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## ABSTRACT

South European man-made reservoirs are essential sources of freshwater with multiple uses such as water supply, electricity, irrigation and recreation. However, pollution especially from agricultural activities is threatening the environmental quality of these water bodies. As modern agricultural practices require the usage of pesticides such herbicides, fungicides and insecticides to prevent losses by pests, possible negative impacts on ecosystems should be evaluated. In Europe, the Water Framework Directive (WFD) has assumed an active role at aiming to preserve and restore the biodiversity of inland water, wetlands, and coastal areas to achieve the "Good ecological status" of water bodies till 2015. This project aimed at (1) identifying the ecological receptors at most risk in the Alqueva reservoir – an impacted water reservoir previously shown to be contaminated with pesticides, and (2) putting forward a tool-box of short-term sub-lethal cost-effective tests to be used in future routine monitoring. Water and sediment samples from five and one sampling stations at the selected contaminated – Alqueva – and reference – Beliche – reservoirs, respectively, were collected during February 2012. Toxicity was evaluated with a battery of laboratory assays including representative species of different taxonomic and functional groups. Assay endpoints for water samples included: luminescence of the marine bacteria *Vibrio fischeri,* growth of the green microalgae *Pseudokirchneriella subcapitata*, survival of the fairy shrimp *Thamnocephalus platyurus*, reproduction and feeding of the planktonic cladoceran *Daphnia magna*, growth and feeding of the zooplanktivorous fish *Danio rerio*. Assay endpoints for sediment samples included: luminescence of *V. fischeri*, growth of the benthic ostracod *Heterocypris incongruens*, growth and feeding of the

benthic midge *Chironomus riparius*. Overall, no toxicity evidence was found at the Alqueva reservoir. This fact was associated to a severe drought registered several months prior to sampling as well as changes in agricultural practices, which might have prevented the input of pesticides through runoff from agricultural fields. The weak anthropogenic pressures at Beliche together with the ecotoxicological evidence of the lack of detrimental effects supported the proposal of this reservoir as a reference site.

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# Chapter 1 Introduction

Freshwater ecosystems have been largely impacted by increasing human population growth and changing global processes. These habitats have lost a significant proportion of their species and might be in greater danger of further losses from intense anthropogenic impacts, such as dams, pollution, overfishing and other threats (McAllister *et al.*, 1997). Pollution from agricultural activities represents an important threat to the water quality of freshwater ecosystems worldwide. According to the United States Environmental Protection Agency, agriculture is responsible for 70% of river and stream pollution (Ehrlich & Ehrlich, 1991). Modern agricultural practices require the usage of pesticides such as herbicides, fungicides and insecticides to prevent losses by pests (Oerke & Dehne, 2004). To keep pace with food demands, global pesticide production dramatically increased from 1945 to 1990 as a result of the doubling of global food production (Fig. 1) (Tilman *et al.,* 2001). Moreover, this trend is very likely to rise due to food demand for a growing population, monocultural production for biofuels and geographical shifts of pests as a consequence of climate change (Tilman *et al.,* 2002; Noyes *et al.,* 2009). By 2050, the increase in pesticide use has been estimated in 2.4 to 2.7-fold the global consumption registered in the year 2000, and comparable increments in nitrogen- and phosphorus-driven eutrophication of terrestrial, freshwater and near-shore marine ecosystems are expected (Table I) (Tilman *et al.,* 2001).

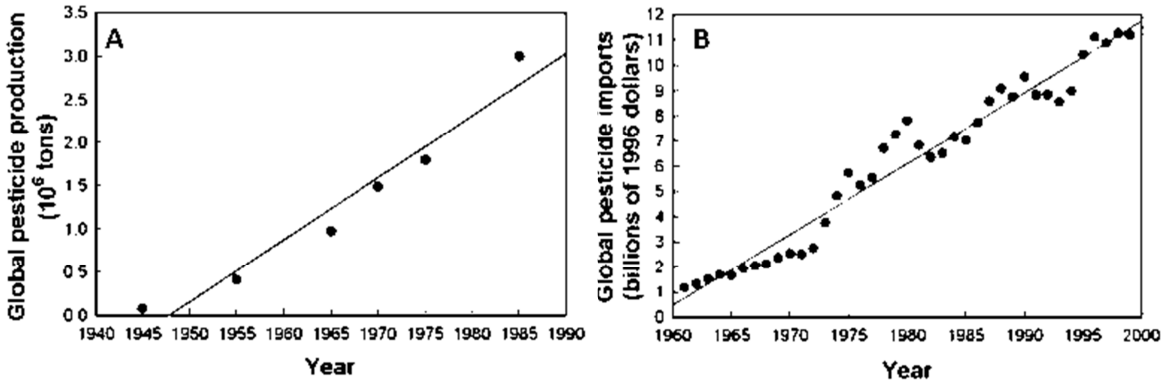


**Figure 1.** (**A**) Trend in global pesticide production rates, measured as millions of metric tons per year (World Health Organization, 1990) and (**B**) trend in expenditures on pesticide imports (Food and Agriculture Organization of the United Nations, 2001) summed across all nations of the world, transformed to constant 1996 U.S. dollars. **Source**: Tilman *et al*. (2001).

**Table I.** Univariate and multivariate forecasts for years 2020 and 2050, based on trends observed in the past 47 to 52 years and their dependence on population and gross domestic product (GDP). Parentheses show $R^2$ values for each regression. Levels of significance: $^{**}P<0.0001$; $^{*}P<0.01$; NS, $P>0.05$. The value in 2000 was based on temporal extrapolation from the latest available data, generally 1998. Mean projections were means of the three univariate and the one multivariate projection. N, nitrogen. P, phosphorus. MT, metric ton. **Source:** Tilman *et al.* (2001)

| | Fertilizer ($10^6$ MT) | | Pesticide | |
|---|---|---|---|---|
| | N | P | Produced ($10^6$ MT) | Imported ($10^9$ 1996 U.S.$) |
| Value in 2000 | 87.0 | 34.3 | 3.75 | 11.8 |
| | | *Mean projections* | | |
| Forecast 2020 | 135 | 47.6 | 6.55 | 18.5 |
| Forecast 2050 | 236 | 83.7 | 10.1 | 32.2 |
| | | *Individual projections for 2050* | | |
| Univariate | | | | |
| By year | 186 (0.986**) | 62.0 (0.927**) | 7.33 (0.946*) | 25.8 (0.957**) |
| By population | 166 (0.980**) | 56.2 (0.910**) | 8.02 (0.990*) | 22.2 (0.951**) |
| By GDP | 343 (0.964**) | 98.3 (0.904**) | 18.1 (0.995*) | 48.8 (0.955**) |
| Multivariate | 249 (0.989**) | 118 (0.979**) | 7.06 ($0.994^{NS}$) | 32.0 (0.960**) |

The use of pesticides in agriculture may lead to the contamination of surface waters by drift, runoff, drainage and leaching (Cerejeira *et al.,* 2003; Schriever *et al.*, 2007; Abrantes *et al.,* 2008). The exposure route of pesticides varies according to the physico-chemical properties of the compound as well as geographical, geological, hydrological, climatic conditions and crop type of a given area (Bach *et al.,* 2001). Once pesticides enter into water bodies, they might be subjected to degradation (e.g. photo-decomposition by sunlight or hydrolysis by water) or be captured on sediment or organic matter (Schäfer *et al.,* 2011). In general, several studies have emphasized that, after pesticide applications, runoff and subsurface flows due to rainfalls greater than 10 mm/day or to intensive irrigation in agricultural areas are the most important entry routes of pesticides to freshwater ecosystems (Liess *et al.,* 1999; Inoue *et al.,* 2002; Nakano *et al.,* 2004; Schulz, 2004). This problem becomes chronic when rivers are fed by many agricultural tributaries which promote a continuos exposure to pesticides and overlapping pesticide inputs are more likely to occur (Schäfer *et al.,* 2011). In addition to pesticides, nutrients coming from agricultural activities can contribute to eutrophication, which can add other stress factors to the pesticide concern, e.g. decrease in oxygen dissolved in water, bacterial growth (Sundbäck *et al.,* 2007).

Pesticides are designed to harm biota, thus, non-target communities might also be affected when these contaminants interact with the abiotic and biotic components of the ecosystem (Van der Werf, 1996). Depending on the physico-chemical properties of pesticides, such as persistence, octanol-water partition coefficient and volatility, they may disperse far from the source point and for prolonged periods of time after their release (Arctic Monitoring and Assessment Program, 1998), and bioaccumulate in food chains,

which in turn might have impacts on the freshwater biotic community (Schäfer *et al.,* 2011) and eventually on human health (Kidd *et al.,* 1995). To address the impacts of pesticides on the ecosystem, ecological receptors can be selected as surrogates for the assessment endpoints (biological responses) (Van Straalen, 1993). These receptors should reflect important ecosystem components and be representative of key ecosystem functions and major trophic levels at the study area (Fisher *et al.*, 2003).

A suitable strategy to evaluate the ecological receptors at most risk integrates the chemical, toxicological and ecological lines of evidence. This integrative strategy contributes to reduce uncertainties in risk assessments by integrating the resulting weight of evidence generated from each approach (Burton *et al.,* 2002). Thus, dominant stressors and their ecological impacts can accurately be determined (Chapman, 2007). In Europe, the Water Framework Directive (WFD) adopted such integrative approach to achieve the "Good ecological status" of water bodies till 2015 (Directive 2000/60/EC; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri =CELEX:32000L0060:en:HTML). Under this legislation, biomonitoring and investigative tools to characterize environmental risks and to estimate environmental quality have to be developed to effectively fulfill such objectives and, thus, to help on management decisions and ecosystem rehabilitation (Burton *et al.,* 2002; Allan *et al.,* 2006).

In the ecotoxicological line of evidence, ecotoxicity tests have been recommended as valuable tools to evaluate the ecological impacts of contaminants (Antunes *et al.,* 2007; Van Straalen & Van Gestel, 2008). Such line of evidence relies mainly on individual level (single-species laboratory) tests/assays, though ecosystem level assessments and experiments related to mechanistic models may also be included (Schäfer *et al.,* 2011).

