceptionally in the snail assay; most frequently from 6 mg/l) to 11 mg/l. Due to the small sample volumes involved, water-column physico-chemical parameters were not measured during the *H. incongruens* growth assay. Although not here presented, physico-chemical parameters in the standard control/reference treatments were within the same optimal levels as in the different treatments.

Table II. Range (minimum-maximum) of water column pH, conductivity (Cond. in µS/cm) and dissolved oxygen (DO in mg/l) levels measured during the laboratory assays with *Pseudokircheneriella subcapitata* (72-houres growth), *Daphnia magna* (24-hours feeding and 21-days reproduction), *Theodoxus fluviatilis* (96-hours postexposure feeding), *Echinogammarus meridionalis* (96-hours postexposure feeding), and *Chrinomus riparius* (96-hours postexposure feeding and 10-days growth) for the ecotoxicity evaluation of five sites at the Alqueva reservoir.

Assays	Site													
	Álamos			Alcarrache			Mourão			Lucefécit		Ajuda		
	pH	Cond. (µS/cm)	DO(mg/l)	pH	Cond. (µS/cm)	DO (mg/l)	pH	Cond. (µS/cm)	DO(mg/l)	pH	Cond. (µS/cm)	pH	Cond. (µS/cm)	DO (mg/l)
Pseudokircheneriella subcapitata [a]	7.62	515	nm	7.59	500	nm	7.52	405	nm	7.62	573	7.59	580	nm
Daphnia magna feeding [a]	7.82	340	9.3	8.07	331	9.4	8.07	330	9.4	8.20	405	7.73	410	8.8
Daphnia magna reproduction [c]	7.46-7.88	321-356	6.4-10.3	7.46-8.38	326-349	6.8-10.6	7.38-8.06	367-388	6.6-10.4	7.42-8.26	405-426	7.45-7.76	409-426	6.9-10.3
Theodoxus fluviatilis [b]	7.33-8.01	339-408	3.7-8.2	7.38-7.99	348-415	3.0-7.7	7.37-7.87	366-372	5.3-7.9	7.31-7.93	402-418	7.20-7.93	420-463	3.0-8.1
Echinogammarus meridionalis [b]	8.20-8.23	389-444	7.3-8.4	8.02-8.03	384-477	7.4-7.9	7.60-7.86	296-340	7.4-8.5	7.77-78.91	345-405	8.03-8.20	450-500	7.3-8.6
Chrinomus riparius feeding [b]	7.96-8.12	353-397	8.0-8.4	7.79-8.24	392-507	7.5-8.3	7.59-7.66	307-344	8.0-8.2	7.58-7.73	354-430	7.76-8.07	428-499	7.5-8.1
Chrinomus riparius growth [b]	7.79-8.36	330-630	8.1-8.3	7.71-8.25	356-543	7.9-8.4	7.49-7.59	317-355	7.4-8.3	7.37-7.64	382-398	7.63-8.34	429-717	7.7-7.9

[a] measured only at start of assay
[b] measured at start and end of assay
[c] measured at all the medium renewal
[d] measured only at end of assay
nm: not measured

The result from one-way ANOVA revealed that the 72-hours growth of *P. subcapitata* was significantly different among treatments ($F_{5,15} = 9.15$, $P < 0.001$). However, the Dunnett's test showed that such difference was simply caused by a significantly higher growth rate (by 5 to 11%) observed at all sites, except Mourão, compared to the control. Whereas the minimum percentage of increase was 7% at Ajuda, the maximum percentage of increase was merely 11% at Álamos (Figure 2).

In the 24-hours *D. magna* feeding assay, a statistically significant differences was observed among all six waters (one-way ANOVA: $F_{5,23} = 11.3$, $P < 0.001$) (Figure 2). The Dunnett's test showed that feeding was significantly lower at Álamos and Alcarrache by a maximum of 11% ($P < 0.01$), but was lower by 14 to 21% at the remaining 3 sites ($P < 0.001$) (Figure 2). Correspondingly, one-way ANOVA revealed that the 21-days reproduction of *D. magna* was significantly different among all six waters ($F_{5,46} = 7.53$, $P < 0.001$) (Figure 2). However, the Dunnett's test showed that such difference was caused by a significantly lower number of juveniles (by 23%) released in the Mourão water compared to the standard control; the second lowest reproduction (by merely 9%) was observed at Ajuda.

