Universidade de Aveiro Departamento de Biologia
Ano 2013

JOANNE RODRÍGUEZ PÉREZ

Toxicidade de químicos em mistura: o caso da albufeira do Alqueva

Chemical mixture toxicity: the case study of the Alqueva dam

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro) e do Professor Doutor Amadeu Mortágua Velho da Maia Soares (Professor Catedrático do Departamento de Biologia da universidade de Aveiro).

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Ao meu principezinho Diogo Alejandro e a Yosvany com todo meu amor.

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Palavras-chave

Avaliação do risco ambiental, misturas de pesticidas, adição da concentração, ação independente, sinergismo, antagonismo, *Chironomus riparius*, *Pseudokirchneriella subcapitata*, *Danio rerio*, Barragem do Alqueva.

Resumo

No meio ambiente o Homem e todos os outros organismos estão geralmente expostos a misturas químicas, e não apenas aos químicos individuais. Por isso, quando se avalia o risco ambiental de produtos químicos, é importante considerar as misturas químicas e suas possíveis interações. O objetivo principal deste trabalho centrou-se na avaliação do risco ambiental de pesticidas encontrados na água da Barragem do Alqueva e as suas combinações binárias. Neste ecossistema aquático vários pesticidas estavam acima dos padrões de qualidade ambiental. Mas, além disso, nalguns pontos de amostragem do reservatório foi observada ecotoxicidade, apesar da presença destes contaminantes ser abaixo das concentrações pré-estabelecidas pela legislação. Neste trabalho foi utilizada uma abordagem baseada em componentes para avaliar os efeitos das misturas de pesticidas. Os efeitos das combinações binárias de quatro herbicidas, atrazina (ATR), terbutilazina (TER), simazina (SIM) e metolacloro (MET) na taxa de crescimento da microalga *Pseudokirchneriella subcapitata* e os efeitos das combinações binárias dos herbicidas *s*-triazinas (ATR e TER) e o inseticida clorpirífos (CPF) no comportamento natatório e a atividade da acetilcolinesterase (AChE) do peixe zebra *Danio rerio* foram avaliados usando os dois modelos de referência, o da adição da concentração (CA) e o da ação independente (IA). Para além disso, foram testados os efeitos combinados dos herbicidas (ATR, TER e MET) e do inseticida CPF no comportamento natatório e na atividade da AChE em larvas de mosquito *Chironomus riparius* após a caracterização das cholinesterases. Na caracterização dos riscos, os quocientes de risco calculados para os herbicidas ATR, TER, SIM e MET foram maiores do que 1, o que significa que estes herbicidas apresentaram um alto risco para o ecossistema do Alqueva. Aqui, a microalga *P. subcapitata* foi a espécie mais sensível aos herbicidas. No entanto, apesar destes herbicidas não representarem um risco para os outros organismos aquáticos testados neste estudo, com valores de EC_{50} superiores às concentrações encontradas neste ecossistema aquático, eles são capazes de aumentar os efeitos tóxicos do CPF quando são testados em misturas binárias. Além disso, os quocientes de risco das misturas destes herbicidas presentes simultaneamente em três locais diferentes do reservatório foram também superiores a 1, pelo que ratifica o facto destes herbicidas, quando presentes em misturas, representarem um risco acrescido para ecossistemas deste tipo.

Keywords Environmental risk assessment, pesticides mixtures, concentration addition, independent action, synergism, antagonism, *Chironomus riparius*, *Pseudokirchneriella subcapitata*, *Danio rerio* and Alqueva reservoir.

Abstract In the environment humans and biota are generally exposed to chemical mixtures rather than individual chemicals. Therefore, when assessing the environmental risk of chemicals, it is important to consider chemical mixtures and their possible interactions. The main objective of this work focused on the environmental risk assessment of pesticides found in the water of the Alqueva reservoir and their binary combinations. In this aquatic ecosystem several pesticides were above of the environmental quality standards. But in addition, there were several sampling points of the reservoir where ecotoxicity was observed despite the presence of these contaminants at low concentrations. Here, a component-based approach was used to assess the effects of the pesticide mixtures. The effects of the binary combinations of four herbicides, atrazine (ATR), terbuthylazine (TER), simazine (SIM) and metolachlor (MET), on the growth rate of the microalgae *Pseudokirchneriella subcapitata* and the effects of the binary combinations of the *s*-triazine herbicides ATR and TER and the insecticide chlorpyrifos (CPF) on the swimming behaviour and acetylcholinesterase (AChE) activity of the zebrafish *Danio rerio* were assessed using the two reference models of concentration addition (CA) and independent action (IA). Moreover, the combined effects of the herbicides (ATR, TER and MET) and the insecticide CPF were also tested on the swimming behaviour and AChE activity of the aquatic midge *Chironomus riparius* after the cholinesterases characterization. In this risk characterization, the calculated risk quotients for the herbicides ATR, TER, SIM and MET were higher than 1, meaning that these herbicides present a high risk for the Alqueva ecosystem. As expected, the microalgae *P. subcapitata* was the most sensitive species to the herbicides. However, despite these herbicides pose no or low risk to other aquatic organisms tested in this study, with EC_{50} values much higher than the concentrations found in this aquatic ecosystem, they are able to increase the toxic effects of CPF when they are tested in binary mixtures. Moreover, the risk quotients of mixtures of these herbicides present simultaneously in three different locations of the reservoir were also higher than 1, so this confirms the fact that these herbicides when present in mixtures, present a greater risk for this ecosystem than the expected considering each single chemical by its own.

Index

Tables Index

Figures Index

Chapter 1
General Introduction

1. General Introduction

Freshwater ecosystems may be affected by a large amount of dangerous substances through inputs from agricultural and industrial activities, sewage discharges, runoff and groundwater leaching. Therefore, the concern regarding the alteration of water quality has led to the development of monitoring programs in the most important waters bodies in order to assess the risks of exposure to these substances, their adverse effects on aquatic ecosystems and also the consequent impact on their functions.

Maximum Admissible Concentrations (MACs) of chemicals are commonly used as the environmental quality standard for the assessments of surface waters. Surface waters are considered to have environmental quality if these concentrations are not overcome. However, many studies have demonstrated that even when these concentrations are not exceeded, the water quality can be compromised by the combined effects of the chemicals. It is known that the joint toxicity of chemicals is often greater or less than their individual toxic effects, i.e. patterns of synergism or antagonism can occur among chemicals when they are together in the environment.

Consequently in the natural environment organisms are not only exposed to the effects of individual chemicals, but also to the effects of their combined action. Nevertheless, environmental risks of chemicals are still often assessed substance-by-substance, ignoring mixture effects. “This may result in risk underestimations, as the typical exposure is toward multicomponent chemical “cocktails”” (Backhaus and Faust, 2012). Therefore, taking into account the abovementioned interactions among chemicals, the toxicity of mixtures and their effects must also be considered in the regulatory risk assessment of chemicals (Syberg et al., 2009).

1.1. Ecological Risk Assessment (ERA)

Ecological risk assessment is defined as: “A process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors” (EPA, 1992). In the European Union is also defined as: “A process intended to calculate or estimate the risk for a given target system following exposure to a particular substance, taking into account the inherent characteristics of a substance of concern as well as the characteristics of the specific target system”. A risk assessment process for new and existing substance follows four steps: 1) the identification of the hazard, followed by 2) the estimation of the Predicted Environmental Concentration (PEC) (exposure assessment), 3) the estimation of a Predicted No Effect Concentration (PNEC) (effects assessment) and finally 4) the characterization of the risk (Figure

1.1). These risk assessment process steps were first developed by the US national research council (EPA, 1992) and were subsequently adopted by the EU in (EC, 2003).

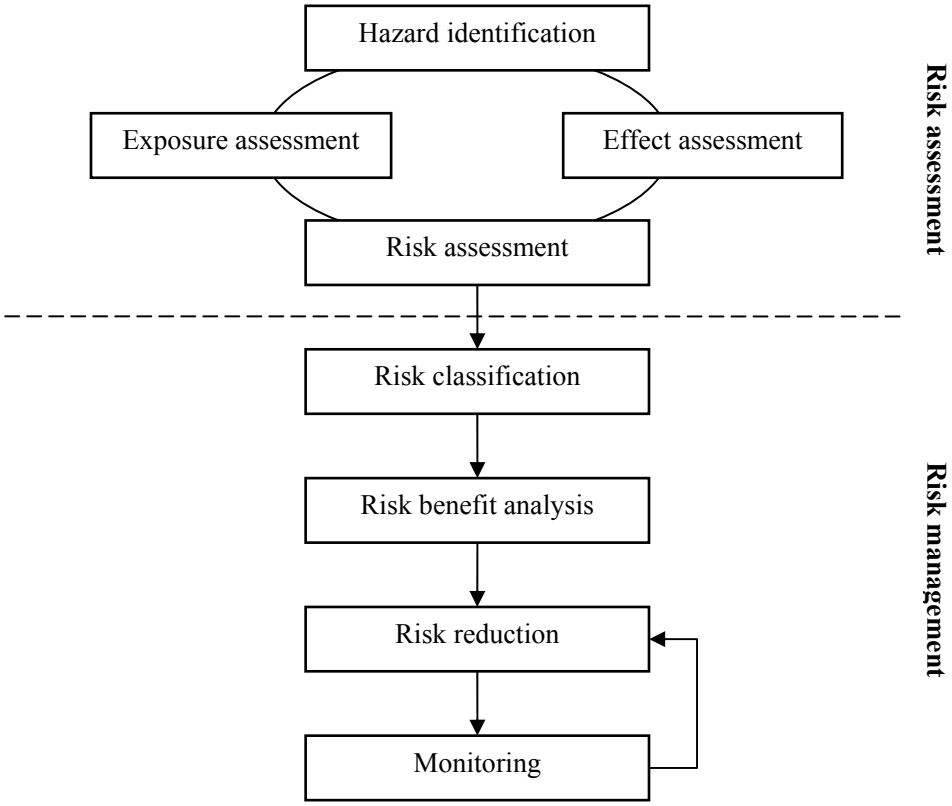

Figure 1. 1. The framework for ecological risk assessment.

The ecological risk assessment of chemical exposure is based on the comparison of the exposure to a chemical of an ecosystem (or fraction of the ecosystem) with the respective sensitivity of the ecosystem (or the same fraction of it) to that chemical (Suter, 1993). The exposure is represented by the Predicted Environmental Concentration (PEC), and can be obtained by estimations using environmental fate models or by real field measurements (e.g. monitoring data). The Predicted No Effect Concentration (PNEC) represents the sensitivity of the ecosystem, and is usually derived from standardised toxicity tests using organisms from major trophic levels (primary producers, primary and secondary consumers). The PEC and PNEC comparison is considered an useful tool to estimate the potential risk of chemicals to ecosystems (EC, 2003).

The PNEC is a fixed threshold value which represents the sensitivity of the environment to a specific toxicant. This value is defined as: “The concentration below which unacceptable effects on organisms will most likely not occur” (EC, 2003).

The PNEC is derived by selecting the most sensitive test (representing the most sensitive trophic level) and applying an appropriate Assessment Factor (AF), which accounts for intra-laboratory and inter-laboratory variation of the data, biological variance, short-term to long-term extrapolation and laboratory to field extrapolation (European Chemicals Agency, 2008) (Table 1.1). Therefore, it is assumed that by protecting the most sensitive trophic level all other organism groups are protected as well and that protecting the structure of an ecosystem also protects ecosystem functions.