Although laboratory toxicity assays are performed under not very realistic conditions in terms of environmental variables and test species, and are subjected to artifacts related to sample collection at the field (Burton *et al.,* 2005), these assays are remarkably successful at precisely evaluating the effects of contaminants on organisms under controlled and standardized conditions (Cairns, 1983; Crane *et al.*, 2007). Other advantages of these assays are that they are least money/time consuming, are more sensitive than when effects are measured at higher levels of biological organization, evaluate responses simple to measure, and their results are easily interpreted (Connell *et al.,* 1999; Liber *et al.,* 2007). Moreover, the outputs from single-species toxicity assays are essential for other research areas such as ecotoxicological modeling (Vann der Brink *et al.,* 2002), trait-based risk assessment (Liess *et al.,* 2008) or assessment of mixture toxicity (Altenburger *et al.,* 2004). Furthermore, uncertainties associated to the extrapolation of results from laboratory toxicity assays to predict contaminant effects under real scenarios of contamination can be lowered by running an ecotoxicological battery of assays comprising representative species of taxonomic and functional groups that are sensitive to pollutants (Allan *et al.,* 2006; Narraci *et al.,* 2009). Although these laboratory ecotoxicity assays measure responses mainly at the organism level, under an ideal extrapolation scenario their responses might be convertible into impacts at higher levels of biological organization in a time-delayed process (Maltby, 1999; Clements & Rohr, 2009).

Endpoints that can be assessed in laboratory assays can be expressed in changes in behavior, biochemistry, physiology, reproduction, and survival of the test individuals (Cairns, 1983; Giesy & Hoke, 1989; Crane *et al.*, 2007). Since contaminants such as pesticides are very likely to provoke changes on the performance of freshwater individuals

long before killing them (Gerhardt, 1996), and, in nature, organisms are not frequently exposed to lethal concentrations of pollutants (Kleerekoper, 1976), endpoints related to sublethal effects (e.g. growth, reproduction) on the test organisms are preferred. It has also been argued that the integration of sublethal effects at the individual level that can unequivocally have direct and immediate effects on ecosystem functioning is of major significance (Maltby *et al.,* 2001; Krell *et al*., 2011; Agostinho *et al*., 2012). This is because such effects may have a stronger ecological relevance than when individual effects are selected assuming that to have effects at the ecosystem level the individual effects (e.g. growth, reproduction,) have to be propagated through successively higher levels of biological organization (Krell *et al*., 2011; Schäfer *et al.,* 2011; Agostinho *et al*., 2012). For instance, if feeding activity is impaired by pollutants, it can directly affect grazing or organic matter decomposition rates at the ecosystem level (Krell *et al*., 2011; Agostinho *et al*., 2012). Moreover, an integration of lethal and sublethal effects has also been suggested of great importance to evaluate the implication of contaminants on ecosystem functioning, namely when sublethal effects occur at concentrations close to lethal concentrations (Forrow & Maltby, 2000; Krell *et al*., 2011; Agostinho *et al*., 2012).

### 1.1 The Alqueva reservoir

The urgent need of water for agriculture and water consumption has led to the construction of artificial lakes or dams worldwide. The scale and extent of the impacts

derived from dams have risen precipitously in the last century. In 1950, there were 5 270 large dams around the world (Abramovitz, 1996). By the end of the 20th century, there were over 45 000 large dams in over 140 countries. At its peak, nearly 5 000 large dams were built in the period from 1970 to 1975 around the world (Bird, 2001). An important environmental concern related to these structures is that they are often located near agricultural fields and receive their runoffs which might contain contaminants and excessive nutrients that can generate environmental and human problems (Carpenter *et al.*, 1998). Within the European Union, the WFD has assumed an active role at aiming to preserve and restore the biodiversity of inland water, wetlands and coastal areas. In many countries, large reservoirs resulting from dam constructions are an essential source of freshwater with multiple uses such as water supply, electricity, irrigation, and recreation. Therefore, the evaluation of the status of these reservoirs is essential to identify their main problems and adapt water management strategies to regional requirements (Burton *et al.,* 2002; Allan *et al.,* 2006; Silva *et al.,* 2011).

The Alqueva aquatic system is an example of a big man-made reservoir. It is located in the Southeast of Portugal, in the Alentejo region, close to the border with Spain, and belongs to the large Guadiana river basin (Fig. 2). This multi-purpose structure was first planned in 1957 and it is in operation since 2002 (Diogo, *et al.,* 2007). The purpose of this reservoir is to provide water for irrigation, drinking, power generation, and leisure activities (World Wide Fund for Nature, 1995). The main concern related to this aquatic ecosystem is the use of the reservoir as water supply to the populations, since agricultural and industrial activities taking place in the area might impact the water and sediment quality generating environmental and public health issues (Palma *et al.,* 2008; Palma *et al.,*

2010a). About 70% of the Alqueva reservoir catchment area is surrounded by agricultural fields, with only a small percentage of seminatural areas, being pesticide inputs one of the main environmental concerns (Morais *et al*., 2007; Palma *et al.,* 2008; Palma *et al.,* 2010a; Rodriguez *et al.,* 2010). Other critical sources of anthropogenic pollution in the area are urban wastewater discharges, pig and cattle breeding farms, and olive oil-press industries (Instituto do Ambiente, 2005). According to Rodriguez *et al.* (2010), in 2006, not only the sum of pesticide concentrations was above 0.5 μg/L for water samples at some sampling stations of the Alqueva reservoir, indicating that they can represent some hazard to human health based on the European Union Council Directive 1998/83/EC on the water quality for human consumption, but some pesticides (atrazine, simazine, terbuthylazine and endosulfan sulfate) were above legislated limits according to the Environmental Quality Standards Directive 2008/105/EC and to the Portuguese law Decreto-Lei n$^{o}$. 236/98 (1998). Also, Silva *et al.* (2011) rated the Alqueva reservoir as being in a "Less than good status" by applying an integrative evaluation following the European WFD 2000/60/EC and the River Basin Management Plan implementation for Portugal (Insituto da Água, 2009); general physico-chemical (e.g. pH, nitrates, phosphorus, dissolved oxygen), biological elements (chlorophyll *a*), specific contaminants and priority substances were used. It has also been reported that the most impacted areas are located at the upstream section of the Alqueva reservoir (Palma *et al.,* 2009; Palma *et al.,* 2010a).

The pesticides most frequently reported in surface waters from the Alqueva reservoir belong to the classes of phenylureas (diuron and isoproturon), triazines (atrazine, simazine and terbuthylazine), chlroacetanilide (metolachlor and alachlor), organophosphorous (chlorpyrifos), organochlorine (endosulfan sulfate) and thiocarbamates

(mainly fungicides) (Palma *et al.,* 2008; Palma *et al.,* 2009; Rodriguez *et al.,* 2010). Six of these pesticides (alachlor, atrazine, diuron, endosulfan sulfate, isoproturon and simazine) are included in the list of the 33 priority substances in the field of water policy defined in Annex X to the WFD (Directive 2000/60/EC; http://eur-lex.europa.eu/LexUriServ /LexUriServ.do?uri=CELEX:32000L0060:en:HTML). Furthermore, Palma *et al.* (2008) stated that the insecticide chlorpyrifos may constitute a potential risk to aquatic animal species, especially to crustacean populations at the Alqueva reservoir. In effect, the concentration of this insecticide in surface waters of the Alentejo region during 2006-2007 (< 0.01-0.36 μg/L) (Palma *et al.,* 2010a) was in the same range as the EC50 (median effective concentration) values obtained for lethal toxicity levels with the crustacean specie *Thamnocephalus platyurus* and *Daphnia magna* (Palma *et al.,* 2008). Together with the two latter crustacean species, the microalgae *Pseudokircheneriella subcapitata* has also been demonstrated to detect toxicity in the Alqueva reservoir surface waters in 2006; growth inhibition, most likely related with herbicides, was reported (Rodriguez *et al.,* 2010), even though potential differences in nutrient levels across sites were not taken into account. Furthermore, Palma *et al.* (2010a) stated that the concentrations of the metals As, Fe and Mn showed a temporal and spatial variability in the Alqueva reservoir surface waters during 2006-2007, occasionally surpassing the stipulated limit values of the Portuguese law Decreto-Lei n$^{o}$. 236/98 (1998). Thus, it is urgent to monitor whether the input of nutrients and chemicals in the area are impacting the aquatic environment.

The Beliche reservoir is located in the Algarve region, belonging also to the Guadiana river basin (Fig. 2). This artificial lake supplies water to the eastern Algarve area and is connected to the Odeleite reservoir by underwater sluices that regulate the flow from

Odeleite to Beliche. Following the application of the River Basin Management Plans for Portugal in compliance with the WFD (Instituto da Água, 2009), Silva *et al.* (2011) rated the Beliche reservoir as being in a "Good ecological status". Thus, the Beliche reservoir may be considered as a potential reference site for water quality assessments in the Alqueva reservoir, even though small differences in water chemistry mainly related with trace elements susceptible of influencing organisms performance should not be neglected when ecotoxicological evaluations are being performed. Also, although the catchment area of Beliche reservoir does not support any significant economic activity that might generate a negative impact on its water or sediment quality (Galvão *et al.,* 2012), relative abundances of cyanobacteria and microcystin concentrations have occasionally been shown to be high in this reservoir, especially during summer and fall (Galvão *et al.,* 2008). According to the latter authors, cyanobacteria concentrations reported for water samples during 2003-2005 were higher than the World Health Organization (WHO) alert level 1 for raw waters (≥ 2 000 cells/ml; initial establishment of cyanobacteria bloom is confirmed), but WHO alert level 2 (≥ 100 000 cells/ml; cyanobacteria bloom with problems in the water quality is confirmed) was never exceeded (World Health Organization, 1998). Moreover, they also reported that microcystin concentrations were always negligible with respect to human health risk, only exceeding WHO guideline value of 1 µg/L in water bottom samples detected in 2004.

The climate at the South of Portugal is Mediterranean, with hot and dry summers and mild and rainy winters. During the hottest months (July and August), the annual average temperature varies from 24 to 28 °C, and from 8 to 11 °C in coldest months (December and January). Most of the precipitation, up to the 80%, occurs from October to

April and the annual average precipitation ranges between 450 and 550 mm for Alqueva and around 644 mm for the Beliche area. The rest of the year the region is severely affected by intense dry periods (Morales, 1993).