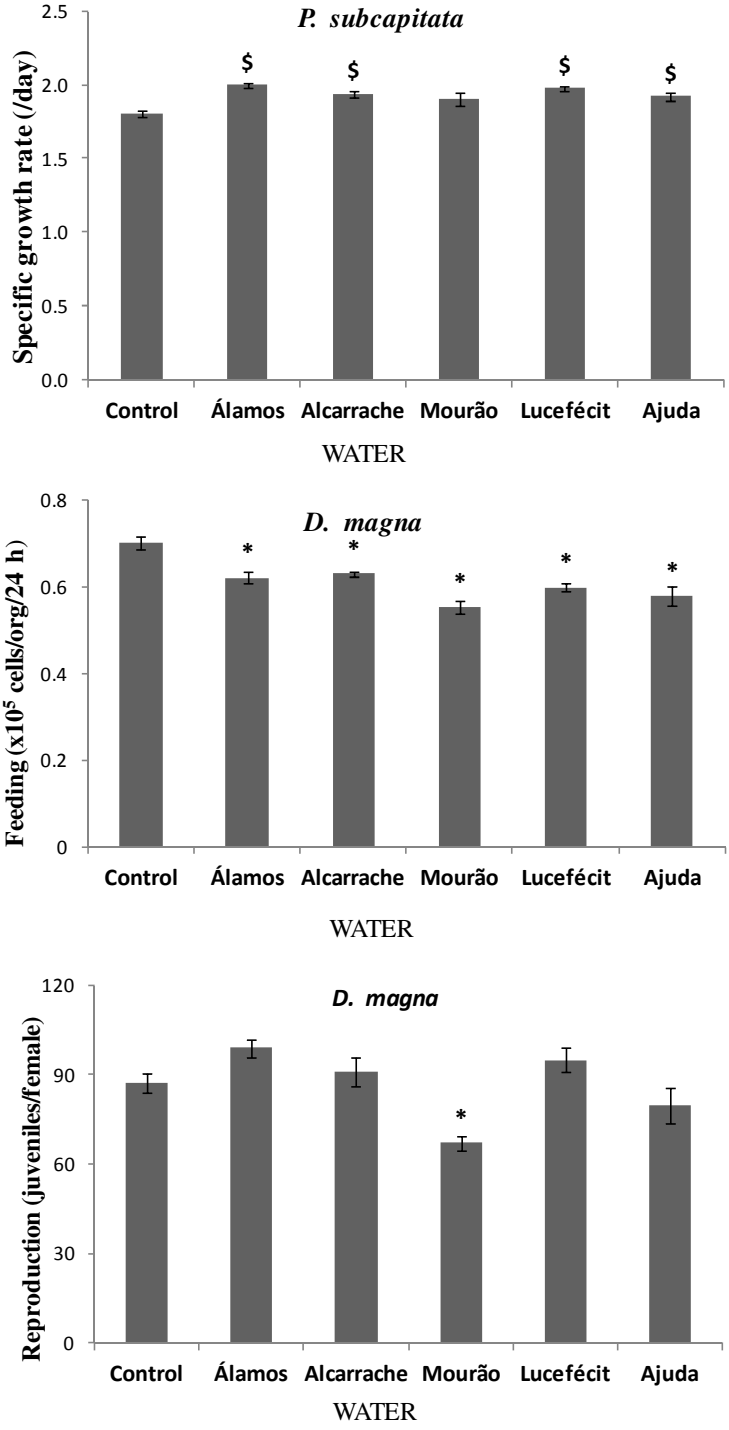

Figure 2. Sub-lethal endpoints measured for water samples collected at five sites of the Alqueva reservoir (Ajuda, Lucefecit, Mourao, Alcarrache, Alamos) with *Pseudokircheneriella subcapitata* (72-hours growth) and *Daphnia magna* (24-hours feeding and 21-days reproduction). Error bars indicate ±1 standard error; asterisks and dollar signs denote means significantly lower and higher than the control, respectively, by Dunnett's multiple comparison test.