For the majority of substances, the data set needed to predict their effects on the ecosystems are very limited. In general, only short-term toxicity data are available. In these conditions, it is accepted that, while not having a solid scientific validity, assessment factors empirically obtained should be used. The purpose of using such factors is to predict a concentration below which an unacceptable effect will not occur (European Chemicals Agency, 2008).

The PNEC is then calculated by dividing the lowest EC_{50}/LC_{50} or NOEC value for three trophic groups of organisms by a suitable assessment factor. The assessment factors are also applied to extrapolate from laboratory single-species toxicity data to multi-species ecosystem effects. When only short-term toxicity data are available, an assessment factor of 1000 will be applied on the lowest EC_{50}/LC_{50} of the relevant available toxicity data, independently of whether or not the species tested is a standard test organism. Moreover, a smaller assessment factor will be applied on the lowest NOEC derived in long-term tests with a relevant test organism (Table 1.1) (European Chemicals Agency, 2008).

Table 1. 1. Assessment factors used to derive PNEC values (European Chemicals Agency, 2008).

Available toxicity data	**Assessment factors**
At least one short-term L(E)C_{50} from each of three trophic levels (fish, invertebrates (preferred Daphnia) and algae)	1000
One long-term EC_{10} or NOEC (either fish or Daphnia)	100
Two long-term results (e.g. EC_{10} or NOECs) from species representing two trophic levels (fish and/or Daphnia and/or algae)	50
Long-term results (e.g. EC_{10} or NOECs) from at least three species (normally fish, Daphnia and algae) representing three trophic levels	10
Species sensitivity distribution (SSD) method	5-1 (to be fully justified case by case)
Field data or model ecosystems	Reviewed on a case by case basis

Subsequently both exposure and effect assessments are integrated in the characterization of the risk. So the risk characterization involves the calculation of the PEC/PNEC ratio, also called, the

Quotient Risk (RQ). If the RQ is less than 1, the substance of concern is considered to present no risk to the environment and there is no need for further testing or risk reduction measures. If, however, the ratio cannot be reduced to less than 1 by the collection of additional information and additional testing, risk reduction measures are necessary and must be implemented.

Risk Quotient has become in fact a standard for the ecotoxicological risk characterization not only for industrial compounds within the context of the European chemicals legislation REACH, but also for biocides and pharmaceuticals (Backhaus and Faust, 2012). A chemical is compatible with the environment if the PNEC, i.e., the concentration that causes no adverse effect on the environment is greater than the predicted environmental concentration (PEC) which is the concentration that one expects to find in the environment.

1.2. Ecological Risk Assessment (ERA) of chemical mixtures

Ecological risk assessment usually focuses on single chemicals. However, it is known that the effects of chemicals on ecosystems and human health mainly come from exposures to mixtures rather than from individual chemicals. In the environment, humans and ecological receptors are often exposed to several chemicals which may or may not interact; i.e. influence each other by physical, chemical or biological means before or after reaching the molecular site of toxic action (Van Gestel et al., 2010).

The risk assessment of chemical mixtures and the risk assessment of the individual substances have some similarities, but also differ in some aspects. Figure 1.2 shows some of the aspects to be additionally considered in the risk assessment of chemical mixtures. In this case the potential environmental risk is due to a chemicals mixture, e.g. the prospective assessment of the net risks of a mixture emission, the retrospective assessment for a site contaminated with a mixture, or the regulatory wish to set a safe exposure level for a mixture (Van Gestel et al., 2010). The hazard identification should account for possible toxicological interactions and joint effects from exposure to combinations of chemicals causing similar or dissimilar toxic effects. As well as for the single substances, the exposure assessment results in a real or predicted exposure level, but in this case for a chemical mixture. Although determination of the exposure level may be more complicated for mixtures, e.g. due to potential chemical interactions between the mixture components that change the mixture composition, the essence is similar to that of single substances. However, the phase of effect assessment may differ considerably between single substances and mixtures. For example, effects may occur even though the single components are below their individual threshold effect levels (Van Gestel et al., 2010). To assess mixture effects, three approaches can be essentially followed:

1. Whole mixture approach for common mixtures: In this case it is a common and frequently complex mixture with more or less constant concentration ratios between the mixture components. A reference value, as a PNEC value or dose-response relationship can be established for the mixture as if it was one complex compound and a safe level can be determined like for single compounds based on toxicity data on the mixture itself or a sufficiently similar mixture. The effect data can later be used for the assessments of mixtures that are identical, for example, originating from the same source or sufficiently similar (read-cross).

2. Whole mixture approach for unique mixtures: In this case it is a totally unknown mixture or with a unique origin and composition. In this case, results of previous effect studies cannot be used to assess the effects of the mixture of concern. Estimating a safe concentration level or a dose-response relationship for these mixtures will be inefficient as the effect data cannot be re-used to assess the risks for other mixtures. The mixture of concern has to be tested directly in the field or in the laboratory, like in Whole Effluent Toxicity (WET) test, resulting in a direct indication of the possible effects.

3. Component-based approach: In this case it is a mixture whose composition can be determined, by chemical analysis, and if a mixture model is available mixture effects can be predicted. The mixture model can either be simple, e.g. summation of PEC/PNEC ratios over all compounds into a Hazard Index (HI), moderately complex e.g. applying the models of Concentration Addition (CA) or Independent Action (IA) with or without taking the shape of the dose-effect curve into account, or complex, e.g. like a special toxicokinetic modeling approach to predict organ-specific concentrations within organisms.

At last, the risk characterization phase merges the information of the two previous steps (i.e. exposure and effect assessment). This stage must consider some matters not tackled in single chemical risk assessments, for example, the assumptions made in order to use a particular risk assessment method (e.g. similarity in chemical composition between whole mixtures, or a shared toxic mode of action for chemical components within a mixture) must be articulated and supported with data (Figure 1.2) (Van Gestel et al., 2010).

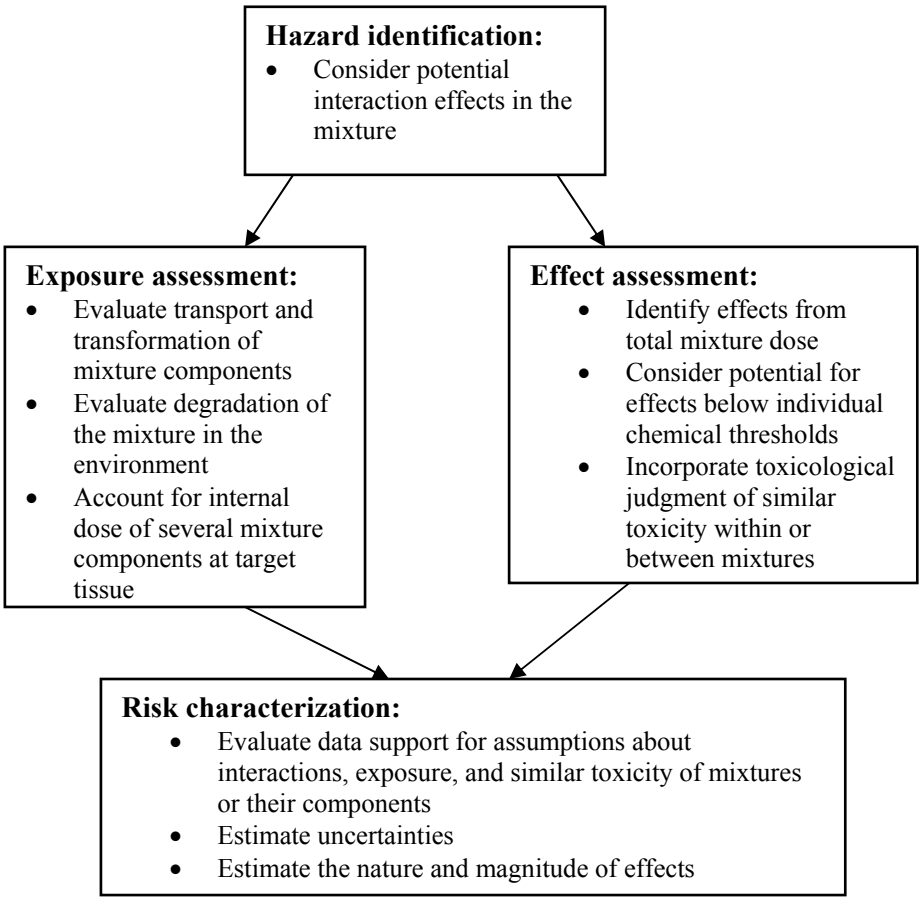

Figure 1. 2. The framework for ecological risk assessment of chemical mixtures [adapted from Van Gestel et al. (2010)].

1.3. The fundamental mixture toxicity concepts

The two reference models commonly used to derive patterns for joint effects of mixtures are the concentration addition (CA) and the independent action (IA) models (Bliss, 1939; Loewe and Muischnek, 1926). Both allow the calculation of expected mixture toxicity on the basis of known individual toxicities of the mixture components. The CA model assumes that the mixed chemicals have the same mode of action (MoA), and consequently can be regarded as dilutions of one another. This conceptual model is defined as a summation of the relative toxicities of the individual components in the mixture (Groten, 2000; Loewe and Muischnek, 1926) and is mathematically defined by:

$$\sum_{n}^{i=1} \frac{C_i}{EC_{xi}} = 1$$

Where C_i gives the concentration (or dose) of the i th component in an n-compound mixture which elicits x% total effect and EC_{xi} denotes the concentration of that substance which provokes x% effect if applied singly. Every fraction C_i / EC_{xi}, also termed a "toxic unit", gives the concentration of a compound in the mixture scaled for its relative potency. In this particular case if the sum of the toxic units equals 1 at a mixture concentration that provokes x% effect, the mixture behaves according to CA. Under these circumstances any mixture component can be exchanged by another chemical without changing the overall mixture toxicity, as long as the size of the concerned toxic unit is constant. Such interchangeability is generally assumed to result from the compounds binding to the same receptor, that is, substances that have a similar mechanism of action, and that do neither interact on a physicochemical level nor in their toxicokinetics and toxicodynamics.

The CA reference model has predicted the effects of mixtures of similar acting compounds in several studies (e.g. Altenburger et al., 2000; Backhaus et al., 2000b; Faust et al., 2001; Junghans et al., 2003; Porsbring et al., 2010). Moreover, CA has been proposed as a possible default model for mixture toxicity assessment schemes due to its predictability power even in mixtures with dissimilar compounds (Syberg et al., 2009).

The alternative model of independent action (IA) is based on the idea of a dissimilar action of mixture components (Bliss, 1939). Under this presumption, this model is usually used if the question asked is whether the probability of toxicity to one chemical is independent from the probability of toxicity exposure to another chemical (Altenburger et al., 2004; Jonker et al., 2004; Jonker et al., 2005). This means that the relative effect of a toxicant remains unchanged in the presence of another chemical. In this case the mathematically formula is expressed as:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(C_i)$$

Where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, q_i (C_i) the probability of non-response, $u_{\max}$ the control response for endpoints and $\prod$ the multiplication function.

This reference model has been able to predict the effects of dissimilar acting compounds in several studies (e.g. Backhaus et al., 2000a; Faust et al., 2003; Jonker et al., 2004; Walter et al., 2002).