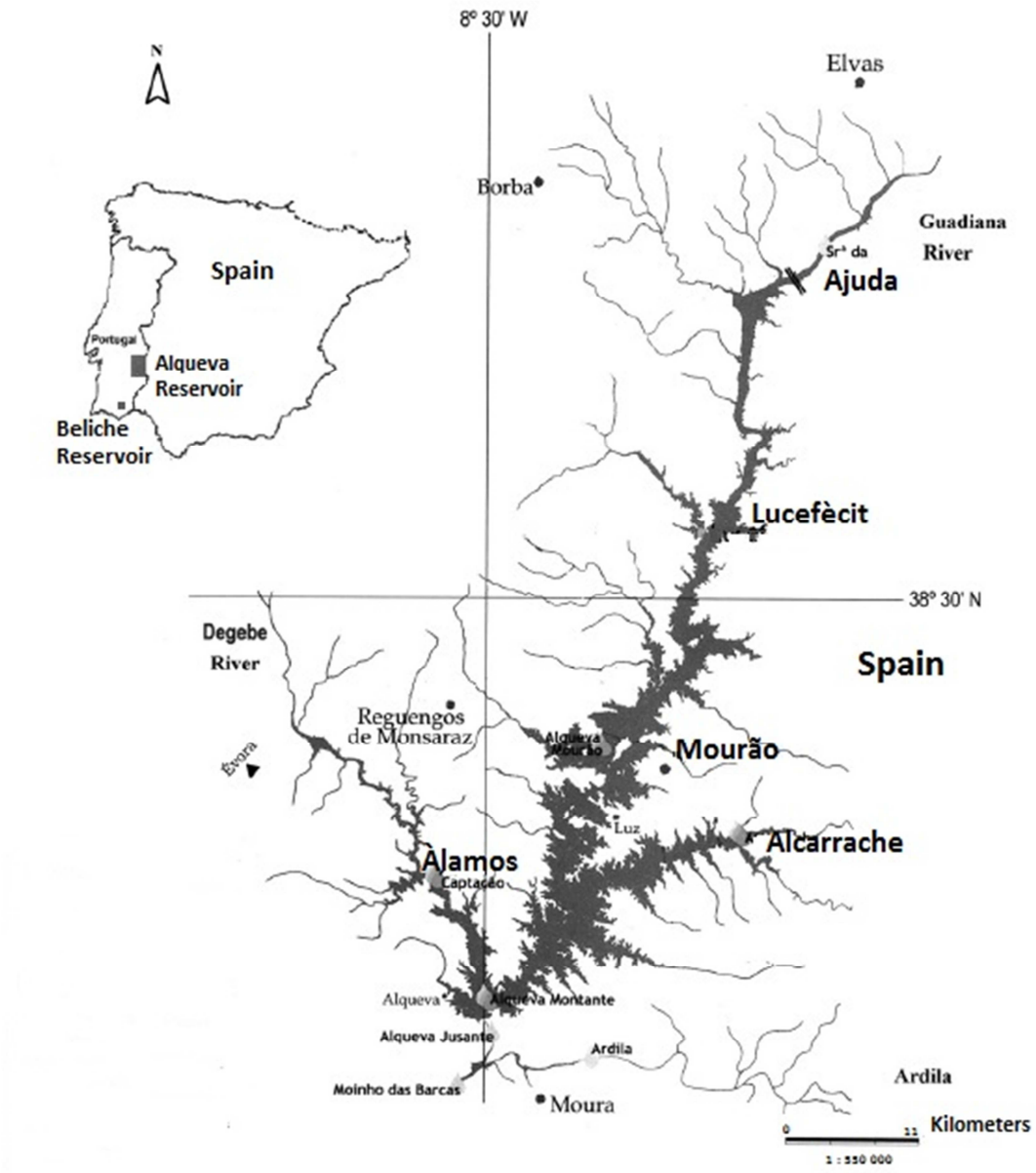


**Figure 2.** Map showing the location of the Alqueva (Alentejo region in Southeast Portugal) and Beliche reservoirs (Algarve region in South Portugal) and of the five study sites at the Alqueva reservoir selected to conduct the present study. Map adapted from Palma *et al.* (2010a).

## 1.2 Objectives

This project aimed at (1) identifying the ecological receptors at most risk in the Alqueva reservoir – an impacted water reservoir previously shown to be contaminated with pesticides, and (2) putting forward a tool-box of short-term sub-lethal cost-effective tests to be used in future routine monitoring. To achieve these goals, a battery of single-species laboratory toxicity assays was performed. The test organisms included in this test battery are species representative of different taxonomic (bacteria, algae, crustaceans, insects, and fish) and functional groups (primary producers, primary and secondary consumers, benthic and epibenthic decomposers). Also, this test battery involved lethal and various sublethal endpoints, so that impacts occurring at lethal and sublethal concentrations could be evaluated. Lethal effects can directly affect the dynamics of populations by increasing mortality rates on some or many populations or even entire groups of organisms and sublethal effects can be measured as biological responses to toxicants since they act on the physiological level of an organism first (Schäfer *et al.,* 2011). Surface water and sediment samples from five sampling stations within the Alqueva reservoir and one sampling station at the Beliche reservoir were collected in February 2012, when the rainy season occurs, to characterize their ecotoxicity. Inputs of pollutants may increase during the rainy season mainly related to runoff events and pesticide regime application on the agricultural fields (Palma *et al.,* 2010b; Rodriguez *et al.,* 2010). Also, highest levels of Fe and Mn were detected during the rainy season due to increases in their reduced forms. The source of such pollutants was associated to the acid mine drainage coming from abandoned mines (Palma *et al.,* 2010b). On the other hand, it is also known that an increase in pollutant concentration

(especially herbicides) may also occur during the dry season placing some organisms of the aquatic community at Alqueva reservoir at risk (Palma *et al.,* 2010a), even though lowest nutrient concentrations in the dry season were associated to high assimilation rates by phytoplankton (Palma *et al.,* 2010b). This master project is part of a bigger initiative under the project "ECOTOXTOOLS - Ecotoxicological tools for assessing agriculture associated environment risks in Southern Europe big man-made freshwater reservoirs" (reference PTDC/AAC-AMB/103547/2008) funded by the Fundação para a Ciência e a Tecnologia (Portugal) in order to develop ecotoxicological tools for assessing agriculture associated risks in Southern Europe reservoirs which outputs would be of use to the local managers.

# Chapter 2 Materials and Methods

## 2.1 Experimental design

To fully evaluate the ecological receptors at most risk in the Alqueva reservoir (Alentejo region, Southeast Portugal), surface water and sediment samples from five sampling stations within the latter reservoir and one sampling station at the Beliche reservoir (Algarve region, South Portugal) (Fig. 2) were collected in February 2012 to characterize their ecotoxicity using a battery of laboratory assays.

The Beliche reservoir was selected as a potential reference site. In effect, although the ecotoxicity of the water/sediment samples may be fundamentally evaluated by comparing the performance of the organisms in the local waters/sediments with the standard control within the respective assay, confounding factors may occur. That is, a response inhibition of the organisms in the former compared to the latter samples may not imply the existence of toxicity but rather that the water/sediment characteristics (e.g. trace elements, micronutrients, organic matter content) are different. Thus, to unravel such factors, it may be necessary to further evaluate the performance of the organisms with a natural reference location with characteristics close to those of the Alqueva reservoir, in the case of the present study the Beliche reservoir (see section 2.2 Study area). All water and sediment samples were tested without applying a dilution factor, i.e. at their 100% concentration, as further testing would be justified only if marked toxicity was detected in the 100% samples.

The laboratory test battery was selected to integrate assays assessing lethal and sublethal responses with species representative of key taxonomic and functional groups. All

laboratory 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. For water samples, the following assays were performed: 5-minutes luminescence of the marine bacteria *Vibrio fischeri* Lehmann and Neumann (decomposer), 72-hours growth of the green planktonic microalgae *P. subcapitata* (Korŝhikov) Hindak (primary producer), 24-hours survival of the planktonic anostracan *T. platyurus* Packard (primary consumer), 24-hours feeding and 21-days reproduction of the planktonic cladoceran *D. magna* Straus (primary consumer), 48-hours feeding and 28-days growth of the zooplanktivorous fish *Danio rerio* (Hamilton) (secondary consumer). The following assays were performed for the sediment samples: 5-minutes luminescence of the marine bacteria *V. fischeri* (decomposer), 6-days growth of the epibenthic omnivorous ostracod *Heterocypris incongruens* (Ramdohr) (primary consumer), 48-hours feeding and 10-days growth of the benthic midge deposit feeder *Chrinomus riparius* Meigen (decomposer). The survival test with *T. platyurus* was included in the selected test battery to evaluate whether contamination levels reached lethal concentrations, as previous tests on water samples from the Alqueva reservoir with this species suggested the presence of lethal toxicity (Palma *et al.,* 2010a; Palma *et al.,* 2012).

## 2.2 Study Area

The Alqueva reservoir is located in the Guadiana river basin in the Alentejo region (Southeast Portugal) (Fig. 2). It has an area of 250 $km^2$ and 1 160 km of margins. The Alqueva reservoir is the largest artificial lake in the Iberian Peninsula with a total volume of 4 150 000 $km^3$ and 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 (Sociedade de Engenharia e Inovação Ambiental, 1995). Major environmental concerns related to this aquatic ecosystem is the use of the reservoir as water supply to the populations, since industrial and intensive agricultural activities in the area might impact the water and sediment quality generating environmental and public health issues (Palma *et al.,* 2008; Palma *et al.,* 2010a).

The Beliche reservoir, also in the Guadiana river basin but in the Algarve region (South Portugal) has a catchment area of 98.47 $km^2$ (Fig. 2). The total volume of the Beliche reservoir is 48 000 $km^3$, with a maximum depth of 52 m and a maximum surface area of 2 920 $km^2$. This man-made reservoir is used mainly for irrigation purpose and for supplying drinking water for approximately 230 000 inhabitants in the Algarve region (Paulino *et al.,* 2009). The only potential environmental problem reported is related with cyanobacteria and microcystin bloom events recorded sporadically during summer and fall due to nutrient resuspension at the mouth of the underground channel between Odeleite and

Beliche reservoirs (Galvão *et al.,* 2008), as the catchment area of the Beliche reservoir does not support significant economic activities affecting water quality (Galvão *et al*., 2012).

To achieve the objectives of the present study, five sampling stations were selected in the Alqueva reservoir, three upstream (Ajuda-Aj, Lucefécit-Lf, Mourão-Mr), and two at the middle (Alcarrache-Ac, Álamos-Al), and one sampling station in the Beliche (Bl) reservoir (Fig. 2 and Table II). All these stations were previously monitored to evaluate environmental risks mainly due to pesticide inputs and were identified as strategic sampling points (Palma *et al.,* 2009; Palma *et al.,* 2010a; Rodriguez *et al.,* 2010; Silva *et al.,* 2011). The Aj station, located close to the Spanish border, was found to be highly affected by contamination due to intensive agriculture and densely populated cities in Spain (Palma *et al.,* 2009; Palma *et al.,* 2010a; Lindim *et al.,* 2011). The Lucefécit stream was characterized as being impacted by cheese-making industries, pig and cattle breeding farms and olive oil-press industries. The Alcarrache and Álamos rivers are also impacted by pig breeding farms (Palma *et al.,* 2010b). The wastewater discharges from these activities are usually made without treatment to the nearest stream (Instituto do Ambiente, 2005). In general, diffuse sources of pollution in the Alqueva reservoir are related to intensive agriculture practices in the surroundings promoted not only by the continuous water supply from the reservoir itself (Palma *et al.,* 2009; Palma *et al.,* 2010a) but by the use of European subsidies that encourage high value intensive crop production (Varela-Ortega *et al.,* 2003).