3.2.2 Sediment-overlying water and sediment assays

In the 6-days *H. incongruens* growth assay, mortality was registered in the Alcarrache and Mourão samples, but it was below the accepted control criterion of 20% (i.e., 9.3 and 4.5%, respectively). The one-way nested ANOVA revealed that the organism size was significantly different among the six treatments ($F_{5,227} = 27.6$, $P < 0.001$). The Dunnett's test comparing each site growth with the control showed that organism size was significantly higher in all Alqueva samples relatively to the control by 18 to 34% (Figure 3), whereas ostracod growth among Alqueva samples differed by a maximum of 13%.

The 96-hours postexposure feeding assay with *T. fluviatilis* showed a statistically significant difference between samples of all study sites (one-way nested ANOVA: $F_{5,95} = 3.63$, $P < 0.05$). However, the Dunnett's test revealed that the significant difference observed was due to a significantly higher feeding rate observed in Ajuda relative to the reference site, an increment as high as 69%, whereas the increase in feeding at all remaining Alqueva sites was of merely 4 to 11% (Figure 3).

For the 96-hours postexposure feeding assay with, *E. meridionalis* a significant effect of treatment, though marginal, was also observed (one-way nested ANOVA: $F_{5,80} = 2.85$; $P = 0.044$). However, the Dunnett's test revealed that there were no significant differences between the reference and any of the tested Alqueva samples (Figure 3).

The one-way nested ANOVA for the 96-hours postexposure feeding assay with *C. riparius* yielded no statistically significant difference between the tested samples ($F_{5,66} = 1.32$, $P = 0.31$), even though feeding was reduced at three sites by a maximum of 27% (at Alcarrache) and increased at the remaining two sites by a maximum of 15% (at Ajuda) (Figure 3). Conversely,

the 10-days growth of *C. riparius* was significantly affected by the tested samples (one-way nested ANOVA: $F_{5,31} = 5.65$, $P < 0.01$). However, the Dunnett's test revealed that a significant positive effect on growth relatively to the control was only observed at Lucefécit (by 110%), even though growth at Ajuda was reduced by 23% (Figure 3).