Deviations from the models can also be tested. Some compounds can interact synergistically becoming more toxic than expected from the toxicity of single compounds or enhancing the probability of effect of one another. Different compounds may also interact antagonistically,

defined as situations where the mixture toxicity is lower than expected from the toxicities of single compounds or where the compounds decrease the probability of effect to one another. The interaction between different compounds may also depend on the mixture dose level or on the dose ratio (Jonker et al., 2005). The dose level-dependency means that observed deviations from the reference models are different at low dose levels and at high dose levels. The dose ratio-dependency means that the proportion of the compounds in the mixture affects the deviation from the reference model in an asymmetric fashion, with one of the chemicals being the major responsible for the observed deviation (Jonker et al., 2005).

1.4. Mixture toxicity in a real scenario: the study site

The case study of this work was the Alqueva reservoir. This reservoir is the largest in Europe, is located in the Alentejo region, southeast of Portugal and is a strategic water reserve in the Guadiana River Basin. With a surface of 250 km^2, a lakeside of 1,200 km (about 1,000 km on Portuguese territory, the rest in Spain), and a length of about 85 km, the "Albufeira do Alqueva" is today the biggest artificial lake in Europe. This reservoir was planned to be an important water supply for human and agricultural consumption, in an especially dry region (World Wide Fund for Nature, 1995). The Alentejo region (South of Iberian Peninsula) has a typical Mediterranean climate, with hot and dry summers and mild and concentrated rains in winter (Morales, 1993).The annual average temperature ranges from 24°C to 28°C in hot months (July/August) and from 8°C to 11°C in cold months (December/January). The average annual precipitation ranges between 450 and 550 mm (Morales, 1993). The region is affected by intense dry periods without precipitation since almost 80% of the precipitation occurs from October to April. The natural inflows of the Guadiana river vary markedly, within the year and from year to year, as a result of seasonal and annual fluctuations in rainfall (Chícharo et al., 2006).

This region is characterized by a highly heterogeneous and complex landscape structure, dominated by agricultural fields (cereal grounds, orchards, and olive groves), with holm oak (*Quercus ilex*) and cork oak (*Quercus suber*) woodlands interspersed within the agricultural landscape. Few patches of forest plantations (eucalyptus and pines) are also found (Sociedade de Engenharia e Inovação Ambiental, 1995).

Considering this, the Alqueva aquatic system is subjected to several pressures, from natural (temperature and rain fluctuations) to anthropogenic, generated by agricultural practices and/ water treatment plants. Therefore, water quality biomonitorization is crucial due to the potential uses of water in this region by men, but also as a complex ecosystem.

In this case, the biomonitorization was carried out during 2006, with water samples collected every two months, and data on chemical concentration (pesticides and metals) and toxicity using bioassays was generated.

1.5. Test organisms

In this study, three representative species of the freshwater ecosystems were used in the several tests performed, namely the green microalgae *Pseudokirchneriella subcapitata*, the midge *Chironomus riparius* and the zebrafish *Danio rerio* (Early Life Stage).

The microalgae *Pseudokirchneriella subcapitata* is an important species within aquatic trophic chains as primary producer and also as food for invertebrates and fish. It is one of the microalgae species widely distributed and can be found in Portugal, including, in this case, the study location. "Although algae may seem insignificant compared to rainforests or other accumulated biomass on land, algae contribute approximately half of the global primary production as well as atmospheric oxygen" (Aruoja, 2011). Moreover, being easily cultivated in laboratory conditions and sensitive to a wide range of chemicals, they are very useful for the ecotoxicological studies. Results of toxicity tests with algae are part of the basic information required for the evaluation of environmental risk of chemicals as recommended internationally by the OECD and required in legislation such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances), by ECHA (European Chemical Agency) and the EU (Aruoja, 2011). Furthermore, algae are frequently included among the species used in biotest batteries for biomonitoring programs of water quality.

Chironomids (order Diptera, family Chironomidae) are widely distributed in the northern hemisphere and can be found in lentic and lotic environments, where they are key species in decomposition processes and also in trophic chains as prey for fish and others aquatic animals. The chironomid life cycle includes several phases: eggs stage, four larval stages, pupal stage (aquatic stage), and adult stage (terrestrial/aerial phase). The ease with which their life cycles can be distinguished, their short life cycle and their suitability for rearing in the laboratory have made chironomids a popular species for ecotoxicity testing. In addition, they live in intimate contact with sediments, feeding on detritus and algae associated providing an important feeding guild and a potentially sensitive tool for aquatic (bio) monitoring (Hudson and Ciborowski, 1995). Despite the fact that chironomids specie used in this work, *Chironomus riparius*, is a useful tool in the study of sediment toxicity, they can also be used in water toxicity tests (Agra and Soares, 2009; Azevedo-Pereira et al., 2011; Faria et al., 2007).

The zebrafish *Danio rerio* early life stages (embryos and larvae) have also been used as test model in this study. The use of this species holds several advantages in chemical toxicity evaluation, such as its rapid development, easy maintenance in the laboratory, large number of offspring, transparency of embryos and access to experimental manipulation (Scholz et al., 2008). Nagel (2002) proposed the embryo test with *D. rerio* as a substitute test for the acute test using adult fish. Moreover, the use of early life stages in toxicity tests has been recommended as an alternative for the acute tests performed with juveniles or adults, in order to overcome the ethical issues of using large numbers of free-feeding larva, juvenile or adult animals (Lammer et al., 2009). The use of alternative tests have also been incorporated into the REACH regulations through strong advocacy for the reduction of testing with live animals (Lilienblum et al., 2008).

1.6. Thesis aims and structure

The fundamental aim of this work focused on the Ecological Risk Assessment of the pesticides found in higher concentrations in the water of Alqueva reservoir and their binary mixtures. To address this, the thesis is structured in six chapters. In this first chapter, the basic principles and concepts supporting this work are drawn. In chapters two to five the work is detailed in the form of four manuscripts that contributed and provided data to the development and enforcement of the basic objective proposed in this work. Finally, Chapter 6 (Final considerations and conclusions) aims to summarize and integrate all the information that was generated with this work, making an environmental risk characterization of the pesticides found in the Alqueva reservoir. The basic aims of each chapter are described below.

Chapter 2: Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays.

Alqueva Reservoir is the biggest artificial freshwater reservoir in Europe and is an important water supply for human and agricultural consumption in the Iberian Peninsula. Pollution can impair environmental and human health status, and to assure water quality and ecological balance, it is crucial to frequently monitor water supplies. In this study, an ecotoxicological test battery was used to identify the potential toxicity of water samples collected from this reservoir. Moreover, in parallel, a physicochemical characterization was also carried out (including total pesticides and metal concentrations) in order to know which contaminants were present and their levels in water to relate it to possible effects on the organisms tested.

This study was published in the journal Environmental Science and Pollution Research (http://dx.doi.org/10.1007/s11356-009-0174-9).
Pérez J.R., Loureiro S., Menezes S., Palma P., Fernandes R.M., Barbosa I.R., Soares A.M.V.M.

(2010) Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays. Environmental Science and Pollution Research 17, 3, 688-702.

Chapter 3: Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures.

Regarding the first results on the Alqueva reservoir, where ecotoxicity of water samples was found due to the presence of the herbicides such as atrazine, simazine, terbuthylazine and metolachlor. In this work a component-based or bottom-up approach study was performed to examine the effects of these herbicides singly and as binary mixtures on the growth rate of the microalgae *Pseudokirchneriella subcapitata*. In this study CA model was selected to evaluate the joint effects of *s*-triazine herbicides on the growth of algae due to their similar mode of action. And, IA reference model was chosen to evaluate the joint toxicity of the chloroacetanilide metolachlor and the *s*-triazine herbicides due to their different mode of action.

This study was published in the journal Ecotoxicology (http://dx.doi.org/10.1007/s10646-011-0661-x).

Perez J., Domingues I., Soares A.M.V.M., Loureiro S. (2011) Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures. Ecotoxicology 20, 6, 1167-1175.

Chapter 4: Cholinesterases' characterization in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity.

The insecticide chlorpyrifos was also previously detected in the water of the Alqueva reservoir. This organophosphate insecticide affects target species by inhibiting acetylcholinesterase (AChE) activity and affecting neuromuscular functions. In this study the toxicity of chlorpyrifos in binary combination with the herbicides atrazine, terbuthylazine and metolachlor was assessed on the swimming behaviour response and AChE activity of *Chironomus riparius* midge (Diptera: Chironomidae). To achieve this objective, a prior biochemical characterization of cholinesterases presents in whole body homogenate of *C. riparius* was performed, using selective inhibitors and different substrates.

This study was accepted in the journal Aquatic Toxicology.

Chapter 5: Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early life stages of the zebrafish *Danio rerio*.

The primary objective of this study was to evaluate the impact of the *s*-triazine herbicides atrazine and terbuthylazine on chlorpyrifos toxicity in early life stages (embryos and larvae) of the zebrafish *Danio rerio* (Hamilton-Buchanan 1822). For that we have first studied the individual effects of the three pesticides and derive the most sensitive endpoint, to be used on the mixture toxicity approach. Additionally, and as the most sensitive endpoint showed to be swimming behaviour, we aimed at examining the variation in AChE activity when organisms were exposed to chlorpyrifos in combination with the herbicides. This component-based approach study was also undertaken in order to provide information about the interactions of pesticide mixtures previously found in the Alqueva reservoir.

This study was published in the journal Environmental Science and Pollution Research (http://dx.doi.org/10.1007/s11356-012-1443-6).

Pérez J., Domingues I., Monteiro M., Soares A.M.V.M., Loureiro S. (2013) Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early life stages of the zebrafish *Danio rerio*. Environmental Science and Pollution Research 20, 7, 4671-4680.

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Chapter 2

Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays

Pérez J.R., Loureiro S., Menezes S., Palma P., Fernandes R.M., Barbosa I.R., Soares A.M.V.M. (2010) Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays. Environmental Science and Pollution Research 17 (3): 688-702.

2. Assessment of water quality in the Alqueva Reservoir (Portugal) using bioassays

Abstract

Background, aim, and scope Alqueva Reservoir is the biggest artificial freshwater reservoir in Europe and is an important water supply for human and agricultural consumption in the Alentejo region (Portugal). Pollution can impair environmental and human health status, and to assure water quality and ecological balance, it is crucial to frequently monitor water supplies. In this study, we used an ecotoxicological test battery to identify the potential toxicity of water from this reservoir.

Materials and methods Water samples from the Alqueva aquatic system were collected bimonthly in 2006 from 11 different water points within the reservoir. Several bioassays were carried out: a 72-h growth test with *Pseudokirchneriella subcapitata*, a 6-day growth test with *Chironomus riparius* larvae, and the luminescence inhibition test with *Vibrio fischeri* (Microtox®).

Results and discussion Algae growth was significantly inhibited in several sampling points and periods throughout the year, mainly due to the presence of pesticides. Although in some sampling points pesticide concentrations (single and sum) were still below the maximum permissible concentrations, water samples showed high toxicities to algae, especially during the summer months. In addition, several sampling points showed pesticide concentrations above the permissible level which can pose a significant risk to humans and the environment. Chironomids showed less sensitivity to the water samples, possibly due to the low concentrations of insecticides present. *V. fischeri* showed no sensitivity when exposed to all the water samples collected throughout the year of 2006.

Conclusions Standardized laboratory bioassays can be useful tools to assess water quality from aquatic systems and can valuably complement chemical analysis evaluation. The results obtained in this study demonstrated that the most sensitive species used in this test battery was the microalgae *P. subcapitata*. The growth of *C. riparius* was less affected, which is probably due to the fact that low insecticide concentrations were measured and, furthermore, since this species lives in the sediment and not in the water column and is, therefore, usually more resistant to pollutants.