**Table II.** Acronyms and geographical coordinates of the sampling stations at the Alqueva (Alentejo region, Southeast Portugal) and Beliche reservoirs (Algarve region, South Portugal).

| Reservoir | Sampling station | Acronym | Latitude (N) | Longitude (W) |
|---|---|---|---|---|
| Alqueva | Ajuda | Aj | 38°46'33'' | 07°10'20'' |
| | Lucefécit | Lf | 38°37'32'' | 07°17'20'' |
| | Mourão | Mr | 38°23'38'' | 07°23'15'' |
| | Alcarrache | Ac | 38°19'00'' | 07°19'30'' |
| | Álamos | Al | 38°20'30'' | 07°34'35'' |
| Beliche | Beliche | Bl | 37°16'28'' | 07°30'38'' |

### 2.3 Collection of environmental samples

Water and sediment samples were collected in the five and single sampling stations at the Alqueva and Beliche reservoirs, respectively, during February 2012, i.e. during the rainy season when pesticide inputs due to runoff events are more likely to occur (Palma *et al.,* 2010b; Rodriguez *et al.,* 2010). Water samples were collected at a depth of 50 cm into polyethylene bottles. For sediment sampling, a stainless steel van Veen grab of 0.05 $m^2$ was used to penetrate the sediment never less than 20 cm deep. The sediment at Aj was collected from the shore. Sediments at Lf, Mr, Ac, Al, and Bl were collected at a water-column depth of 25, 48, 37, 27, and 20-30 m, respectively. All sediment samples were packed in aluminium dishes. After collection, all water and sediment samples were immediately placed at 4 °C in darkness to be transported to the laboratory, where they were stored at -18 °C in darkness. All toxicity assays were carried within four months after sample collection. No rain episodes were registered the days before or during the collection of samples.

### 2.4 Ecotoxicology tests

Given that samples were tested without being diluted (at 100%), unless otherwise stated, all collected samples were tested simultaneously, i.e. each assay consisted of a total of seven treatments, five water/sediment samples from the Alqueva reservoir and one from the Beliche reservoir plus a control. The 24-hours *T. platyurus* survival assay was conducted according to the Thamnotoxkit F standard operating procedure (Creasel, 1999) for water samples. All necessary materials were provided in the commercial kit. Live organisms to perform the test were obtained from cyst hatching conducted in diluted (1:8 with deionized water) reconstituted moderately hard water (ASTM, 2002) at 24 to 26 °C under continuous cool-white fluorescent illumination (approximately 75 $\mu E/m^2/s$) for 24 hours. For testing, three 1-ml replicates of each water type and the control (ASTM moderately hard water) were set up in 24-well microplates, ensuring at least one control replicate per microplate to verify for homogeneous environmental conditions within the tested area, and ten 24-hours larvae were added into each well. The assay was incubated in a temperature controlled chamber at 25 °C in darkness. The assay endpoint estimated after the 24-hours exposure period was survival.

The 5-minutes *V. fischeri* luminescence assay was conducted following the Microtox 81.9% basic test protocol for water samples and Microtox solid-phase test protocol for sediment samples (Azur Environmental, Carlsbad, CA, USA). According to these protocols, maximum tested concentrations are 81.9% for water and 197 400 mg/L for sediment (on a wet weight basis). Water samples were tested in a single replicate whereas

sediment samples were tested in triplicate. The Microtox toxicity analyzer model 500 (Strategic Diagnosis, Newark, DE, USA) was used to measure the light emission (in Lt) of the luminescent marine bacteria after exposure periods of 5 and 15 minutes, but only the 5 minutes results will be presented as no differences were found between the two exposure periods. A single reading was measured for the water samples and three replicates with two sub-replicates each were read for the sediment samples.

The 72-hours *P. subcapitata* growth assay was performed following the OECD (1984) and EC (1992) guidelines. The *P. subcapitata* (strain Nr. WW 15-2521) was 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 $B_1$, 0.5 µg/L $B_{12}$ and 0.5 µg/L biotin), at 19 to 21 °C, under continuous cool-white fluorescent illumination (100 µE/$m^2$/s). For testing, the six natural water samples were vacuum-filtered (0.45 µm) to exclude the natural microalgae community that might compete with the test organisms. The MBL growth medium (not supplemented with vitamins) was diluted 2.5 times to be used as control medium according to the N/P specifications. All waters were tested as plain and supplemented with nutrients in the same amounts as the control medium to discriminate potential toxic effects from those due to differences in nutrient levels among the natural waters. The assay was conducted in 24-wells microplates (Coastar, Cambridge, MA) with each replicate well consisting of 900 µl test solution plus 100 µl of algal inoculum; three replicates per water type plus six for the control were set up with an initial algal concentration of $10^4$ cell/ml. 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. After the 72-hours exposure period, the final cell densities were counted from well-mixed aliquots of each replicate under a microscope (×400 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 & Källqvist, 1989).

Organisms for both assays with *D. magna* (Clone A originated from IRCHA in France; OECD, 1998) were obtained from cultures maintained at 19 to 21 °C, under a 14:10-hours light:dark photoperiod, in reconstituted hard water (ASTM, 2002) supplemented with vitamins (7.5 μg/L $B_1$, 1 μg/L $B_{12}$, and 0.75 μg/L biotin) and Marinure extract (Glenside, Stirling, UK; 7.5 ml/L of a suspension with an absorbance of 620 units at 400 nm), fed daily with *P. subcapitata* ($3 \times 10^5$ cells/ml; 25 and 15 daphnids/L up to the first brood and from there onwards, respectively), and with medium renewal every other day. The 24-hours *D. magna* feeding assay was based on the methodology developed by McWilliam & Baird (2002). The assay was performed using 4-days old juveniles. The control medium was the same used for the stock cultures, except that no vitamin supplement was provided. For each tested water and control five replicates were set up, each consisting of 175-ml glass vessels filled with 120 ml of test solution plus food ($3.5 \times 10^5$ cells/ml of *P. subcapitata*) and five organisms. A blank treatment, consisting of control medium without organisms, was also run to control for algal growth during the test period. The test was incubated at 19 to 21 °C in darkness. After a 24-hours exposure period,

the test endpoint was estimated as feeding rate (number of 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.

The 21-days *D. magna* reproduction assay was carried out according to the OECD (1998) guideline, using less than 24-hours old neonates from a third-brood. For each water type and control (same medium used for stock culturing), 10 replicates were set up in 60-ml glass beakers filled with 50 ml of the tested waters (natural waters were also supplemented with Marinure extract at the same concentration as the control medium, to better ensure direct comparison of results with the control). A semi-static approach was followed in which the test solutions were renewed on Mondays, Wednesdays and Fridays. 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 alive parent animal.

Organisms for both assays with *D. rerio* were obtained from a local supplier and maintained in an aquarium filled with 50 L of aerated dechlorinated tap water, at 19 to 21 °C, under a 8:16-hours light:dark photoperiod and fed either on a commercial flake food diet (Tetramin, Tetrawerk, Melle, Germany) or on live juveniles (less than 72-hours old) of the cladoceran *D. magna*, at least times per week. Fish were maintained under these acclimation/quarantine culture conditions for at least two weeks to ensure that individuals fulfill the criteria to accept the batch for testing (OECD, 2000). The 48-hours *D. rerio* feeding assay was based on the methodology developed by Abdel-Moneim (2011) using

fish with a mean wet weight (± standard deviation [SD]; *n*) of 0.26 g (± 0.033; *n* = 12). For testing, three replicates with three fish each were set up in transparent polyethylene terephthalate vials (8 cm diameter) filled with 750 ml of tested water under continuous aeration for control and the five water samples; control medium was the same used for fish culture. All test vials were aerated for 1 hour prior to the start of the test, i.e. fish introduction. During the exposure period, no food was provided and the test was incubated at 19 to 21 °C under a 14:10-hours light:dark photoperiod. After the 48-hours exposure period, test organisms were individually transferred into 175-ml glass vials filled with 100 ml of the exposure medium for a 1-hour acclimation period during which all vials were wrapped with white paper to isolate the fishes from external stress factors. After the 1-hour acclimation, 10 live *D. magna* juveniles (aged less than 48-hours old) were provided to each fish, which were allowed to feed for 1 hour. The assay endpoint – feeding rate – was estimated as number of juveniles/fish/hour, calculated from the initial and final number of daphnids.

The 28-days *D. rerio* growth assay followed the OECD (2000) guideline under appropriate semi-static conditions using fish with an initial mean (± SD; *n*) wet weight of 0.26 g (± 0.043; *n* = 12). For testing, four replicates with two fish each were set up in transparent polyethylene terephthalate vials (8-cm diameter) filled with 450 ml of test solution under continuous aeration for control and tested waters; control medium was the same used for the feeding test. All test vials were aerated for 1 hour prior to fish introduction. During the exposure period, the medium was partially renewed three times per week, 50% on Mondays and 32% on Wednesdays and Fridays. Two food types were provided during testing in proportions corresponding (in dry or wet weight depending on

the food type) to 4% of the initial wet weight of the fish. Fish were fed on Tetramin on Mondays, Wednesdays and Fridays, and on live newly hatched *nauplii* of the brine shrimp *Artemia franciscana* Kellog (assuming a wet weight of 15 ug per *nauplii*; Jacques *et al.,* 1998), while no food was provided on Sundays. Hatching of *A. franciscana* cysts (Creasel, Deinze, Belgium) was carried out in standard reconstituted seawater (salinity and pH of 35 and around 7.6, respectively) at 24 to 26 °C under continuous cool-white fluorescent light (75 μE/$m^2$/s) during 24 hours (Soares *et al.*, 2005). The food was supplied once a day early in the morning. For feeding aeration was stopped for 20 minutes and water levels were adjusted with distilled water. The test was incubated at 19 to 21 °C under a 14:10-hours light:dark photoperiod. At the end of 28-days exposure period, the wet weight of each test organisms was recorded. The assay endpoint was estimated as the specific growth rate, calculated from the initial and final fish wet weight (OECD, 2000; Abdel-Moneim, 2011).

The 6-days ostracod *H. incongruens* growth assay was conducted according to the Ostracodtoxkit F standard operating procedure (Creasel, 2001) for sediment samples. All necessary materials were provided in the commercial kit. The test organism were obtained from cysts, which were incubated for hatching in reconstituted moderately hard water (ASTM, 2002) at 24 to 26 °C under continuous cool-white fluorescent illumination (approximately 75 μE/$m^2$/s), for 52 hours; after the first 48-hours pre-feeding of the freshly hatched organisms was carried out with *Spirulina* powder. The test was conducted in 6-wells microplates. For each tested sediment and control (kit-provided reference sand), five replicates were set up each consisting of 1 ml of sediment plus 4 ml of ASTM moderately hard water inoculated with fresh microalgae as food (approximately $3.75 \times 10^6$ cells/ml) and 10 newly hatched ostracods. The test was incubated at 24 to 26 °C in

darkness. After the 6-days exposure period, survival and final length (in μm) were recorded, being the assay endpoint the specific growth rate, calculated from the initial (estimated at the start of the assay from a subsample of 12 newly hatched organisms) and final body length.