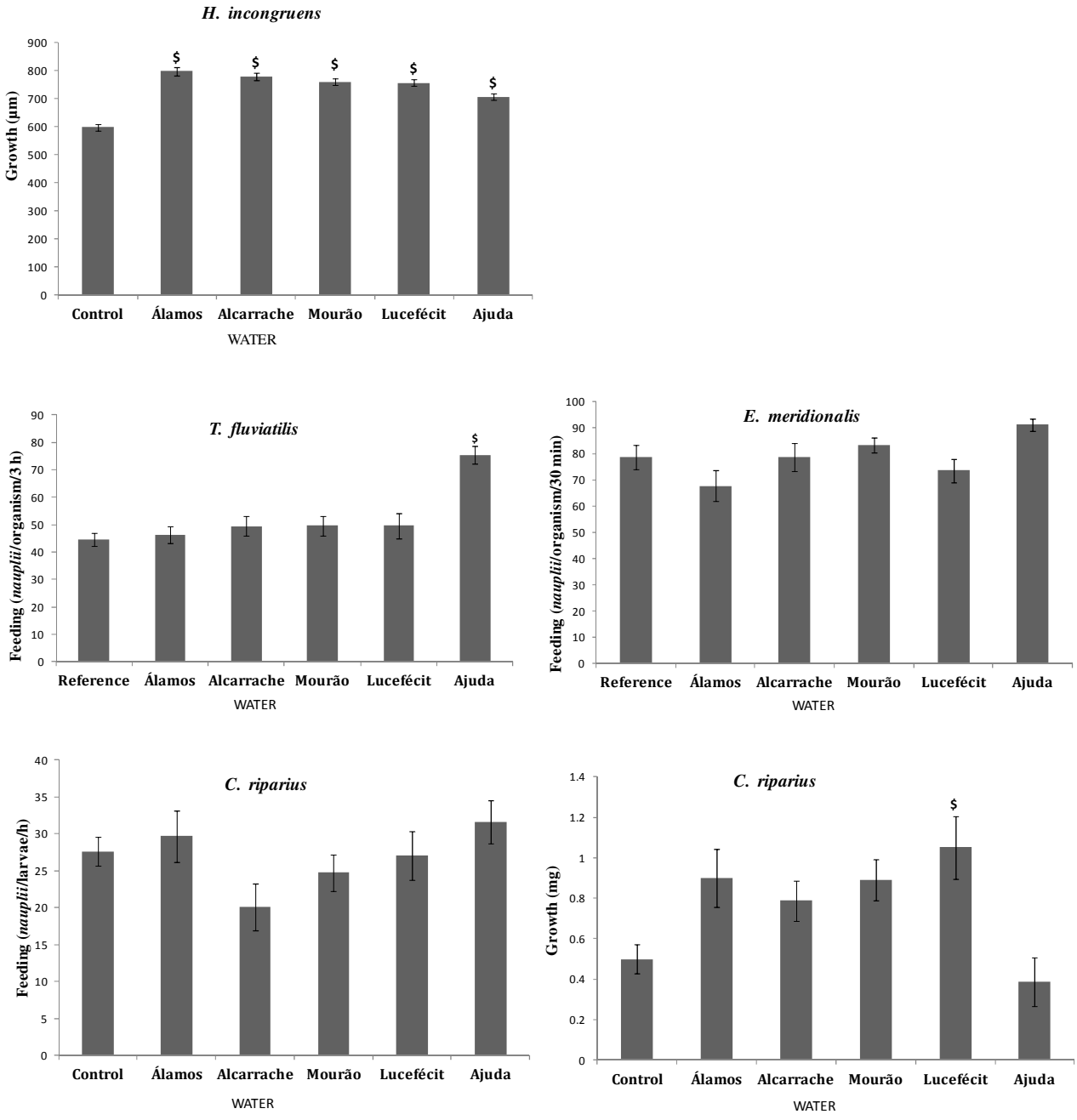

Figure 3. Sub-lethal endpoints measured for water samples collected at five sites of the Alqueva reservoir (Ajuda, Lucefecit, Mourao, Alcarrache, Alamos) and a reference site/control, with *Heterocypris incongruens* (6-days growth), *Theodoxus fluviatilis* (96-hours postexposure feeding), *Echinogammarus meridionalis* (96-hours postexposure feeding) and *Chrinomus riparius* (96-hours postexposure feeding and 10-days growth).Error bars indicate ±1 standard error; asterisks and dollar signs denote means significantly lower and higher than the control/reference, respectively, by Dunnett's multiple comparison test.

3.3 *In situ* assays

3.3.1 Water column assays

Results of the physico-chemical parameters measured during the *in situ* assays at each study site are summarized in Table III. Overall, temperature ranged between 15 and 29 ºC with small variations within each site and the dissolved oxygen was more than above saturation level in each site. The dissolved oxygen of the Anços site was comparable to the study sites on the Alqueva reservoir, whereas the conductivity was a bit higher.

Table III. Range (minimum-maximum) of water column pH, conductivity (Cond. in µS/cm), dissolved oxygen (DO in mg/l), and temperature (T in ºC) levels measured during the *in situ* assays at a reference site and at five sites at the Alqueva reservoir.

Site	Parameter			
	pH	Cond. (µS/cm)	DO (mg/l)	T °C
Reference[a]	nm	554	10.0	16
Álamos[c]	7.71 - 7.84	300-321	11.0-10.5	16-29[b]
Alcarrache[c]	8.35 - 8.77	305-326	11.7-13.4	20.6-22.5
Mourão[c]	8.06 - 8.24	397-407	10.6-11.3	15-33[b]
Lucefécit[c]	8.25 - 8.96	397-407	11.8-16.6	15-29[b]
Ajuda[c]	7.57 - 7.77	409-413	10.4-10.9	17.4-20.6

[a] measurements only once at deployment
[b] measurements with data loggers during all in situ exposure
[c] measurements once at deployment and once at retrieval
nm = not measured

Results from a one-way ANOVA on the 8-days *P. subcapitata in situ* growth showed marginal significant differences among control treatments at all sites ($F_{4,10} = 3.73$, $P = 0.042$), and the Tukey multiple comparison test showed only a significant decrease in growth by 13% at Mourão relatively to Ajuda. Due to chamber lost at Alcarrache, this site was excluded from further analysis. Results from the *t*-tests revealed that only at Álamos (by 15%) and Ajuda (by

21%) growth was significantly inhibited in treatment LW+N relatively to the control ($t_4 = 2.78$, $P < 0.01$ (Figure 4).

For the 96-hours postexposure feeding assay with *D. longispina* no significant difference in feeding was observed among sites (one-way ANOVA: $F_{4,10} = 2.31$, $P = 0.13$), most likely due to the high variability associated to the mean values, though due to a logistic problem the assay control was not performed.(Figure 4).