Recommendations and perspectives On its own, chemical analysis is not enough to derive conclusions on the water quality and/or status, which can be valuably complemented by laboratory bioassays. Single chemical, maximum permissible values and the sum of pesticide concentrations do not take into account possible patterns of synergism, antagonism, dose level dependencies, or even the dominance of several chemicals within a mixture. In addition, several species from

different levels in trophic chains are recommended due to differences in species' sensitivities to chemical compounds that are present.

Keywords: Algae, Biomonitorization, Chironomidae, Metallic elements, Microtox and Pesticides

2.1. Background, aim, and scope

Nowadays, the increased need of higher water supplies for agriculture or human consumption has lead to the construction of artificial lakes or dams. Some of them are big water bodies located near agricultural fields and receive their runoffs, which can become both an environmental and human problem.

One of the major sources of water pollution is agriculture practices which create an input of pesticides and nutrients to freshwater ecosystems. In addition to contamination problems, nutrients can also contribute to eutrophication phenomena, which can add other stress factors to the pesticide cocktails (e.g., decrease in oxygen dissolved in water, bacterial growth).

Several legislations have been implemented in Europe to protect water status within river basins, taking into consideration the vulnerability and equilibrium of aquatic ecosystems [e.g., Directive 2000/60/EC of the European Parliament and of the Council (23 October 2000), establishing a framework for Community action in the field of water policy; Council Directive 98/83/EC (3 November 1998) on the quality of water intended for human consumption]. For artificial water bodies, these directives advise the use and evaluation of quality elements applicable to other natural surface water categories which include biological elements, chemical and physicochemical elements supporting the biological elements, specific pollutants, among others. For the setting of chemical and environmental quality criteria, they also advise the use of standards which include the collection of acute and chronic data on several aquatic taxa (e.g., daphnids, algae, and fish).

To fulfill these requirements, biomonitorization processes should be implemented and applied to natural and artificial aquatic ecosystems with the aim of maintaining better water quality and sustainability.

Chemical analysis on its own is usually a bad predictor of stressor effects in organisms because it does not take into account the interaction between chemicals, the heterogeneity of the environment, chemical bioavailability, and behaviour and life style/stage of organisms. On the other hand, bioassays can complement chemical analysis by providing more accurate information on the effects produced by the stressors' exposure.

Bioassays have been used as biomonitoring tools to provide an integrated picture of overall toxicity in real contaminated scenarios, with the aim of obtaining a realistic prediction of the behaviour of substances in the environment.

One single bioassay is not able to provide a full picture of the quality of the environment, so a representative, cost effective, and quantitative test battery should be applied in biomonitorization processes (Bierkens et al. 1998; Isooma and Lilius 1995). They should include organisms of several trophic levels, like bacteria, protozoan, algae, invertebrates, and fish (Repetto et al. 2001).

In this work, we used an ecotoxicological test battery to identify the potential toxicity of the water of Alqueva Reservoir (southeast of Portugal) as a case study and example of a real scenario. The Alqueva Reservoir is the biggest artificial freshwater reservoir in Europe and is an important water supply for human and agricultural consumption in the Iberian Peninsula. It is located near agricultural fields and it is, therefore, questioned whether the inherent runoffs are impairing the aquatic environment. To answer this general question, three species were chosen to be used in the biomonitoring process: the green algae *Pseudokirchneriella subcapitata*, the midge *Chironomus riparius*, and the luminescent marine bacterium *Vibrio fischeri*. *C. riparius* (Chironomidae) is widely distributed in the northern hemisphere and can be found in lentic and lotic environments, including this aquatic system, where they are key species in decomposition processes and also in trophic chains as prey for fish and birds. Additionally, this species has also been used as a monitoring species in Portuguese aquatic systems (Faria et al. 2008). The algae *P. subcapitata* is also an important species within aquatic trophic chains as primary producer and also as food for invertebrates and fish. It is one of the microalgae species widely distributed and can be found in Portugal, including, in this case, the study location. The *V. fischeri* kit was used due to its simplicity, sensitivity, and low cost as an ecotoxicological bioassay.

In parallel, a physicochemical characterization was also carried out (including total pesticides and metal concentrations) in order to know which contaminants were present and their levels in water and to relate it to possible effects on the organisms tested.

This monitoring process was designed to provide a coherent and comprehensive overview of the ecological and chemical status in the Alqueva Reservoir during the year 2006 and can be used as a foundation for future biomonitorization procedures.

2.2. Materials and methods

2.2.1. Study site, water sampling, and physicochemical parameters

The Alqueva dam is a reservoir located at the Guadiana river in the Alentejo region, southeast of Portugal (Figure. 2.1). With a surface of 250 km^2, a lakeside of 1,200 km (about 1,000 km on Portuguese territory, the rest in Spain), and a length of about 85 km, the "Albufeira do Alqueva" is today the biggest artificial lake in Europe.

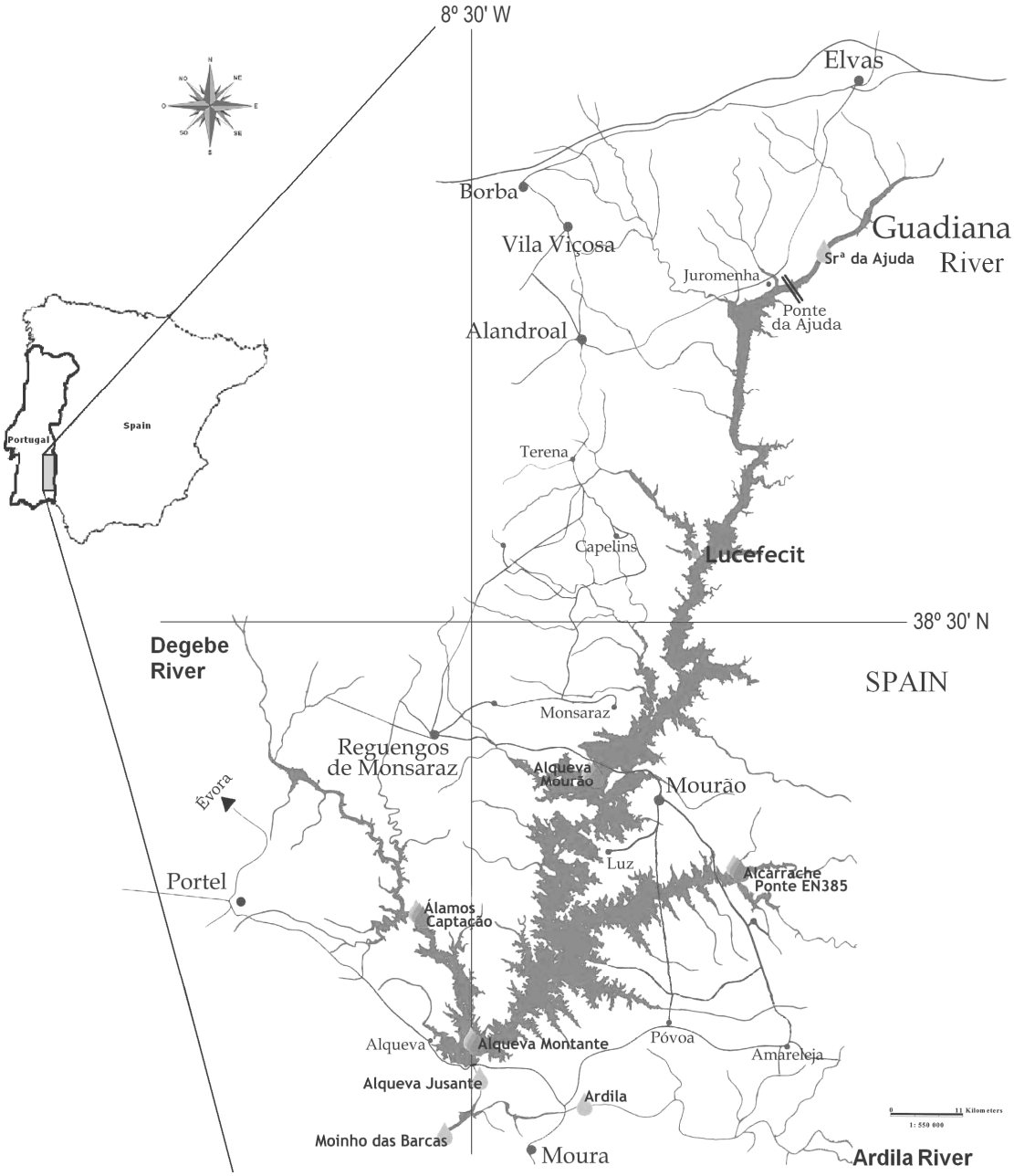

Figure 2. 1. . Map of the study area with the sampling locations in the Alqueva Reservoir, Portugal adapted from (Palma et al. 2009)

Water samples were collected bimonthly from 11 different water points in this aquatic system (see Figure. 2.1). In each sampling point, 2 L of water were collected for general chemical analysis (physicochemical characterization and metals) and 1 L for pesticide analysis. In this last case, brown glass bottles were used to store water samples collected using a Van Dorn bottle at 50 cm depth.

Samples were collected upstream of the reservoir [Sra Ajuda (38°46′54″ N, 07°10′38″ W); Alcarreche (38°19′56″ N, 07°16′25″ W); Álamos (38°20′30″ N, 07°34′41″ W)], in the middle of the aquatic system [Montante (38°12′55″ N, 07°29′ 28″ W); Mourão (38°23′69″ N, 07°23′15″ W); Lucefécit (38°37′49″ N, 07°26′1.6″ W)], and downstream [Jusante (38°11′69″ N, 07°29′64″ W); Ardila (38°10′03″ N, 07°25′05″ W); Moinho das Barcas (38°09′47″ N, 07°30′21″ W)] (see Figure. 2.1). In addition, two extra points outside the Alqueva aquatic system were also monitored in Odivelas Reservoir (38°11′6″ N, 8°6′54.5″ W) because this reservoir also receives a water input from the Alqueva dam.

A pooled sample was collected in each site in February, March, May, July, September, and November of 2006. The samples were collected in plastic containers and were kept at 4°C in a refrigerator during transport until reception in the laboratory. Then, the samples were stored at −20°C until processing. For the algae bioassay, water was additionally filtered through a cellulose nitrate filter paper (Albet®; 0.2μm porosity; 47 mm diameter).

Physicochemical parameters like dissolved oxygen, temperature, pH, conductivity, and redox potential were measured in situ using portable measurement devices (Jenway® 970, Jenway® 370, and Jenway® 470, respectively). Biological oxygen demand (BOD) and chemical oxygen demand (COD) were measured by the manometric method and potassium dichromate method, respectively, as described in Clesceri et al. (1998). Chemical parameters like nitrates, nitrites, and total phosphorus were measured by ultraviolet-visible spectrophotometry.

Several chemical compounds were analysed in the water samples using chromatographic methods. For the pesticides atrazine, simazine, terbuthylazine, metolachlor, chlorpyrifos (CPF), and endosulfan sulfate, analysis was performed using gas chromatography–mass spectrometry. Metallic elements were analysed by graphite furnace atomic absorption spectrometry (Pb, Cr, Ni, As, Fe, and Mn).