Organisms for both assays with *C. riparius* larvae were obtained from laboratory cultures held inside a 40 × 60 × 120 cm closed transparent acrylic box to allow adult swarming and copulation. Cultures were reared in crystallizing dishes containing 185 g of quartz sea sand (0.1 – 0.4 mm particle size; Merck, Darmstadt, Germany) and 300 ml of reconstituted hard water (ASTM, 2002), fed a suspension of ground Tetramin (Tetrawerk) every other day (0.1 g/dish, with 30 and 15 larvae/dish up to day seven and from there onwards, respectively), and maintained at 19 to 21 °C under a 14:10-hours light:dark photoperiod (100 $\mu E/m^2/s$) with 90-minutes dawn and dusk periods (50 $\mu E/m^2/s$) (for further details see Castro *et al.*, 2003). The 48-hours *C. riparius* postexposure feeding assay was based on the methodology developed by Soares *et al.* (2005) for sediments using third-instar larvae (aged 10 days old). For each treatment, three replicates were set up in 175-ml glass vials, each with sediment and 120 ml of overlying ASTM hard water on a 1:4 height ratio and five larvae. Sediment amounts consisted of 40 ml of each tested sediment and 50 g of control sediment, which was the same sediment used for cultures. The glass vials were prepared 12 hours prior to the beginning of the test and left with continuous aeration. To add the larvae, 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 testing. After the 48-hours exposure period, each larva was individually transferred to a 60-ml glass vial filled with 30 ml ASTM hard water and

100 defrosted newly hatched *nauplii* of *A. franciscana*, allowed to feed at 19 to 21°C in darkness for 1 hour, time after which larvae were retrieved and the remaining *nauplii* were counted, following procedures described in Soares *et al*. (2005); cyst hatching was as above described for the *D. rerio* growth assay. Hatched *nauplii* were counted and frozen into 1.5-ml eppendorfs filled with ASTM hard water (ASTM, 2002) before the assay was started. The test endpoint – postexposure feeding rate – was estimated as number of *nauplii*/larva/hour, calculated as the difference between the initial and the final number of *nauplii*.

The 10-days *C. riparius* growth assay was conducted according to the EC (1997) and OECD (2004) guidelines using first-instar larvae. The test procedures and incubation conditions were similar to those of the postexposure feeding assay except that four and three replicates, each with three larvae, were set up for the control and tested sediments, respectively, 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 (Tetrawerk) 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 individually dried at 60 °C for 72 hours, to estimate the test endpoint growth as the body dry weight (in mg).

Measurements of pH (Wissenschaftlich Technische Werkstätten, WTW 537 pH meter, Weilheim, Germany), conductivity (WTW 315i/SET conductivity meter) and dissolved oxygen (WTW OXI 92 oxygen meter) were taken in the water column at the start (whole sample) of the *P. subcapitata* growth and *D. rerio* feeding assays and for the latter assay also at the end (two replicates), and in two replicates at the beginning and at the end of both *C. riparius* assays and *D. magna* feeding assay. For the *D. magna* reproduction and

*D. rerio* growth assays, the same physico-chemical parameters were measured in two replicates of old and fresh medium at all medium renewals. For the *T. platyurus*, *V. fischeri* and *H. incongruens* assays, water-column physico-chemical parameters were not measured during testing due to the small sample volumes involved.

### 2.5 Data Analysis

For all assays, the estimated endpoints were first explored for significant differences between the standard assay control and the five stations of the Alqueva reservoir to (1) evaluate the ecotoxicity of the Alqueva stations and (2) potentially identify the ecological receptors at most risk within the suggested battery of laboratory assays. For the *T. platyurus* survival assay, percentage of lethality was calculated by pooling the results from the three replicates (Creasel, 1999). For the *V. fischeri* basic test no statistical analysis were performed as the test design does not include replication. For all sublethal assays, except the *P. subcapitata* assay, this analysis was performed through one-way analysis of variance (ANOVA) or one-way nested ANOVA. When significant differences were detected, the Dunnett's test was performed to compare each water/sediment with the control. For the *D. magna* feeding test, a paired Student's *t*-test comparing algal cell densities in the standard control at the start and end of the assay confirmed that no algal growth took place during the 24-hours exposure. For the microalgae assay, a two-way ANOVA was performed to verify that potential differences among waters were irrespective

of nutrient levels and only after one-way ANOVA was performed using the set of waters enriched with the same nutrient levels as the standard control.

Because the *V. fischeri* solid-phase assay signaled the sediment at all stations of the Alqueva reservoir as potentially toxic, significant differences between the Beliche and the five stations of the Alqueva reservoir were also evaluated to unravel potential confounding factors associated to the Microtox control determining an overestimation of toxicity (e.g. color). All *V. fischeri* solid-phase assay analyses were made after verifying with a Student's *t*-test that no significant differences occurred among control values and thus pooling all control replicates, since the Microtox analyzer does not test all samples simultaneously, resulting in a different control reading for each sample set.

Although only the *V. fischeri* solid-phase assay results called for the need to use the Beliche as a potential reference to study the ecotoxicity of water and sediment stations at the Alqueva reservoir, all estimated endpoints were further explored for significant differences between the standard assay control and the Beliche reservoir station to evaluate the suitability of the water/sediment samples from this reservoir to serve as a natural reference site for the Alqueva reservoir. Such analyses were made through the Student's *t*-test; the absence of significant differences between the standard control and Beliche would support the use of Beliche as a reference station.

The assumptions of normality and homoscedasticity were verified using Shapiro–Wilk's and Bartlett's tests, respectively. All statements of significant difference were set at the 0.05 level. All statistical analyses were conducted using Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

# Chapter 3 Results

All the laboratory assays fulfilled the validity criteria for control performance required in the respective guidelines/(standard) operational procedures. Although the guideline for the *D. rerio* growth assay does not specify the criterion for weight increase in the control (OECD, 2000), the observed mean weight increase of 46% was within the range of previously reported values (Smolders *et al.,* 2002; Rosa *et al.,* 2010; Abdel-Moneim, 2011). Also, in all assays mortalities in the Alqueva and Beliche waters were occasional and below 10%, except for the 6-days ostracod *H. incongruens* growth assay (see below).

Results of the overall range of pH, conductivity and dissolved oxygen measurements taken in the water column during the assays are presented in Table II for the five treatments of the Alqueva reservoir and in Table III for the standard control and the Beliche reservoir. Due to the small sample volumes involved, water-column physico-chemical parameters were not measured during the *T. platyurus*, *V. fischeri* and *H. incongruens* assays.

**Table II.** Range of pH, conductivity (Cond.) and dissolved oxygen (DO) levels measured in the water column of the Alqueva (five stations) treatments during seven out of the 11 assays comprising the battery of laboratory assays selected for the present study. *nm* – not measured.

| | Assay | Ajuda | | | Lucefécit | | | Mourão | | | Alcarrache | | | Álamos | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 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) | DO (mg/l) | pH | Cond. (µS/cm) | DO (mg/l) |
| **Water** | *Pseudokirchneriella subcapitata growth*[a] | 7.38 | 522 | *nm* | 7.48 | 624 | *nm* | 7.56 | 532 | *nm* | 7.47 | 535 | *nm* | 7.49 | 533 | *nm* |
| | *Daphnia magna* feeding[b] | 7.57-7.82 | 502-518 | 7.6-8.1 | 7.74-7.94 | 425-440 | 8.5-9.7 | 7.68-7.89 | 323-341 | 8.5-10.0 | 7.68-7.97 | 297-313 | 8.4-10.0 | 7.69-7.86 | 334-346 | 8.6-10.3 |
| | *Daphnia magna* reproduction[c] | 7.42-7.90 | 482-559 | 5.6-9.9 | 7.42-7.99 | 435-475 | 6.5-11.2 | 7.40-7.96 | 370-401 | 5.8-11.2 | 7.43-7.93 | 326-374 | 6.8-11.4 | 7.27-7.99 | 337-369 | 6.7-11.1 |
| | *Danio rerio* feeding[d] | 7.71-7.82 | 518-520 | 8.6-8.7 | 7.89-7.90 | 450-452 | 8.8 | 7.85 | 383-390 | 8.6-8.9 | 7.82 | 329-333 | 8.8-9.0 | 7.86-7.87 | 341 | 8.8-8.9 |
| | *Danio rerio* growth[c] | 7.48-7.95 | 469-614 | 7.3-9.1 | 7.67-8.41 | 295-497 | 7.4-10.2 | 7.64-8.48 | 325-470 | 7.0-9.8 | 7.53-8.06 | 288-436 | 7.4-10.3 | 7.62-7.98 | 291-437 | 6.8-10.4 |
| **Sediment** | *Chrinomus riparius* feeding[b] | 7.92-8.21 | 594-678 | 8.6-8.9 | 7.74-7.86 | 633-727 | 7.74-7.86 | 7.90-8.04 | 603-682 | 8.0-9.1 | 7.78-8.05 | 566-574 | 8.0-9.2 | 7.47-7.64 | 555-565 | 8.0-8.8 |
| | *Chrinomus riparius* growth[b] | 7.79-7.97 | 610-790 | 7.9-8.9 | 7.83-7.94 | 627-695 | 8.2-9.0 | 7.78-7.96 | 617-772 | 8.6-9.0 | 7.62-7.82 | 468-688 | 8.1-8.9 | 7.19-7.59 | 523-550 | 8.2-9.0 |

[a] measured only at start of assay
[b] measured at start and end of assay
[c] measured at all medium renewals
[d] measured only at end of assay

**Table III.** Range of pH, conductivity (Cond.) and dissolved oxygen (DO) levels measured in the water column of the standard control and Beliche (single station) treatments during seven out of the 11 assays comprising the battery of laboratory assays selected for the present study. Standard control medium: anostracan – reconstituted moderately hard water; microalgae – Woods Hole MBL (not supplemented with vitamins, diluted 2.5 times); cladoceran – reconstituted hard water supplemented with vitamins and Marinure extract; fish – dechlorinated tap water; midge larvae – reconstituted hard water (quartz sea sand as sediment). *nm* – not measured.

| Assay | | Standard Control | | | Beliche | | |
|---|---|---|---|---|---|---|---|
| | | pH | Cond. (µS/cm) | DO (mg/l) | pH | Cond. (µS/cm) | DO (mg/l) |
| **Water** | *Pseudokirchneriella subcapitata growth*[a] | 6.80 | 253 | *nm* | 7.38 | 365 | *nm* |
| | *Daphnia magna* feeding[b] | 7.77-7.82 | 557-566 | 8.6-9.0 | 7.30-7.50 | 140-152 | 8.0-10.4 |
| | *Daphnia magna* reproduction[c] | 7.37-8.01 | 567-594 | 6.7-9.6 | 7.08-7.88 | 144-166 | 6.9-11.3 |
| | *Danio rerio* feeding[d] | 7.21-7.26 | 180-181 | 8.6-8.8 | 7.26-7.56 | 148-151 | 8.7-8.8 |
| | *Danio rerio* growth[c] | 6.86-7.53 | 121-230 | 7.1-9.1 | 7.25-7.78 | 137-281 | 6.9-10.1 |
| **Sediment** | *Chrinomus riparius* feeding[b] | 7.90-8.05 | 559-576 | 8.6-9.0 | 7.14-7.41 | 424-463 | 8.4-8.7 |
| | *Chrinomus riparius* growth[b] | 7.56-7.81 | 570-623 | 8.7-9.0 | 6.66-7.40 | 441-476 | 8.0-8.8 |

[a] measured only at start of assay
[b] measured at start and end of assay
[c] measured at all medium renewals
[d] measured only at end of assay

## 3.1 Ecological receptors at most risk in the Alqueva reservoir

### 3.1.1 Assays for water samples

In the 24-hours *T. platyurus* survival assay, the percentage of organism survival was above 96% for all five water samples from the Alqueva reservoir, which is above the control validity criterion of 90%. Figure 3 presents the results of all sublethal laboratory assays performed with the five water samples of the Alqueva reservoir. As shown in Figure 3, for the 5-minutes *V. fischeri* assay the bacteria luminescence was not inhibited in either of the Alqueva waters relatively to the Microtox control since the maximum percentage of luminescence decrease was 4%.