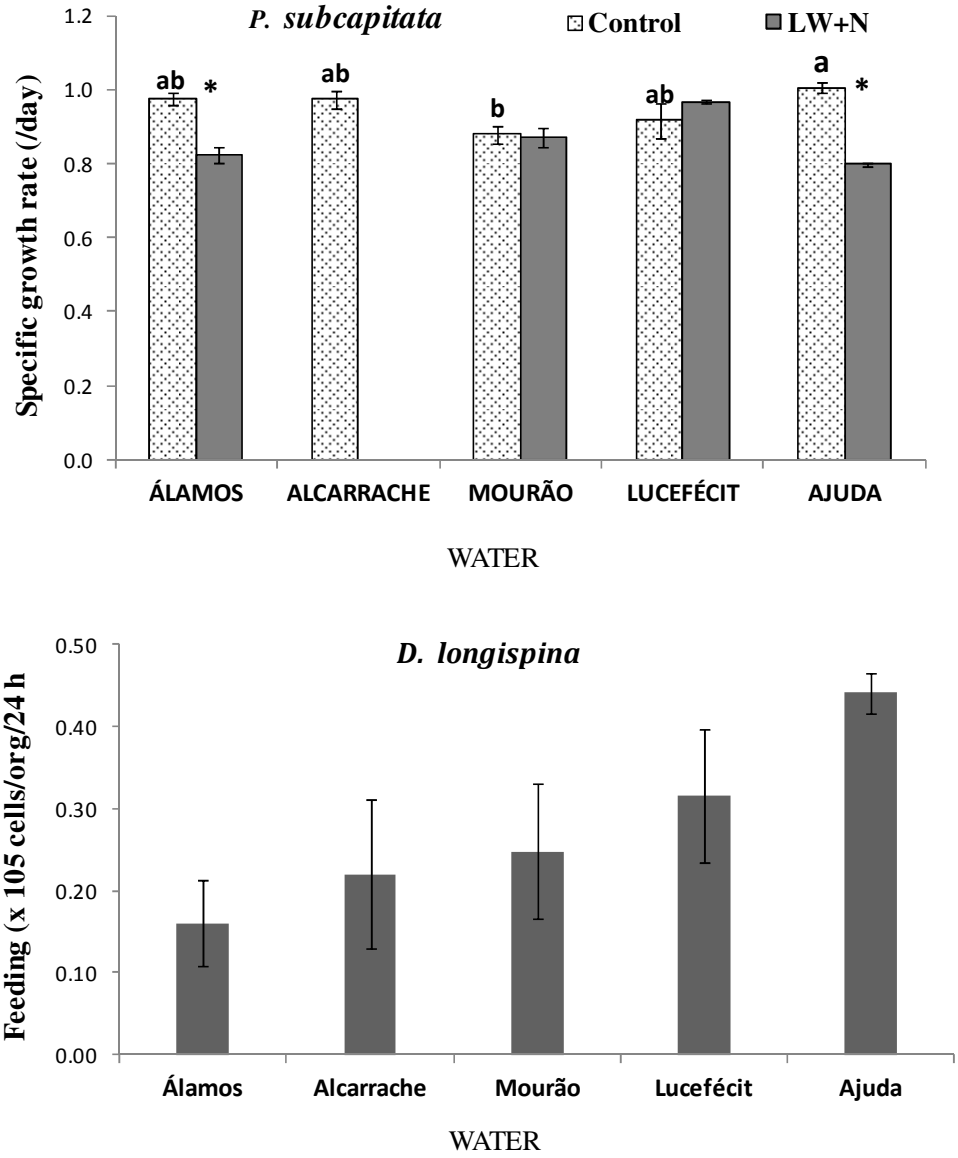

Figure 4. Sub-lethal endpoints measured at the five sites of the Alqueva reservoir (Ajuda, Lucefecit, Mourao, Alcarrache, Alamos) with *Pseudokircheneriella subcapitata* (8-days growth) in local water with nutrients (LW+N) and in control treatment, and *Daphnia longispina* (96-hours postexposure feeding). Error bars indicate ±1 standard error; similar letters above columns of control treatment denote means not significantly different within this treatment by Tukey HSD multiple comparison test; asterisks signs denote means significantly lower compared to the control by t-test.