2.2.2. Test organisms

The microalgae *P. subcapitata* is currently recommended as a standard species for algal toxicity tests (OECD 2006). It was obtained from nonaxenic batch cultures with Woods Hole MBL medium at 20±2°C and with a 16:8-h light/dark photoperiod.

For the maintenance of the laboratory cultures and the start of new cultures, algae were harvested while still in the exponential growth phase (5–7 days old) and inoculated in fresh medium.

The midge *C. riparius* was obtained from a controlled laboratory culture where the whole life cycle of the chironomids was taking place (OECD 2004). Cultures were maintained at 20±2°C with a 14:10-h light/dark photoperiod. At the start of a new culture, approximately 30 first-instar larvae (3–4 days post hatch) were introduced into a new glass beaker containing ASTM hard water (ASTM 1980) and sea sand. An ad libitum suspension of TetraMin® was them added as a single food source. Each beaker was also aerated. Seven days later, larvae were either used in test or transferred to new culture beakers with fresh media until emergence occurred. Adults were fed on a sucrose solution wetted paper placed inside the culture unit. Fresh laid egg masses were transferred into new beakers for a 3- to 4-day period until hatching occurs. The new-born larvae (first instars) were then used to start a new culture.

The marine bacteria *V. fischeri* was purchased from Azur Environmental (Carlsbad, CA, USA), reconstituted with a sodium chloride solution (NaCl, 0.01%), and then used in the Microtox® apparatus.

2.2.3. Bioassays: experimental procedure

In this work, we used an ecotoxicological test battery to identify the potential toxicity of water in this region. Several bioassays were carried out: a 6-day growth test with *C. riparius* larvae, a 72-h growth test with *P. subcapitata*, and the luminescence inhibition test with *V. fischeri* (Microtox®).

2.2.3.1. Growth inhibition test with Chironomus riparius larvae

In this bioassay, second-instar larvae of *C. riparius* were used. They were obtained from laboratory cultures established at the Biology Department, University of Aveiro.

Initially, it was made as a pre-exposure of 50 mg of sediment (sea sand) to 100 mL of water samples for 7 days in glass flasks (volume of 200 mL) using five replicates per water sample. A negative control of ASTM medium was added to the experience, also with five replicates (ASTM 1980).

After this 7-day period, second-instar larvae were exposed to aerated water samples and to the sediment for 6 days at 20°C and 16:8-h light/dark photoperiod and fed every 2 days with TetraMin® fish food (Tetrawerke, Germany). During the experience period, several physicochemical parameters were measured: temperature, pH, dissolved oxygen, and conductivity. At the end of the test, the survival was determined and the length of living organisms was measured using a stereomicroscope (Leica MS5, Leica Microsystems, Germany) fitted with a calibrated eyepiece micrometer.

Organism growth rate was then calculated using the equation:

$$Gr = \frac{(L_f - L_i)}{L_i}$$

Where Gr is the growth rate, L_f is the organism's length after 6 days of exposure, and L_i is the organism's length at the beginning of the test.

2.2.3.2. Growth inhibition test with Pseudokirchneriella subcapitata algae

The growth inhibition test with the unicellular algae *P. subcapitata* has been used to evaluate the bioavailability and toxicity of water-borne contaminants. In this study, the microalgae was exposed to the test medium (water samples) for 72 h and the algae growth was determined based on protocol 201 from OECD (2006). The MBL medium was used as a control (Carvalho et al. 1995). In the beginning of the test, *P. subcapitata* was exposed to the water samples (40 mL) in an Erlenmeyer flask with a capacity of 100 mL and covered with permeable gauze using three replicates per water sample. Test vials were randomly incubated in an orbital shaker for 72 h at 21±2°C and with a constant luminous intensity (60–120μE/m^2/s, equivalent to 6,000–10,000 lx).

The initial concentration of algae was of 3×10^4 cells per milliliter, and after 24, 48, and 72 h, the algae concentration was measured by spectrometry (440 nm; Jenway, 6505 uv/vis spectrophotometer), using the equation:

$$C = -17107.5 + ABS \times 7925350$$

Where C is the algae concentration (cells per milliliter) and ABS is the absorbance obtained at 440 nm.

The average specific growth rate for a specific period was calculated as the logarithmic increase in biomass after 72 h from the equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i}$$

Where μ_{i-j} is the average specific growth rate from time i to j, ti is the time for the start of the exposure period, t_j is the time for the end of the exposure period, B_i is the biomass concentration at time i, and B_j is the biomass concentration at time j.

The inhibition of algal growth was estimated as percentage of reduction of growth rate with respect to the control:

$$\%I = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

Where %*I* is the mean percentage of inhibition for specific growth rate, μ_c is the mean value for the growth rate in the control, and μ_t is the mean value for the growth rate in the water samples.

2.2.3.3. Luminescence inhibition test with Vibrio fischeri bacterium (Microtox®)

The Microtox® system is a bioassay to test acute toxicity in environmental samples and pure compounds based on the natural bioluminescence of the marine bacteria *V. fischeri*. The test principle considers that the bacteria luminescence is reduced when exposed to stressors and the toxicity is expressed as the concentration of the sample or of the specific agents which produces a 50% reduction of the initial luminescence.

In this study, the basic test was used. Water samples were pipetted into glass cuvettes and the salinity was adjusted with MOAS (Azur Environmental, Carlsbad, CA, USA); a series of dilutions were made as indicated in the protocol of the manufacturer (MicrobicsCorporation 1992). Five and 15 min after transferring the bacteria to the vials, water sample toxicity was evaluated and a 50% reduction luminescence was computed using Microtox Data Collection and Reduction software (MicrobicsCorporation 1992) and reported as the percentage of inhibition.

Simultaneously, it was performed as a positive control test using a solution of zinc sulfate (ZnSO4) to validate the results.

2.2.4. Statistics

One-way analysis of variance (ANOVA) using the SigmaStat statistical package (Systat 2006) was used to test for statistical differences between the 11 sampling points and the control for the algae

and chironomids test. Whenever significant differences between them were found, the post hoc multiple comparison Dunnett's method was performed (Zar 1996). Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on ranks was performed (Zar 1996), followed by a Dunnett's or Dunn's method when significant differences were found.

Multivariate statistical analysis has been used in this study as a powerful tool to investigate the main environmental parameters/characteristics that account for the variability among different sampling places, giving an easy summarization of the data and facilitating its comprehension and communication (Leps and Smilauer 2003). Principal component analysis (PCA), a linear ordination technique, was performed to help in summarizing the differences between subareas, emphasizing the importance of the different environmental variables for those differences. The relationship between algae and chironomids growth rates and environment variables were also investigated through an ordination technique, the redundancy analysis (RDA), which ordinates response data in which the axes are constrained to be linear combinations of environmental variables; this technique presents the advantage of being applied to any number of species and sampling sites needed. These analyses were developed using the CANOCO software (version 4.5; terBraak and Smilauer 2002).

2.3. Results

Data on pesticide, metallic elements, and other physicochemical properties are shown in Tables 2.1, 2.2, and 2.3, respectively.

The growth inhibition test with *P. subcapitata* showed to be the most sensitive bioassay when testing the water samples tested in this study. All the monitored places showed a growth inhibition of the microalga after 72 h of exposure (Table 2.4).

In February, a significant decrease of algae growth was observed in Alcarreche and Lucefecit (Kruskal–Wallis one-way ANOVA on ranks, H=32.66, df=11, p<0.05). Although no significant differences were found in any sampling sites, with the exception of Ajuda, Ardila, and Odivelas 2, an inhibition in algae growth higher than 50% was observed.

In March, all the sampling sites showed a significant decrease in the algae growth rate (one-way ANOVA, $F_{11,24}$=93.60, p<0.05). This inhibition was more pronounced (>50%) in Alcarreche, Álamos, Montante, Mourão, and Lucefecit.

In May, the algae growth pattern was similar from the one in February, with a significant decrease in algae growth in all sampling sites, compared with the MBL control (one-way ANOVA, $F_{11,24}$=329.17, p<0.05; Dunnett's method, p<0.05; Figure. 2.2), with the exception of Ajuda, Ardila, and Odivelas 2. In July, all sampling points, with the exception of Ardila, showed algae growth inhibitions higher than 50% (one-way ANOVA, $F_{11,24}$=121.17, p<0.05; Dunnett's method, p<0.05; see Figure. 2.2).

Table 2. 1. Pesticide concentration (in micrograms per liter) per sample collected in each point and time in the Alqueva aquatic system

Pesticides (µg/L)	season	location										
		Ajuda	Alcarreche	Álamos	Montante	Mourão	Lucefécit	Jusante	Ardila	Moinho das Barcas	Odivelas I	Odivelas II
Atrazine	Februar	0.07	0.02	0.30	0.06	0.04	<LOQ	0.06	0.02	0.04	0.09	0.06
	March	<LOQ	0.03	0.50	<LOQ	0.04	<LOQ	0.03	0.02	0.03	0.07	<LOQ
	May	0.64	1.40	0.50	0.70	1.30	1.30	<LOQ	0.10	1.50	<LOQ	0.09
	July	2.60	1.96	0.45	0.46	0.53	1.15	0.53	0.22	1.50	<LOQ	<LOQ
	September	0.16	0.15	0.26	0.07	0.23	0.17	0.24	0.26	0.11	0.14	<LOQ
	November	0.02	0.03	0.03	<LOQ	0.04	0.02	0.03	0.04	0.02	0.01	0.02
Simazine	February	0.35	0.02	0.20	0.03	0.03	0.02	<LOQ	<LOQ	0.02	0.01	0.03
	March	<LOQ	0.02	0.30	<LOQ	0.04	0.03	<LOQ	<LOQ	0.03	0.03	<LOQ
	May	0.38	0.3	1.80	1.05	0.32	0.32	0.15	<LOQ	0.26	0.07	0.14
	July	<LOQ	0.03	0.05	0.05	0.06	0.05	0.05	0.03	0.05	0.27	0.05
	September	0.62	0.33	0.61	0.55	0.39	0.03	0.08	1.05	0.98	1.17	<LOQ
	November	0.41	0.02	0.02	0.02	0.02	0.01	0.01	0.07	0.03	0.05	0.04
Terbutilazyne	February	0.03	0.02	0.20	0.03	0.02	0.02	0.03	<LOQ	<LOQ	<LOQ	<LOQ
	March	<LOQ	0.02	0.30	0.03	0.01	0.02	0.03	<LOQ	<LOQ	<LOQ	<LOQ
	May	<LOQ	<LOQ	0.50	0.02	0.02	0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	July	<LOQ	0.02	0.04	0.03	0.04	0.04	0.03	0.02	0.02	0.2	0.03
	September	0.45	<LOQ	0.67	0.35	0.55	0.42	0.53	0.34	0.52	0.6	<LOQ
	November	0.03	<LOQ	0.05	0.01	0.05	0.04	0.04	0.02	0.02	0.03	0.02
Metolachlor	February	0.08	0.02	0.10	<LOQ	0.05	<LOQ	0.03	0.02	<LOQ	0.02	<LOQ
	March	<LOQ	0.01	0.00	<LOQ	0.03	<LOQ	<LOQ	0.01	<LOQ	<LOQ	<LOQ
	May	<LOQ	<LOQ	0.19	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	July	<LOQ	0.06	0.00	<LOQ	<LOQ	0.04	0.05	<LOQ	0.04	0.03	<LOQ
	September	0.09	<LOQ	0.22	0.17	0.12	0.12	0.12	0.14	0.18	0.06	<LOQ
	November	0.16	<LOQ	0.03	0.02	0.07	0.08	0.02	0.01	0.05	0.01	0.01
Chlorpyriphos	February	0.03	0.18	0.03	0.1	0.06	0.05	0.02	0.02	0.1	0.04	0.05
	March	<LOQ	0.15	0.01	0.02	0.05	0.04	0.02	0.03	0.07	0.03	<LOQ
	May	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	July	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	September	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	November	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Endosulfan sulfate	February	0.02	0	<LOQ	0.04	0.02	<LOQ	<LOQ	0.02	<LOQ	0.03	0.03
	March	<LOQ	0	<LOQ	0.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.01	<LOQ
	May	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.78	<LOQ
	July	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	September	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	November	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

In September, there was a decrease in the algae growth similar to what happened in July for all water samples (see Figure. 2.2). In this month, it was not possible to test the water from Odivelas 2 because there was no water available to be collected due to the intensive drought observed in the region on this period.