Results from a two-way ANOVA on the 72-hours *P. subcapitata* growth assay showed that growth was significantly influenced by the two main factors, nutrient levels (plain waters versus waters supplemented with nutrients; $F_{1,5} = 1729$; $P < 0.001$) and Alqueva reservoir waters ($F_{1,5} = 2.94$; $P < 0.05$). However, the interaction effect was not significant ($F_{5,24} = 2.50$; $P = 0.0589$), showing that potential differences among waters were irrespective of the nutrient levels during testing. Thus, to be able to discriminate potential toxic effects from effects due to differences in nutrient levels across stations, remaining analysis were carried out only on nutrient enriched water samples. No significant differences were found among the standard control and Alqueva waters (one-way ANOVA: $F_{5,15} = 1.40$; $P = 0.28$); maximum percentage of difference between specific growth rate in control and Alqueva waters was 3% (Fig. 3).

In the 24-hours *D. magna* feeding assay, no significant differences were found among all six water treatments (one-way ANOVA: $F_{5,24} = 1.68$; $P = 0.18$), being the maximum percentage of inhibition in Alqueva waters relatively to the control of merely 13% (Fig. 3). On the contrary, the one-way ANOVA revealed that the 21-days reproduction of *D. magna* was significantly different among all six water samples ($F_{5,51} = 46.8$; $P < 0.001$) (Fig. 3). However, the Dunnett's test showed that such difference was simply caused by a significantly higher number of juveniles released in the Aj water compared to the control (by 38%); maximum percentage of inhibition in Alqueva waters relatively to the control was of merely 8%.

In the 48-hours *D. rerio* feeding assay, the mean percentage of food eaten in the standard control and five Alqueva waters was equal to or higher than 99%, and thus no statistical analysis were performed (Fig. 3). Results from the 28-days growth assay with *D. rerio* showed that the fish specific growth rate was similar among the control and Alqueva waters (one-way nested ANOVA; $F_{5,18} = 0.466$; $P = 0.80$), with the maximum percentages of increase and inhibition of 22 and 10%, respectively (Fig. 3).

### 3.1.2 Assays for sediment samples

Regarding the *V. fischeri* soild-phase assay, results showed significant differences among the 5-minutes luminescence of the bacteria in the control and five sediment samples of the Alqueva reservoir (one-way ANOVA; $F_{5,13} = 48.9$; $P < 0.001$). Luminescence was markedly inhibited at all sites relatively to the control, i.e. by 40, 58, 95,

99, and 99% at Aj, Ac, Mr, Lf, and Al, respectively (Fig. 4). Further analysis using the station at Beliche as a reference relatively to the Alqueva also showed significant differences in the 5-minutes luminescence across the six water samples (one-way ANOVA; $F_{5,12} = 20.4$; $P < 0.001$). However, the Dunnett's test revealed these differences to be due to the significantly higher luminescence at Aj and Ac relatively to Beliche, and, thus, the bacteria luminescence in Alqueva waters was not affected compared to the bacteria activity in the Beliche water.

In the 6-days *H. incongruens* growth assay, mortality was registered in the control, Lf, Mr and Al, but always well below the accepted control criterion of 20% (i.e. 6, 10, 2, and 2%, respectively). The one-way nested ANOVA revealed that means among control and Alqueva samples were significantly different ($F_{5,24} = 39.5$; $P < 0.001$). However, only at Al the specific growth rate was significantly lower than in the control and the percentage inhibition was of merely 10% (Fig. 4); in all the other Alqueva waters growth rate was significantly higher than in the control by 12 to 24% (Fig. 4).

For *C. riparius*, both the 48-hours postexposure feeding and the 10-days growth assays showed significant differences among the control and the five Alqueva waters (one-way nested ANOVA: $F_{5,10} = 9.48$; $P < 0.01$ and $F_{5,13} = 5.52$; $P < 0.01$, respectively). However, such differences corresponded only to responses significantly higher than in the controls; postexposure feeding was significantly higher in Mr by 77% and growth was significantly higher in all except Ac waters by 66 to 161% (Fig 4).

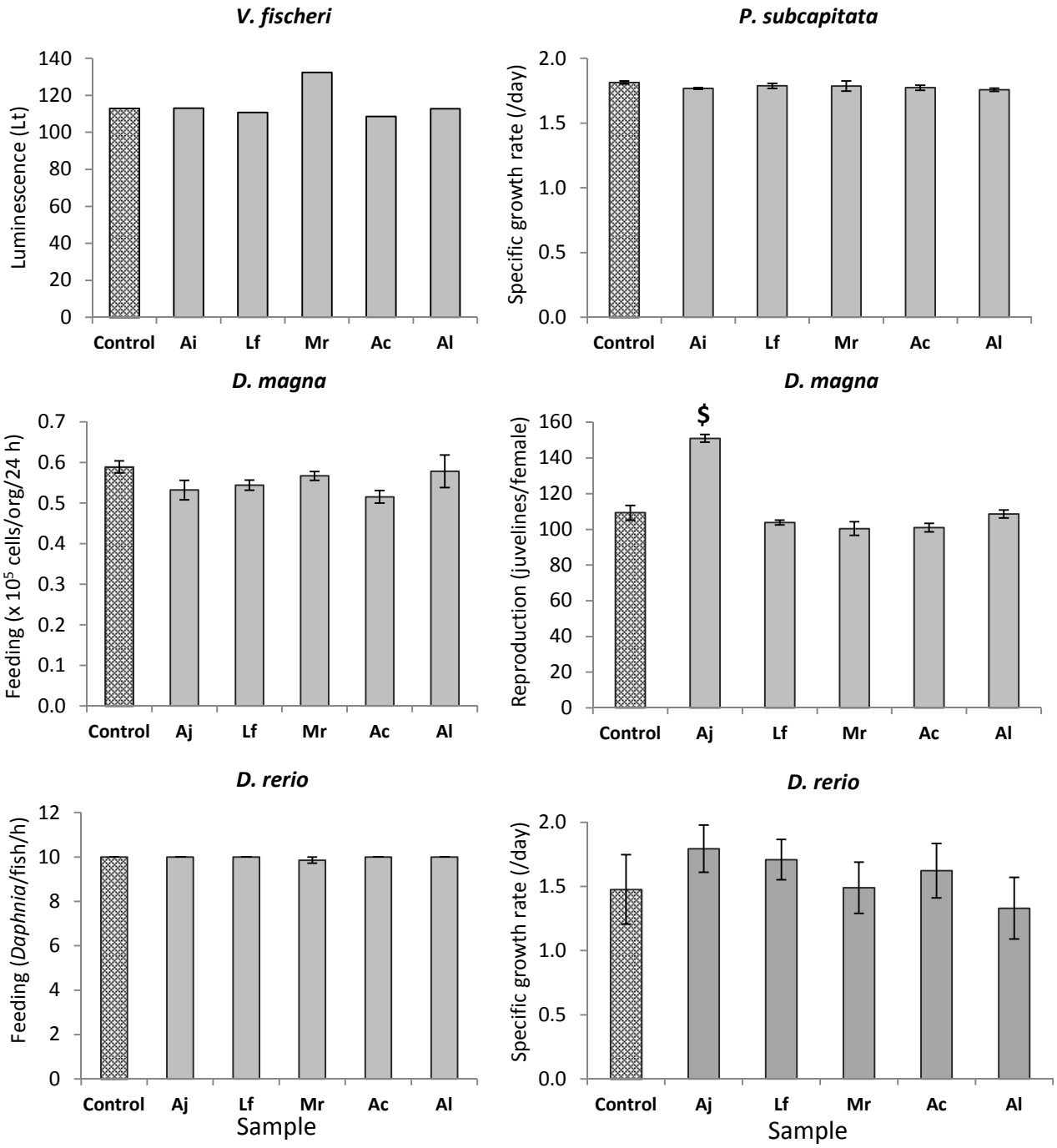


**Figure 3.** Sublethal effects of water samples collected at five stations of the Alqueva reservoir (Aj – Ajuda, Lf – Lucefécit, Mr – Mourão, Ac – Alcarrache, Al – Álamos) , on *Vibrio fischeri* (5-minutes luminescence), *Pseudokirchneriella subcapitata* (72-hours growth), *Daphnia magna* (24-hours feeding and 21-days reproduction), and *Danio rerio* (48-hours feeding and 28-days growth). Error bars indicate ± 1 standard error (no error bars for *V. fischeri* results as the test design does not include replication); asterisks and dollar signs denote means significantly lower and higher than the control, respectively, by Dunnett's test.