3.3.2 Sediment-overlying water and sediment assays

The 96-hours *T. fluviatilis* postexposure feeding assay showed a significance effect of the study sites (one-way nested ANOVA: $F_{4,90} = 4.33$; $P < 0.05$), whereas the Dunnett's test revealed that feeding at all four tested sites (due to vandalism at Ajuda none of the three replicate chambers were retrieved) was significantly higher relatively to Anços (by 36 to 45%); at Lucefécit two chambers were also lost most likely due to the wind action on the last day (Figure 4). Except for organism losses due to vandalism and weather organism retrieval was 100% and no mortality was observed.

Regarding the 96-hours *E. meridionalis* assay, the one-way ANOVA revealed a statistically significant effect of site on postexposure feeding ($F_{3, 35} = 7.36$, $P < 0.001$) and the Dunnett's test showed that such difference was caused by a significant increase in feeding at Álamos, Mourão and Lucefécit relatively to the reference site (by 39, 53 and 88%, respectively) (Figure 4). Due to the vandalism at Ajuda the chambers were lost, whereas in Alcarrache the chambers was retrieved without organisms most likely due to the existence of some coarse sediment in deeper layers that turned chamber retrieval without loosing organisms a difficult task. Except for these losses, organism retrieval was 100% with 20% mortality at Lucefécit.

The one-way nested ANOVA for the 96-hours postexposure feeding assay with *C. riparius* yielded no statically significant difference among the reference, Álamos, Alcarrache, and Ajuda sites ($F_{3,34} = 3.11$, $P = 0.11$), even though feeding was increased by 62, 17 and 37% compared to the reference (Figure 4). Whereas organisms at Ajuda were lost due to vandalism, organism retrieval at Mourão was zero a fact that cannot be readily explained, though at this site

local Chironomids were rarely present compared to a high abundance at all other sites. Except for these two sites, organism retrieval was 100% and no mortality was observed.

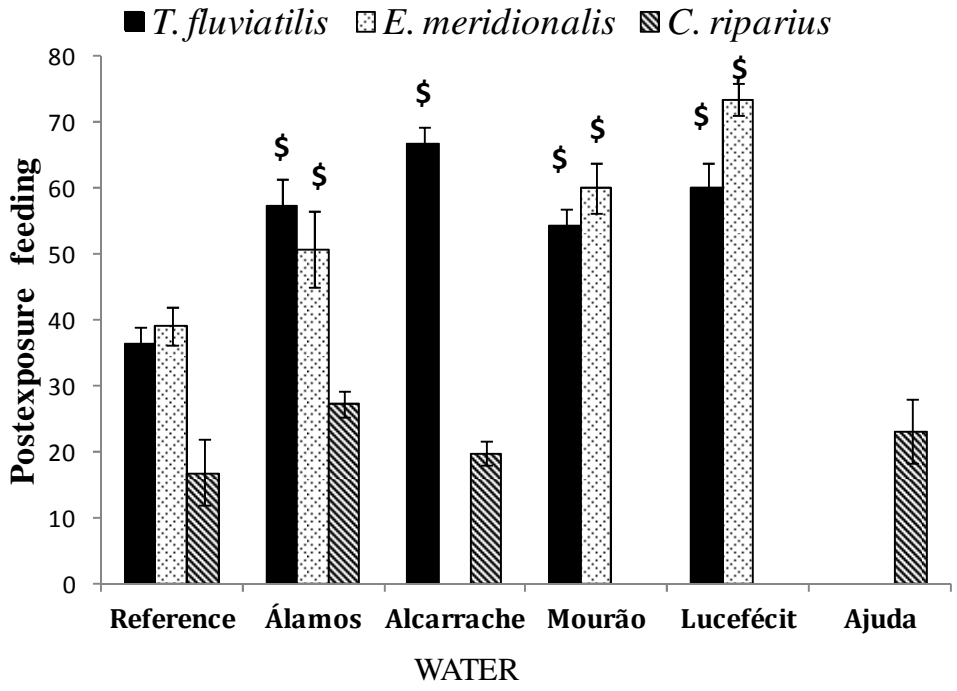

Figure 5. Sub-lethal endpoints measured at the five sites of the Alqueva reservoir (Ajuda, Lucefecit, Mourao, Alcarrache, Alamos) and a reference site (Anços), with *Theodoxus fluviatilis* (96-hours postexposure feeding)*, Echinogammarus meridionalis* (96-hours postexposure feeding) and *Chrinomus riparius* (96-hours postexposure feeding). Error bars indicate ±1 standard error; dollar signs denote means significantly higher than the reference site, by Dunnett's multiple comparison test.