In November, it was observed that algae exposed to water samples showed an inhibition on their growth lower than 35%, with the exception of the samples from Alcarreche, Montante, and Odivelas 1.

Table 2. 2. Metallic elements concentration (in micrograms per liter and milligrams per liter) per sample collected in each point and time in the Alqueva aquatic system.

Metallic elements (mg/L)	Season	Location										
		Ajuda	Alcarreche	Álamos	Montante	Mourão	Lucefecit	Jusante	Ardila	Moinho das Barcas	Odivelas I	Odivelas II
Pb	February	0	0	0	0	0	0	0	0	0	0	0
	March	3.00	0.15	0.52	0.42	0.00	1.36	1.45	2.31	6.16	0.91	4.58
	May	0.33	0.78	1.45	2.89	0.85	8.41	0.62	2.23	1.37	0.00	4.65
	July	1.87	1.71	3.32	2.11	2.13	1.35	1.29	3.00	1.58	3.03	2.19
	September	2.19	0.00	1.34	0.00	1.34	0.00	0.00	1.41	0.00	0.00	0.00
	November	4.29	0.40	0.93	1.52	1.09	1.78	1.40	0.98	2.37	1.43	0.53
Cr	February	2.12	0.00	0.00	0.00	0.91	0.00	0.00	0.79	0.00	0.56	2.20
	March	4.39	1.08	1.02	1.84	0.90	0.87	1.83	2.50	1.29	1.52	7.89
	May	1.18	0.78	1.12	1.23	0.92	0.84	0.91	1.36	0.89	0.85	5.03
	July	2.04	0.78	2.27	1.54	2.09	1.59	1.42	4.84	1.47	3.24	2.84
	September	1.79	1.38	1.47	1.99	1.31	0.10	1.24	1.42	1.36	1.54	0.00
	November	8.00	1.08	1.91	6.27	0.76	3.74	0.00	0.94	1.83	1.42	6.06
Ni	February	1.92	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.00	4.92	1.09
	March	2.69	0.00	1.72	0.80	0.63	0.84	0.00	1.94	0.00	0.69	8.92
	May	1.20	0.00	1.50	0.00	0.00	0.00	0.00	0.81	1.48	0.00	2.49
	July	2.52	4.36	3.74	1.64	3.16	2.06	1.87	3.66	2.16	4.64	3.71
	September	1.37	0.99	2.21	1.64	1.62	0.00	1.97	1.99	1.81	1.88	0.00
	November	13.67	0.66	1.51	0.69	1.88	4.80	2.74	1.12	4.53	1.27	8.00
As	February	5.63	1.20	1.59	2.51	3.43	2.05	2.56	2.36	2.51	1.55	5.80
	March	7.17	3.44	2.24	3.65	3.93	5.62	4.52	7.10	5.18	2.32	7.44
	May	7.20	2.02	1.25	2.16	1.73	4.16	3.68	9.08	3.71	1.98	11.76
	July	6.74	1.59	0.96	1.14	1.79	5.12	2.19	3.81	2.55	1.93	7.79
	September	1.87	3.74	3.11	0.98	2.52	1.41	1.21	3.03	1.51	4.02	0.00
	November	0.00	1.70	3.40	2.24	3.90	4.82	3.55	3.28	3.89	2.41	4.92
Fe	February	0.90	0.04	0.04	0.03	0.07	0.06	0.04	0.47	0.12	0.21	1.03
	March	1.78	0.06	0.07	0.05	0.05	0.15	0.13	0.65	0.08	0.50	5.46
	May	0.29	0.05	0.07	0.05	0.08	0.11	0.11	0.29	0.07	0.09	2.16
	July	0.28	0.14	0.11	0.00	0.10	0.00	0.00	0.15	0.06	5.06	0.98
	September	0.43	0.10	0.07	0.07	0.07	0.00	0.07	0.10	0.00	0.19	0.00
	November	4.00	0.06	0.90	0.07	0.08	1.95	0.67	0.30	0.86	0.30	0.58
Mn	February	1.10	0.00	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.00	2.98
	March	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	May	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.47
	July	0.02	0.10	0.02	0.01	0.06	0.02	0.00	0.01	0.03	0.01	0.01
	September	0.07	0.03	0.04	0.00	0.02	0.03	0.02	0.10	0.02	0.07	0.00
	November	0.15	0.01	0.03	0.01	0.02	0.08	0.30	0.12	0.18	0.01	0.17

In all water samples tested, mortality rates for *C. riparius* were not higher than 10%. Compared with the negative control, there were no observed differences on the size of larvae exposed to the water samples collected in February, July, and November (Figure. 2.3). In September, there was an increase on the growth of larvae exposed to water samples from Odivelas 1, Jusante, and Lucefecit (Kruskal–Wallis one-way ANOVA on ranks, H=35.342, df=10; Dunn's method, p<0.05).

In March, there was a significant decrease on the larvae size exposed to water samples collected in Montante, Mourão, and Alcarreche (Kruskal–Wallis one-way ANOVA on ranks, H=32.24, df=12,

$p<0.05$). In May, a decrease was observed in larvae development exposed to the samples of Alamos and Odivelas 1.

Table 2. 3. Physicochemical parameters collected in each point and time in the Alqueva aquatic system.

Parameter	season	location										
		Ajuda	Alcarreche	Álamos	Montante	Mourão	Lucefecit	Jusante	Ardila	Moinhodas Barcas	Odivelas I	Odivelas II
pH	February	9.29	8.16	7.79	7.9	8.03	8.13	7.86	7.94	7.95	8.2	7.9
	March	7.57	8.15	8.07	8.16	8.07	8.15	8.3	8.85	8.55	8	7.6
	May	6.92	8.07	8.79	8.2	8.26	8.72	8.63	8.44	8.62	8.3	8
	July	6.9	7.95	7.92	8.19	8.06	8.21	8.08	7.62	8.03	7.9	7.55
	September	8.67	8.93	8.36	8.75	9.4	9.36	8.76	8.76	8.76	8.3	0
	November	6.57	6.92	7.15	8.02	7.15	6.7	7.5	8.12	7.14	7	7
Temperature (ºC)	February	8.8	10.5	10.31	10.06	10.21	10	10.44	10.15	10.41	14	15
	March	11.3	12.12	10.83	11.17	11.14	11.67	11.72	12.16	12.31	14	13
	May	21.08	21.39	21.88	21.62	20.1	21.13	21.9	23.18	22.53	22	20.4
	July	27.90	27.75	28.05	27.43	27.3	27.41	26.75	26.81	27.54	25.3	24.8
	September	27	27.4	25.66	26.57	28.1	26.6	27.61	29.04	27.46	28.2	0
	November	15.4	19.2	18.3	18.7	19	17.5	17.9	17.6	18	19	19.3
Dissolved oxygen (%)	February	99.8	82	64.8	71.7	74.7	82.5	67.6	84.2	71.7	99.8	82.5
	March	66.7	88.7	75.4	84.1	84.6	88.8	86.7	92.5	104.4	66.7	82.5
	May	42.7	78.6	90.8	87.8	88.9	113.2	127	125.8	127.1	66.7	98.2
	July	38.9	93.4	95.9	91.5	95.3	83.9	100.2	55.2	99.1	96.2	100.2
	September	73.2	110.5	81.3	94.2	167.3	202	130.6	130.6	114.7	99.3	0
	November	112.2	157	71.4	105.8	75	64.5	63	158	70	71	80
Redox	February	204	219	38	128	216	220	104	112	93	230	150
	March	205	183	100	136	160	198	170	163	164	280	230
	May	358	265	12	-15	248	294	20	22	69	210	290
	July	-97	369	75	-24	-32	-23	22	29	38	180	280
	September	151	138	149	190	134	164	184	184	147	290	0
	November	200	243	218	222	218	275	210	188	220	222	225
Conductivity (µS cm^{-1})	February	676	493	462	498	506	546	493	385	503	431	581
	March	368	492	475	485	513	547	501	522	490	405	275
	May	570	493	437	495	514	526	487	501	482	453	638
	July	610	517	508	507	525	520	530	523	529	400	543
	September	580	518	518	522	499	501	498	498	598	457	0
	November	416	482	354	492	508	286	349	354	330	432	511
BOD (mg O_2/L)	February	17	0	0	9	0	0	1	4	0	6.5	9.5
	March	8	0.5	1.5	0.5	1.5	1.5	5	8.5	9.75	1	4.5
	May	7.5	1.5	5	4	1.5	5	5.5	7.5	6	5	5
	July	15	7.5	7	7	8.5	10	7	13.5	6.5	7	11

	September	19.5	8.5	10.5	7.5	11	15.5	10	13.5	10	4.5	0
	November	4	2	3	1	4	4	3	6	2.5	3	2
COD (mg O_2/L)	February	60.83	4.67	28.27	28.70	38.20	17.00	51.43	34.00	34.43	133.07	57.77
	March	114.67	38.40	19.53	38.57	9.13	57.57	66.00	95.13	38.17	19.23	53.33
	May	57.17	51.37	28.00	38.23	72.27	57.07	19.30	57.27	8.27	20.25	57.85
	July	37.91	50.21	47.21	16.67	30.33	28.44	18.26	101.68	51.71	20.92	58.45
	September	20.74	8.89	0.00	17.94	8.89	17.30	26.92	44.86	41.87	4.32	0.00
	November	30.55	17.39	31.20	6.03	10.69	13.27	23.24	28.56	24.59	38.35	74.91
	February	1.62	0.11	0.45	0.23	0.48	0.74	0.19	0.80	0.18	0.17	0.80
	March	3.98	0.00	0.35	0.00	0.11	3.33	0.55	1.19	0.86	1.51	2.10
	May	4.73	0.00	0.00	0.00	0.39	1.63	0.00	0.13	0.00	1.16	0.72
Nitrates (mg/L)	July	2.44	0.41	0.42	0.49	0.26	0.56	0.26	1.31	0.79	1.00	5.75
	September	0.82	0.06	0.02	0.05	0.11	0.14	0.17	1.79	0.31	0.39	0.00
	November	11.26	0.67	2.79	0.34	2.82	7.36	9.09	9.33	6.33	1.19	5.97
	February	0.30	0.03	0.04	0.01	0.04	0.14	0.02	0.17	0.06	0.12	0.25
	March	0.35	0.02	0.02	0.01	0.04	0.12	0.05	0.13	0.07	0.08	0.15
Nitrites (mg/L)	May	0.00	0.17	0.25	0.16	0.33	0.87	0.33	0.43	0.64	0.35	0.32
	July	0.11	0.01	0.01	0.01	0.01	0.05	0.01	0.00	0.01	0.00	0.00
	September	0.04	0.03	0.01	0.01	0.00	0.00	0.02	0.00	0.02	0.01	0.00
	November	0.18	0.03	0.04	0.02	0.11	0.27	0.24	0.25	0.42	0.13	0.27
	February	951.79	51.27	78.81	104.66	104.18	78.91	66.62	1124.22	238.03	79.60	714.52
	March	1101.39	51.00	63.04	51.00	62.63	63.36	167.68	925.11	205.37	74.57	1244.43
Phosphorous (µg/L)	May	1777.30	25.93	77.13	26.96	26.22	447.39	102.37	968.97	205.25	64.07	625.63
	July	329.38	33.35	37.40	27.86	40.13	112.78	47.14	527.71	58.40	173.09	338.92
	September	336.25	25.19	49.04	10.88	11.33	48.09	17.56	93.89	22.33	19.47	0.00
	November	883.94	62.21	328.61	27.48	141.98	500.75	455.33	677.84	420.22	159.16	239.31

BOD- biological oxygen demand; COD- chemical oxygen demand

Table 2. 4. Algae growth inhibition (in percentage, compared with the MBL control) of all samples collected throughout the year of 2006 in all sampling points.