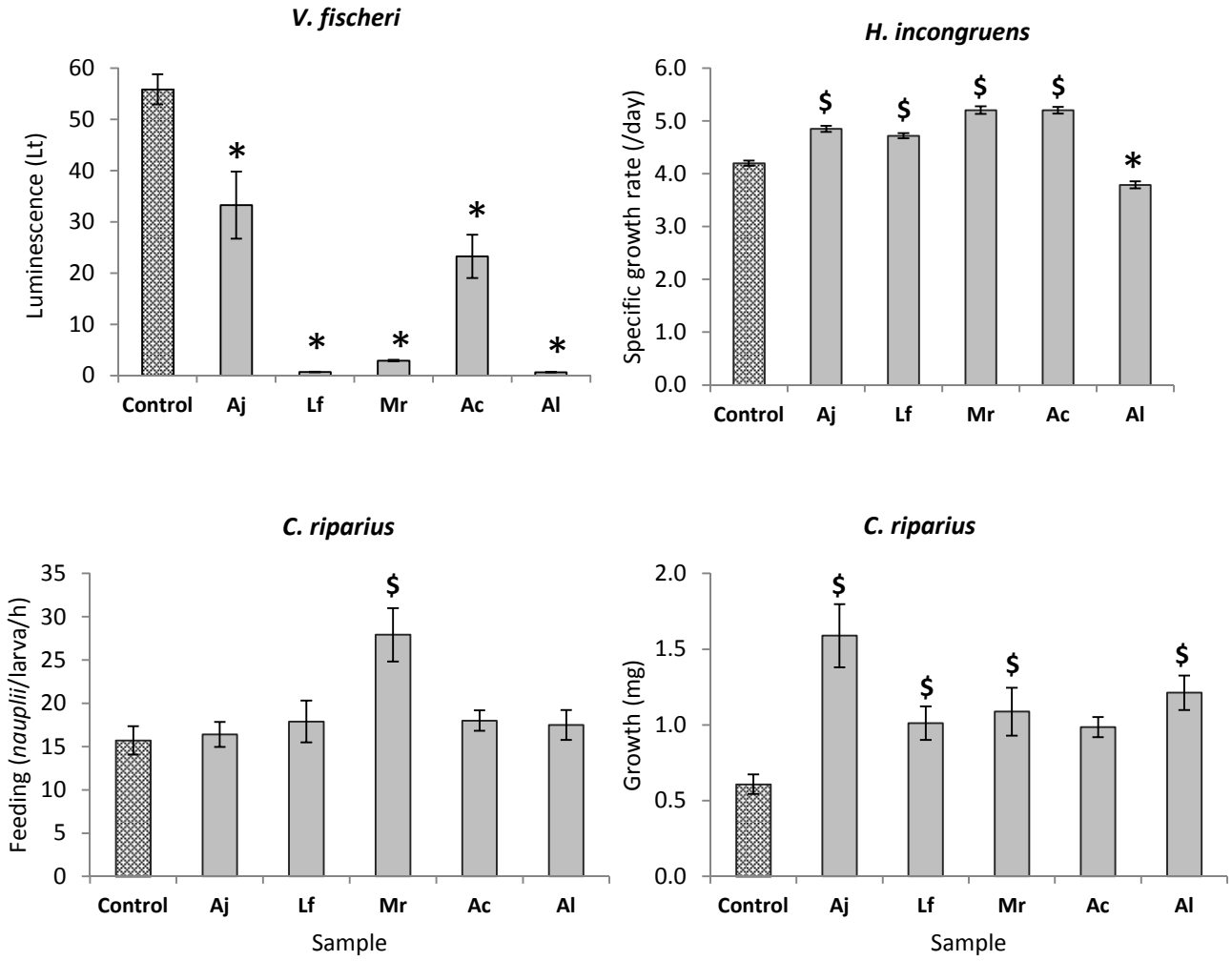


**Figure 4.** Sublethal effects of sediment samples collected at five stations of the Alqueva reservoir (Aj – Ajuda, Lf – Lucefécit, Mr – Mourão, Ac – Alcarrache, Al – Álamos) , on *Vibrio fischeri* (5-minutes luminescence), *Heterocypris incongruens* (6-days growth) and *Chrinomus riparius* (48-hours 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, respectively, by Dunnett's test.

### 3.2 Beliche reservoir as a potential reference for the Alqueva reservoir

Although only the solid-phase *V. fischeri* assay indicated toxicity, determining the need to proceed with further analysis using a reference, the potential future use of Beliche as a reference for ecotoxicological studies at the Alqueva reservoir was explored for all the 11 assays performed in the present study. Results from Student's *t*-tests revealed that only for two sediment assays – the 5-minutes *V. fischeri* luminescence and the 6-days *H. incongruens* growth – organism's responses in Beliche samples were significantly lower than in the control by 99 and 29%, respectively ($t_5$ = 15.9; $P$ < 0.001 and $t_8$ = 6.47; $P$ < 0.001) (Fig. 5). For all other assays, responses in control and Beliche were either similar (*T. platyurus* survival, *V. fischeri* luminescence in water, *P. subcapitata* growth, *D. magna* feeding and reproduction, and *D. rerio* feeding; $t_{7\text{-}18}$ > - 0.178; $P$ > 0.13) or significantly higher in Beliche than in the control (*D. rerio* growth and *C. riparius* postexposure feeding and growth; $t_{4\text{-}6}$ < - 3.06; $P$ < 0.05) by 37, 35 and 112%, respectively.

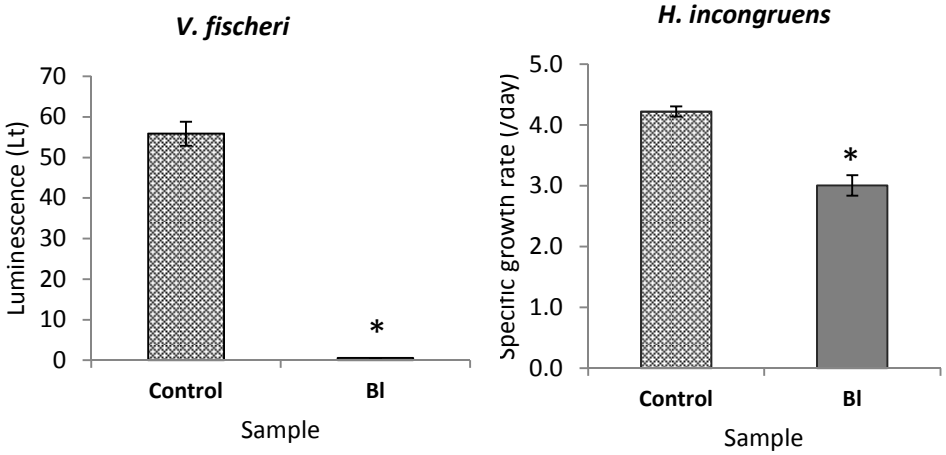


**Figure 4.** Sublethal effects of sediment samples collected at a single station of the Beliche reservoir (Bl), on *Vibrio fischeri* (5-minutes luminescence) and *Heterocypris incongruens* (6-days growth). Error bars indicate ± 1 standard error; asterisks denote means significantly lower than the control by Student's *t*-test.

# Chapter 4 Discussion

### 4.1 Ecological receptors at most risk in the Alqueva reservoir

The toxicity of water and sediment samples collected in February 2012 was evaluated with a battery of laboratory assays comprising test species from different taxonomic and functional groups to identify the ecological receptors at most risk in the Alqueva reservoir. Overall, the results from the present study did not reveal toxic effects for the ecological receptors evaluated, except for the *V. fischeri* solid-phase assay on sediment samples for all study sites. Although there was a general lack of toxicity, i.e. there was no inhibition of the organisms response in the Alqueva samples relatively to the standard control treatment, some organism responses were significantly higher in Alqueva samples than in controls (*D. magna* reproduction at Ajuda, *H. incongruens* growth at all stations except Álamos, *C. riparius* postexposure feeding at Mourão, and *C. riparius* growth at all stations except Alcarrache). These differences in responses across stations were expected, since distinct areas within the large Alqueva reservoir are known to have different biological and physico-chemical characteristics. For instance, in a previous study to evaluate the ecological status of Alqueva reservoir, six sampling stations within the reservoir with available historic data were assessed and four stations were classified as in "Good ecological status" and two as in "Less than good status" (Silva *et al.,* 2011). Furthermore, previous studies have concluded that the most impacted areas corresponded to the upper half of the Alqueva reservoir, close to the Spanish territory, since this receives the major pesticide and nutrient inputs (Palma *et al.,* 2009; Palma *et al.,* 2010a; Lindim *et al.,* 2011). Based on this statement, Ajuda, Lucefécit and Mourão were indeed stations were organism responses were stimulated (Robinson *et al.,* 1994), mainly at Ajuda (*D. magna*

reproduction, *H. incongruens* and *C. riparius* growth). Moreover, in a recent survey from 2011, only at the Ajuda station the nutrient levels surpassed the guide values recommended on the Freshwater Guidelines Quality (Palma *et al.,* 2012).

In the present study, the toxicity assessment of water samples from Alqueva reservoir revealed no lethal toxicity in the assay with *T. platyurus*, even though this assay was efficient at discriminating toxicity among water samples collected during 2006-2007 in the Alqueva reservoir, especially during the dry season (Palma *et al.,* 2010a). Regarding the sublethal assays, the absence of toxicity in water samples pointed by the *V. fischeri* assay in the present study is in agreement with previously results on water samples collected throughout the year 2006 at Alqueva reservoir (Rodriguez *et al.,* 2010). Moreover, *V. fischeri* was the test organism less sensitive to the main pesticides reported for water samples from Alqueva reservoir (Palma *et al.,* 2008), and has being cited as an assay not as sensitive to insecticides and herbicides as other aquatic organisms (Strachan *et al.,* 2001). However, in a recent study at the Alqueva reservoir, *V. fischeri* was the most sensitive species compared to *T. platyurus* and *D. magna* for water and sediment elutriates (decanted supernatant after centrifugation) of samples collected in 2011 (Palma *et al.,* 2012). Relatively to the *P. subcapitata* growth assay, contrary to the present study, toxicity toward this species has been previously reported and correlated with pesticide levels for water samples collected in the summer of 2006 (Rodriguez *et al.,* 2010), even though in the latter study potential differences in nutrient levels across sites were not taken into account. For the test species *D. magna,* Palma *et al.* (2010a) reported that the chronic exposure to surface waters from Alqueva reservoir did not promote lethal toxicity to *D. magna*, however a significant decrease in the number of juveniles per female was observed, mainly

at the dry period. Moreover, *D. magna* was less sensitive than *T. platyurus* to common pesticides reported for this water body (Palma *et al.,* 2008), suggesting, thus, not to be a good indicator of neither lethal nor sublethal toxicity for ecotoxicological monitoring programs in the Alqueva reservoir (Palma *et al.,* 2008; Palma *et al.*, 2010a). Yet in a recent study, *D. magna* showed slight sublethal toxicity toward sediment elutriates collected in 2011, but was again unresponsive to the water samples from this reservoir (Palma *et al.,* 2012). To our knowledge, it is the first time that a secondary consumer – the fish *D. rerio* – was used to evaluate feeding and growth rates from water samples at the Alqueva reservoir, showing no toxicity evidence for the selected endpoints.