Chapter 4 Discussion and Conclusion

4.1 Ecological receptors at most risk in the Alqueva reservoir

The major aim of this study was to identify the ecological receptors at most risk in the Alqueva reservoir and to set an ecologically relevant and cost-effective (easy based on short-term and sub-lethal responses) tool-box that will be used for future water quality assessment and eventually monitoring purposes of the reservoir. Thus, several laboratory and *in situ* assays were performed, comprising test species from different taxonomic and functional groups that are surrogates to the ecological receptors in the study catchment.

Overall, the laboratory assays did not reveal toxicity effects for the ecological receptors evaluated, except for the water column assays at Mourão. Mourão was the only site where the growth of the microalgae was not significantly increased relatively to the control and the reproduction of the cladoceran *D. magna* was significantly inhibited. Although a statically significant feeding depression of *D. magna* was observed across all the sites, the highest percentage inhibition was observed at this site (by more than 20%). However, the observed feeding depression of *D. magna* across all sampling sites might be linked with the fact that site waters contain other microalgae (though in extremely low amounts) and other suspended organic particles which are also filtered and ingested by the daphnids. Yet, the observed inhibition in reproduction might be associated with presence of toxicants at that specific site. Previous studies have concluded that the most impacted areas corresponded to the upper half of the reservoir (Palma *et al.,* 2009; Palma *et al.,* 2010a; Lindim *et al.,* 2011), classifying Mourao site as one of the impacted sites (Robinson *et al.,* 1994). Moreover, Palma *et al.* (2009) observed that during the dry season the biological oxygen demand, chlorides, herbicides, and the endosulfan sulfate concentrations increased in the reservoir, specifically in the upstream area related to high agricultural input. Furthermore, it is observed that in the same drainage basin endosulfan sulphate

showed a high acute toxic effect to *D. magna* (48 h EC_{50} = 0.92 mg/l). This might be the reason for the observed slight toxic effects with Mourao water, which had already shown strong anthropogenic impacts compared to the other sites (Silva *et al.,* 2011; Palma *et al.,* 2010a).

Although there was a general lack of toxicity for the rest of the laboratory assays (i.e., no observed deleterious effects on the test organisms), some organism responses were significantly higher in Alqueva samples than in controls/reference (*D. magna* reproduction at Alamos, *H. incongruens* growth at all sites and *C. riparius* growth at all sites except Ajuda). These test species responses across the sampling sites for the laboratory assays were in agreement with a recent study done by Ordonez (2012). This indicates the distinct areas within the large Alqueva reservoir might have different biological and physico-chemical characteristics, which resulted in variation in biological response across the sampling sites. Silva *et al*. (2011) based on historical data on ecological status evaluation criteria have classified four sampling stations at Alqueva as in Good ecological status" and two as in "Less than good status". Thus, the observed biological effect difference across the sampling sites in the reservoir might have been associated with the inherent spatial variability of the water quality in the reservoir.

The overall results of the *in situ* assays were in agreement with the laboratory observations. Moreover, they did not corroborated the suggestion of slight toxicity at Mourão. No toxicity effect on the surrogate test species was detected across the study sites. However, the 8-days *P. subcapitata* growth assay showed a significant growth inhibition at Álamos (by 15%) and Ajuda (by 21%) sites compared to the control treatment. Although toxicity is suggested, which is very likely specially at Ajuda for the reasons above discussed, the possibility that the fouling of the chambers meshes was not equal across sites cannot be excluded.

In most cases, *in situ* assay are recommended as tools that are more ecologically relevant than laboratory assays and as such can provide important complementary information (Moreira-Santos *et al.,* 2004; Burton *et al.,* 2005). In the present study, the *in situ* assays did not corroborate results of all laboratory assays as overall they point toward the absence of marked toxic effects at the Alqueva reservoir.