Samples	February	March	May	July	September	November
Ajuda	17.29	23.74	9.73	56.28[a]	43.47	11.02
(st. error)	0.43	3.7	0.18	2.82	1.04	0.55
Alcarreche	70.27[a]	70.82[a]	68.63[a]	100[a]	85.23[a]	62.34[a]
(st. error)	0	1.58	1.16	-	2.15	1.99
Alamos	58.88[a]	68.56[a]	69.38[a]	87.02[a]	77.11[a]	33.91
(st. error)	1.5	3.03	1.86	4.03	3.09	0.13
Montante	61.28[a]	59.76[a]	54.25[a]	78.02[a]	73.89[a]	60.2[a]
(st. error)	0.73	0.9	1.69	1.36	3.41	0.75
Mourao	59.16[a]	65.89[a]	57.85[a]	100[a]	57.31[a]	47.69
(st. error)	0.91	1.15	0.87	13.00	0.41	0.39
Lucefecit	65.36[a]	68.93[a]	56.56[a]	85.45[a]	72.8[a]	15.8
(st. error)	0.91	2.63	0.82	2.27	1.96	0.53
Jusante	61.3[a]	44.95	51.62[a]	78.02[a]	75.94[a]	11.45
(st. error)	1	5.28	0.6	1.36	1.49	0.55
Ardila	21.85	25.33	17.51	29.76	51.14[a]	12.8
(st. error)	0.94	0.29	0.4	6.62	0.32	0.54
Moinho das Barcas	51.16[a]	58.17[a]	52.06[a]	100[a]	69.18[a]	20.92
(st. error)	0	1.15	1.7	7.38	1.14	0.26
Odivelas 1	61.34[a]	69.04[a]	64.37[a]	71.21[a]	73.41[a]	55.04[a]
(st. error)	3.25	3.03	1.48	0.58	0	0.31
Odivelas 2	28.18	22.77	12.43	57.86[a]	-	28.82
(st. error)	0.78	1.22	1.33	0.88	-	2.07

[a] These cells stress out inhibitions higher than 50 %

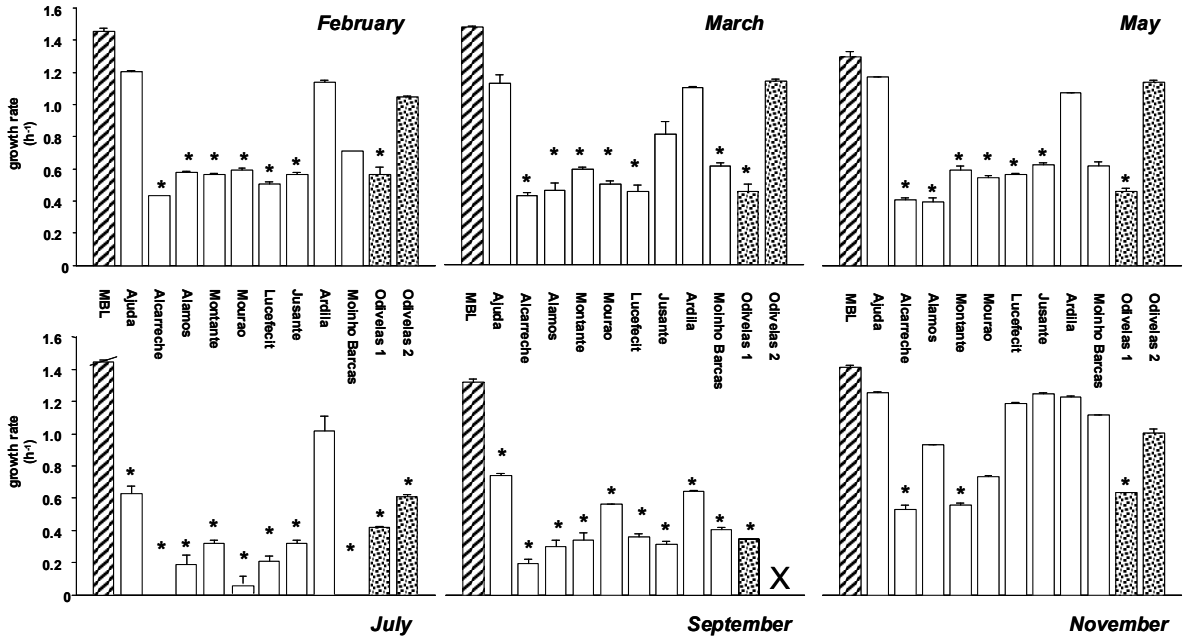

Figure 2. 2. Growth rates (per day) of the microalgae *P. subcapitata* after 72 h of exposure to the sampling points of the Alqueva aquatic system during 2006 (bimonthly sampling). *p<0.05, Dunnett's or Dunn's test, compared to the control. Control of MBL (dashed bars), samples from sites that receive water from the Alqueva aquatic system (pointed bars), samples collected from the Alqueva aquatic system (white bars), no data available (X).

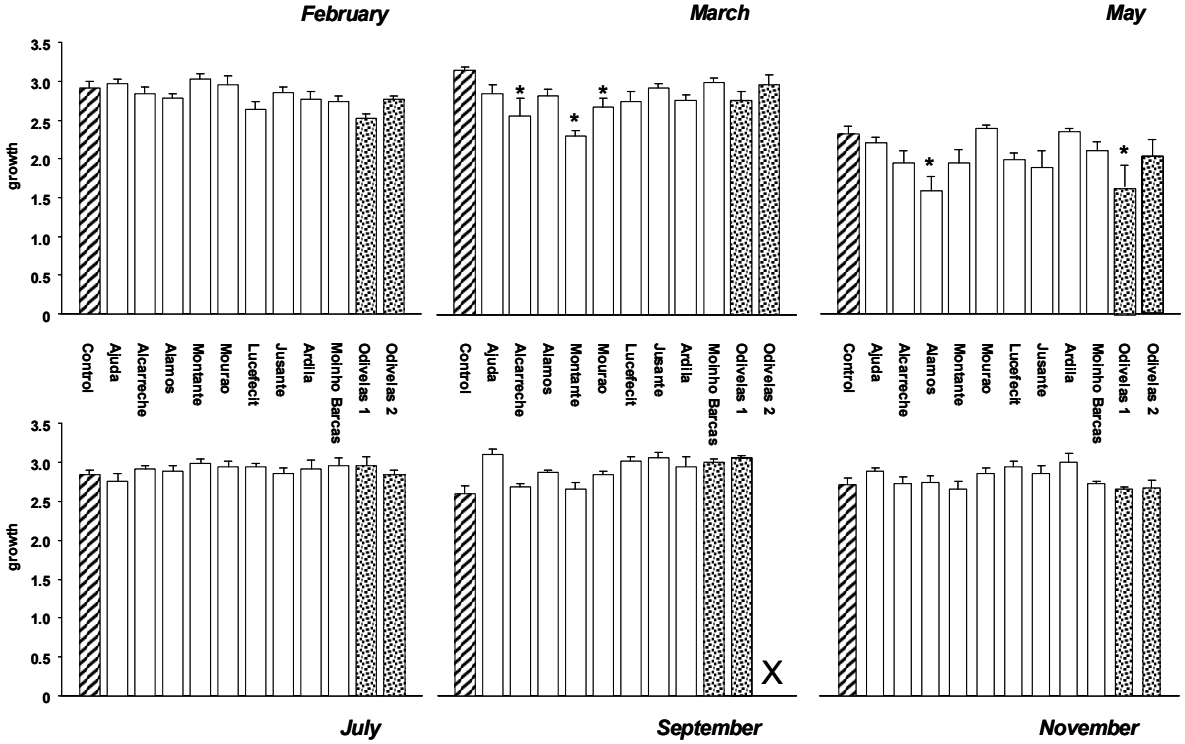

Figure 2. 3. Growth rates (per day) of the microalgae *P. subcapitata* after 72 h of exposure to the sampling points of the Alqueva aquatic system during 2006 (bimonthly sampling). *p<0.05, Dunnett's or Dunn's test, compared to the control. Control of MBL (dashed bars), samples from sites that receive water from the Alqueva aquatic system (pointed bars), samples collected from the Alqueva aquatic system (white bars), no data available (X).

The basic test of Microtox® showed no effects on the luminescence of *V. fischeri* when exposed to all water samples in all sampling periods. There was no decrease in the luminescence of the bacterium after 5 and 15 min of exposure when samples were diluted to 45%. Additionally, every time a water sample was tested, a positive control test with $ZnSO_4$ was carried out, as a reference test; the results obtained in this reference test were within the limits advised to validate the test.

Multivariable analysis was carried out to integrate physicochemical analysis and ecotoxicological results. A linear ordination technique, PCA (Figure. 2.4), was performed to summarize the differences between the sampling points on pesticides and heavy metals concentrations.