In the toxicity assessment of sediment samples from the Alqueva reservoir conducted in the present study, only the *V. fischeri* luminescence solid-phase assay showed potential toxic evidence as indicated by strong luminescence inhibitions relatively to the control. Compared with all other assay results this finding was somehow unexpected, in as much as no toxic effects were revealed when the Alqueva samples were compared with the sediment sample from the Beliche reservoir (potential reference reservoir). These results may be due to the fact that practically all sediments were rich in fine particles, which might be associated to: (1) high contaminant levels due to their relatively large surface area and transport (Burton, 1991), (2) bacterial adhesion affecting light emission (Ankley *et al.* 1994; Parvez *et al.,* 2006), and (3) dark-brownish sediments which may lead to non-specific reductions in light levels by absorbing light of the wavelength emitted by the bacteria (Western Canadian Microtox Users Committee, 1994). Given that the sediment samples collected within the present study were rich in fine particles and showed reddish-brown colours, colour corrections should be performed to establish if the reported inhibition

effects were actually linked to the presence of contaminants. As for the low luminescence levels found for the Beliche sediment samples, also rich in fine particles but the lightest in colour, it may be associated with high natural levels of metals since this reservoir is located in the Iberian pyrite belt (González *et al.,* 2011), and *V. fischeri* is known to be particularly sensitive to metals (Salizzato *et al.,* 1998). Further studies related to sediment particles and presence of other contaminants (e.g. polycyclic aromatic hydrocarbons, metals) have to be addressed to explain these high luminescence inhibitions with the sediment samples.

To our knowledge, this is the first time the *H. incongruens* growth assay was applied to assess the toxicity of sediment samples from the Alqueva reservoir. Although this organism responses can also be influenced by fine particles (e.g. affecting respiratory structures) (Lemly, 1982), growth was affected by none of the tested sediment samples. Regarding the *C. riparius* assays, results of the present study are in agreement with those of Rodriguez *et al.,* (2010) and were somehow expected, since this test organism is an opportunistic species that can be resistant to contaminants (Burton, 1991; De Hass *et al.,* 2002) and the availability of nutrients on sediments might increase *C. riparius* growth rates (De Haas *et al.,* 2002); in the present study the midge larvae grew significantly more at all sediment samples (except Alcarrache) than in the control.

The general lack of toxicity at all Alqueva sampling stations selected within the present study might be explained by a decrease in pesticide levels promoted by a severe drought in Autumn 2011 and Winter 2011-2012, as well as by a change in the agricultural practices in the region. In effect, 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).

The meteorological station at Reguengos, located close to the Alqueva reservoir, registered precipitation levels during Autumn 2011 and Winter 2011-2012 lower than normal for these seasons. For instance, the average monthly precipitation in February 2012 of 0.6 mm dramatically contrasts with the historic average monthly precipitation of 67 mm for this month. Also, average monthly temperature was 8.3 ˚C for February 2012 which was 1.7 ˚C lower than the historic average monthly temperature for the area, leading to less water evaporation within the reservoir (Sistema Nacional de Informação de Recursos Hídricos website: http://snirh.inag.pt). Several authors have emphasized rainfalls greater than 10 mm/day are the most important driven-cause of input of pesticides to freshwater ecosystems as a result of runoff and subsurface flows (Liess *et al.,* 1999; Inoue *et al.,* 2002; Nakano *et al.,* 2004; Schulz, 2004). Therefore, extreme low precipitation events during Autumn 2011 and Winter 2011-2012 might have prevented the entry of pesticides from surrounding agricultural fields.

Another factor preventing the input of pesticides from agricultural fields is associated to changes in agricultural practices in the Alentejo region driven by the need to adopt environmentally friendly techniques (Pinheiro, 2004). Olive orchards represent 40% of the area of agricultural fields, in which a vegetal coverage approach, instead of the traditional tillage technique, has demonstrated highest levels of water infiltration, prevention of soil erosion and soil disturbance which in turn might prevent runoff (Pinheiro *et al.,* 2002). The erosive forces of rain drops and the wind can be tackled with a vegetation coverage technique allowing their root systems to stabilize the soil, reduce water loss and

improve soil water intake at the olive orchards (Pinheiro, 2004). Moreover, the drop by drop irrigation system adopted in the area also prevents runoff by reducing water demands for cultivation, thus efficiently using local water resources (Pinheiro *et al.,* 2002).

### 4.2 Putting forward a tool-box of short-term sub-lethal cost-effective tests

The test battery used in this study comprised several species from different taxonomic and functional groups as well as lethal and sublethal endpoints aiming to set a cost-effective tool-box for assessment and monitoring of water quality at the Alqueva reservoir. A battery of test species to perform ecotoxicological evaluations is essential mainly because: (1) sensitivity of test species varies depending on the chemical properties of the pollutant and there is no single most sensitive species (Van der Brink *et al.,* 2006), (2) potential effects of contaminants on the population dynamics might be extrapolated (Schäfer *et al.,* 2011), (3) functional responses can be measured (Giesy & Hoke, 1989), and (4) a general evaluation of the effects from all water and sediment components, including those due to unknown substances and synergic, antagonistic or additive effects can be evaluated (Brack *et al.,* 2007). Therefore, the development of 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 selected to be included in the test battery for the present study were: luminescence of the marine bacteria *V. fischeri,* growth of the green microalgae *P. subcapitata*, survival of the fairy shrimp *T. platyurus*, reproduction and feeding of the planktonic cladoceran *D. magna*, growth and feeding of the zooplanktivorous fish *D. rerio* for water samples and luminescence of *V. fischeri*, growth of the benthic ostracod *H. incongruens*, growth and feeding of the benthic midge *C. riparius* for sediment samples. The decomposer marine bacteria *V. fischeri* is an essential representative of the microbial community involved in degradation processes (Parvez *et al.,* 2006). Another decomposer is the midge larvae *C. riparius*, a benthic organism widely distributed and abundant insect in stream ecosystems that breaks down dead plant into nutrients to make them available for other organisms (Soares *et al.,* 2005). The microalgae *P. subcapitata* plays a major role as primary producer in most ecosystems by producing food using sun's energy. Primary consumers included the species *T. platyurus*, *D. magna* and *H. incongruens,* invertebrates that also serve as food source for secondary consumers on the aquatic food chain. Finally, the fish *D. rerio* represent the secondary consumer pelagic fish of this test battery, a key species in the trophic chain of lakes (Cabral *et al.,* 1998). 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 is, to our knowledge, an innovative step as it is composed of 11 assays, chiefly based on sublethal responses and integrates a wide range of taxonomic and functional groups.

Defining a cost-effective tool-box for assessing impacts at the Alqueva reservoir, or other South European big-man made reservoirs, due to agricultural and wastewater discharges is a promising approach for ecological monitoring. In the present study, no

toxicity evidence was recorded for water and sediment samples from Alqueva reservoir, most likely due to the severe drought during Autumn 2011 and Winter 2011-2012 as well as changes in agricultural practices in the area, which probably reduced contaminant concentrations in the reservoir. Therefore, the selection of assays to be included in the tool-box could not be achieved. However, based on the potential toxicity evidence detected in the *V. fischeri* assay on sediment samples in the present study and in a recent study by *Palma et al.* (2012) for sediment elutriates, it is highly recommended to include laboratory assays with benthic/epibenthic test organisms on a future tool-box.

### 4.3 Beliche reservoir as a potential reference for the Alqueva reservoir

The Beliche reservoir was proposed as a potential reference site that can be useful to unravel confounding factors involved in the biological responses of ecotoxicity assays at the Alqueva reservoir. The advantages of setting reference sites include: (1) a baseline against which anthropogenic pressures can be assessed, (2) a biological community goal for setting directions to restoration efforts and (3) a description of spatial and temporal variability that characterizes healthy ecosystems in the area (Schmutz *et al.,* 2002).

Among the criteria to establish reference sites, areas with least amount of human disturbance would be preferred (Schmutz *et al.,* 2002), as it has been advocated for the Beliche reservoir, which is in fact not likely to present high levels of contaminants due to

no economic or anthropogenic activities in the surroundings (Galvão, *et al.,* 2012); it has been classified as in "Good ecological status" (Silva *et al.,* 2011). The only environmental challenge associated to the Beliche reservoir is related to the abundance of cyanobacteria and microcystins, even though WHO guidelines (World Health Organization, 1998) with respect to human health risk have not been exceeded for these organisms (Galvão *et al.,* 2008). Moreover, it has been proposed that harmful algal blooms can be prevented or alleviated by reducing water residence time in the reservoir (Galvão *et al.,* 2012).

In the present study, evidences of toxicity were found neither for water nor sediment samples from the Beliche reservoir. The only exception was relatively to the luminescence of the bacteria *V. fischeri* (inhibited by > 98%), even though, as previously discussed, the presence of fine particles and of high metal levels in this reservoir located in the Iberian pyrite belt region might be confounding factors at the origin of these results which deserve to be further investigated. Moreover, although Beliche and Alqueva reservoirs are part of the same river basin and both are located at the South of Portugal facing slightly similar environmental conditions, other geographical differences should also be addressed such as geomorphological, hydrological and biogeographic conditions to ensure that communities of the freshwater ecosystems can be truly comparable. Reservoir type differences should be fully addressed as well, since this variation might also reflect to biological differences (Schmutz *et al.,* 2002), namely in what regards dissimilarities in hydrological conditions (Silva *et al.,* 2011).

### 4.4 Conclusion

South European big-man made reservoirs are essential sources of freshwater that are being threatened by pollution mainly coming from agricultural activities (e.g. pesticides). In the present study, water and sediment samples from five and one sampling stations at the selected contaminated – Alqueva – and reference – Beliche – reservoirs, respectively, were collected during February 2012 to be ecotoxicologically evaluated through a battery of 11 laboratory assays. Assay endpoints for water samples included: luminescence of the marine bacteria *V. fischeri,* growth of the green microalgae *P. subcapitata*, survival of the fairy shrimp *T. platyurus*, reproduction and feeding of the planktonic cladoceran *D. magna*, growth and feeding of the zooplanktivorous fish *D. rerio*. Assay endpoints for sediment samples included: luminescence of *V. fischeri*, growth of the benthic ostracod *H. incongruens*, growth and feeding of the benthic midge *C. riparius*. Results obtained allowed to conclude that no toxicity evidence was found at the Alqueva reservoir. This fact was associated to a severe drought occurred months prior to sampling as well as changes in agricultural practices (e.g. drop by drop irrigation system and a vegetal coverage approach), which might have prevented the input of pesticides through runoff from agricultural fields, limiting the input of pesticides to the reservoir. As the test organisms used to evaluate ecological receptors at most risk did not show such harmful effect on their biological responses, it is suggested that aquatic communities might not be at risk during such scenario, at least presently. Due to absence of toxicity in the samples, a cost-effective tool-box could not be put forward. Finally, the weak anthropogenic pressures at Beliche together

with the ecotoxicological evidence of the lack of detrimental effects supported the proposal of this reservoir as a reference site.

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