To our knowledge, this was the first time the *E. meridionalis* and *T. fluviatilis feeding* assay was applied to assess the toxicity of Alqueva reservoir. Although these organisms were shown to be sensitive to contaminants (Agostinho *et al*., 2012; Correira *et al*., 2012), there was no observed feeding depression in the laboratory as well *in situ* assay. Moreover, it was also the first time, to our knowledge, that various species were deployed simultaneously in the same chambers. This design allows to conduct assays simultaneously with different species, reducing confounding factors, while the number of chamber deployed per site dramatically decreases, thereby minimizing effort and time lost deploying and retrieving many chamber per site.

Generally, the observed lack of toxicity for the *in situ* as well as the laboratory assays at all Alqueva sampling stations might be explained by a decrease in agricultural contaminant levels promoted by a change in the agricultural practices in the region, as agricultural contaminants were the main water quality violator of the reservoir (Rodriguez *et al.,* 2010). Change in irrigation and farming system coupled with increased in vegetation cover around the catchment areas might have intercepted the contaminants supposed to reach to the reservoir (Pinheiro, 2004). Besides this, comparative surveys conducted at the Alqueva reservoir during 2006-2007 and 2011 showed that the levels of nutrients and of total pesticides decreased during the last sampling period, except for nutrients at Ajuda which values surpassed the recommended limits of the Freshwater Guidelines Quality for nutrients (Palma *et al.,* 2010a; Palma *et al.,* 2012).

Furthermore, a recent study done to characterize the ecotoxicity of the reservoir with the same sampling stations showed no toxic effect on the test battery used, except on *V. fischeri* luminescence solid-phase assay (Ordonez, 2012). For the observed effect on on *V. fischeri* the author concluded that it might be associated with the presence of interference to the assay (e.g. dark-brownish sediments rich in fine particle was tested which leads to reduction in light emission of the bacteria).

4.2 Setting an ecologically relevant and cost-effective tool-box

Knowing that there is no universally sensitive test species that can reliably predict the potential hazards associated with contaminated aquatic ecosystems, a multi-trophic battery of tests incorporating a number of different test species is advocated to reduce uncertainty in the toxicity assessment of contaminated system. In the present study, a battery of assays covering a wide range of organisms representative of species of different taxonomic and functional groups as well as of different environmental compartment was evaluated to set an ecologically relevant, easy and cost-effective tool-box. In most cases, a tool-box approach is important to reduce uncertainties and to increase robustness and reliability in ecological risk assessment (Allan *et al.,* 2006; Narraci *et al.,* 2009, Palma *et al.,* 2010a). The assays evaluated for this purpose were generally using test species widely used and recommended for (standard) toxicity testing. To select and recommend for feature risk assessment tool, assays with positive correlation with toxicity and ecological relevant should be given priority.

Compared to all previous test batteries applied to assess contaminant effects at the Alqueva reservoir (Palma *et al.,* 2010a, Palma *et al.,* 2012, Rodriguez *et al.,* 2010), the one selected for the present study was, to our knowledge, an innovative step as it is was composed by

both *in situ* and laboratory assays, chiefly based on sub-lethal responses and integrating a wide range of taxonomic and functional groups. However, due to the absence of toxicity effect on the test species from the laboratory as well as from the *in situ* assays made impossible the selection of assays to be included in a tool-box. But, based on the potential toxicity evidence detected from the laboratory reproduction assay with *D. magna*, this study recommends this assay to be further evaluated in the field at the different season before use.

4.3 Conclusions and recommendations

Based on the result obtained from the *in situ* as well as the laboratory assays, it is possible to conclude that, the Alqueva reservoir water and sediment pose no toxic effect to the ecological receptors. However, further temporal variability in toxicity has to be studied to see if there will be change in different season of the year. The surrogate organism used to evaluate ecological receptors at most risk did not show such harmful effect on their biological responses, it is suggested that aquatic communities in Alqueva reservoir might not be at risk during the dry season. To set ecologically relevant and cost-effective toolbox, the assay must have a good correlation with toxicity and be ecologically relevant with reduced time- and effort- demanding. However, the absence of toxicity effect on the test species from the laboratory as well as from the *in situ* experiments, the selection of assays to be included in the toolbox could not be achieved. But, based on the potential toxicity evidence detected from the laboratory reproduction assay with *D. magna*, this study recommends to evaluate further the species response in the field before use for risk assessment of the reservoir.

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