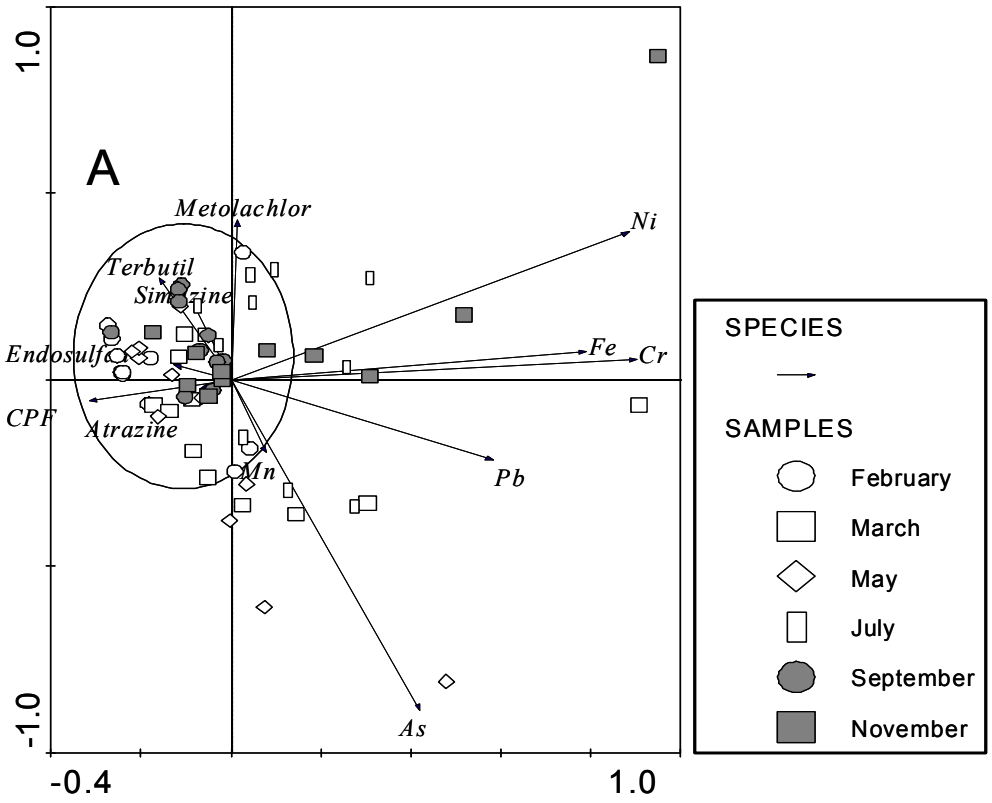

Figure 2. 4. PCA biplot of the sampling points and environmental variables (pesticides and heavy metals concentrations). In every month, there is a representation of the 11 sampling points (Ajuda, Lucefecit, Mourão, Álamos, Alcarreche, Montante, Jusante, Ardila, Pedrogão, Odivelas 1, and Odivelas 2).

The biplot of the PCA analysis discriminated one main group (A) correlated with the negative part of axis 1 (see Figure. 2.4) including the majority of the sampling points of the Alqueva Reservoir, which seemed to share the same chemical properties, particularly in pesticide concentrations, mainly during February, May, and September. The eigenvalues for the first three axes were 0.521, 0.275, and 0.105.

The first two axes explained 79.6% of the total significance in environmental parameters (pesticides and heavy metal concentrations) between the sampling points. The three axes explained 90.1% of this variation. Ni, Fe, Cr, and Pb total concentrations were the variables which correlated with the positive part of axis 1, while total concentrations of the pesticides atrazine, simazine, terbuthylazine, metolachlor, CPF, and endosulfan sulfate correlated with the negative part of the same axis and the positive part of axis 2.

The relationship between growth rates on both species (*P. subcapitata* and *C. riparius*) and the concentrations of pesticides and heavy metals were also analysed through an ordination technique, the RDA (Figure. 2.5). This analysis is an ordination of response data in which the axes are constrained to be linear combinations of environmental variables (Leps and Smilauer 2003). The triplot resulting from the RDA based on the growth rates, sampling points, and environmental variables (pesticides and heavy metals) clearly express the relationship between growth rates of *P. subcapitata* and the pesticides. Considering the plot showed in Figure. 2.5, algae growth rates are not affected by the presence of metals (e.g., Cr, Fe, Mn, Ni, and As), but are inhibited when pesticides like simazine, atrazine, CPF, metolachlor, and terbuthylazine are detected at higher concentrations. The eigenvalues based on the growth rates for the RDA axes 1, 2, and 3 were 0.282, 0.237, and 0.272, respectively (see Figure. 2.5). The first two axes explained 51.9% of the growth rates' environmental variable (pesticides and heavy metals) relationships and, together with axis 3, explained 79.1% (terBraak and Smilauer 2002).

The ordination of the growth rates indicated that low growth rates for both species were associated with sampling points which were characterized by high concentrations of pesticides, mainly from May, July, and September where high concentrations of the herbicides like atrazine, simazine, and terbuthylazine were detected (see Figure. 2.4). These triazines had significantly higher correlations with the decrease of the algae growth rates (F=5.39, p=0.006; F= 4.63, p=0.022; and F=12.19, p=0.002, respectively). Also, in these months, the growth rates were significantly low (see Figure. 2.2). From this statistical analysis, it was depicted that the insecticide endosulfan sulfate was significantly correlated with the decrease of chironomids growth rates (F=6.73, p=0.010).

Additionally, the highest growth rates of *P. subcapitata* were significantly correlated with the concentrations of As (F=8.33, p=0.002) and Ni (F=4.64, p=0.012) found in the water samples; on the other hand, it was not correlated with Fe, Cr, and Mn (F=0.09, p=0.902; F=1.30, p=0.256; and F=1.84, p=0.198, respectively). Moreover, the triplot resulting from the RDA showed that the sampling points belonging to November did not have any correlation with high pesticide concentrations (see Figure. 2.5).

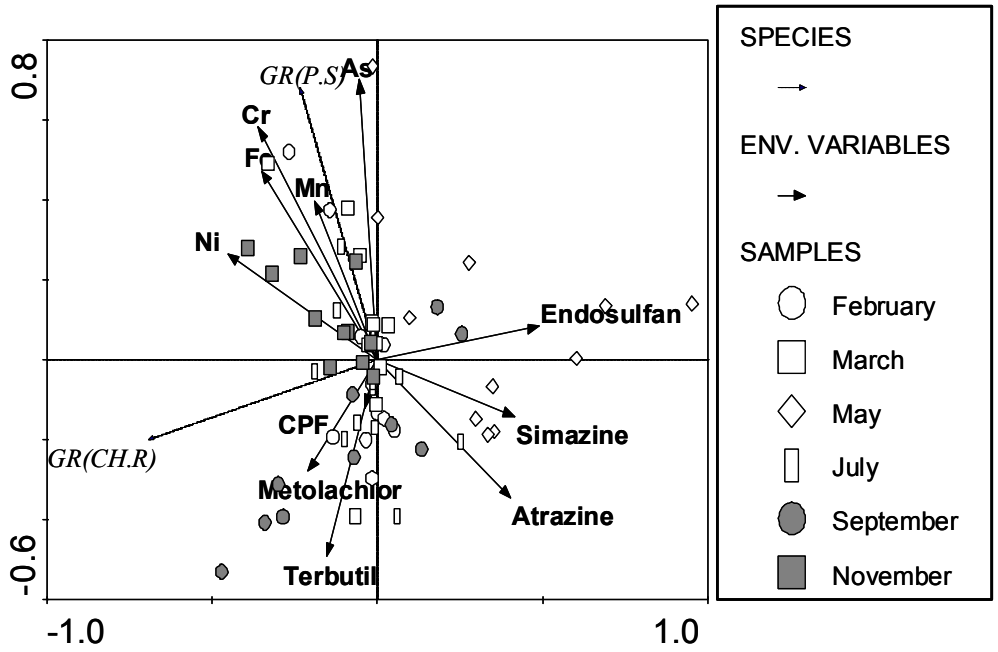

Figure 2. 5. RDA triplot of the growth rates of *C. riparius* and *P. subcapitata* sampling points and environmental variables (pesticides and heavy metals concentrations). In every month, there is a representation of the 11 sampling points (Ajuda, Lucefecit, Mourão, Álamos, Alcarreche, Montante, Jusante, Ardila, Pedrogão, Odivelas 1, and Odivelas 2). *GR (CH.R)* growth rate of *C. riparius*, *GR (P.S)* growth rate of *P. subcapitata*.

2.4. Discussion

In some sampling spots in the Alqueva aquatic system, the sum of the pesticide concentration is above 0.5µg/L, which can represent some hazard to human health, according to the Council Directive 98/83/EC on the quality of water for human consumption. In addition, atrazine, simazine, CPF, endosulfan sulfate, and Pb are considered priority hazardous substances in the list no. 2477/2001/EC of priority pollutants from the EU Water Framework Directive (2000/ 60/EC). For environmental water quality (accordingly to the EU Directive 2008/105/EC and to the Portuguese law DL 236/38 and its annex documents), pesticide total concentration should be below 2.5µg/L and individual pesticide concentration should never be above 0.5µg/L. In our study, these requirements were fulfilled for the total pesticide concentration (sum of concentrations), but several pesticides were above the individual limits: atrazine (May and July), simazine (September), terbuthylazine (September), and endosulfan sulfate (May).

Precisely, with this chemical information, one could conclude that the Alqueva Reservoir water quality is not acceptable for environmental purposes and some caution and monitorization has to be performed for water consumption by man. However, in addition, there were several sampling points where toxicity was observed despite the presence of contaminants in low concentrations. One example can be seen on the February sampling period where almost all chemical endpoints are

within permissible limits and algae growth inhibition was reported for a total of eight out of the 11 sampling sites (>50% inhibition).

Information from the Meteorology Institute I.P. of Portugal states that the year of 2006 was awkward regarding the usual precipitation and temperatures of the four seasons observed annually in a Mediterranean country like Portugal (mainly the southern part). Unexpectedly, in January and February 2006, there were few rainy periods with significant increase of precipitation by the end of March. April showed low precipitation periods and May was a fully dry month. June, July, August, and September were months with low precipitation (less than 20 mm), while in October and November, precipitation was higher than the normal ranges with values between 130 and 160 mm (accumulated precipitation). Temperature showed significant daily non-normal values when compared with the period between 1961 and 1990. Temperatures reached maximum differences of 12°C and 9°C in some days in May and August, respectively. In October and November, the same pattern was observed (6°C and 8°C, respectively). These climate changes are somehow related to the results obtained in this study. During the sampling months where low precipitation and high temperature occurred, water samples toxicity was higher (May, July, and September), mainly due to higher concentrations of pesticides where the presence of herbicides like triazines contributed to a marked decrease in the growth of *P. subcapitata*. During this period and considering the Council Directive 98/83/EC, the concentration of the pesticides (total) exceed 0.5µg/L and their individual concentrations also exceed the value of 0.1µg/L, possibly representing some hazard to human health.

The physicochemical analysis revealed that the BOD was frequently higher than the maximum permissible values for environmental quality in freshwater, which is 5 mg/L according to the Portuguese law (DL 236/38, Annex XXI). BOD above this value indicates the presence of pollutants and usually indicates the presence of organic matter and bacteria (i.e., high decomposition process rates). On the other hand, these values are usually related to low dissolved oxygen concentrations which was not the case in this study. Nitrate concentrations were considered acceptable for the water quality status, always below the maximum recommended value (MRV) by the European and Portuguese legislations for water of human and agricultural consumption (25 mg/L). Nitrites overcame the maximum admissible value (0.1 mg/L) in several sampling periods and points. In addition, the phosphorous concentrations were much higher than the MRV by the European and Portuguese legislation (400µg/L) in the sampling points of Ajuda, Ardila, and Odivelas 2 (see Table 2.3). These high concentrations of nutrients like phosphorous or nitrogen compounds could have influenced and stimulated the growth of *P. subcapitata* in these sampling places (see Figure. 2.2).

The algae bioassay showed to be the most sensitive bioassay used in this study. The commonly used herbicides triazines are known to inhibit electron transport mechanisms of photosystem II in target plants (Wacksman et al. 2006). For this reason, the presence of triazines was expected to affect algae status.

The sum of the concentrations of herbicides like atrazine, simazine, terbuthylazine, and metolachlor overcame the values recommended for water for human and agricultural use (0.5µg/L) in May, July, and September; coincidently, algae growth rates were low in this period (see Figure. 2.2).

Previous studies demonstrate a notable sensitivity of the microalgae *P. subcapitata* to triazines like cyanazine and to acetanilide metolachlor (Fairchild et al. 1997). The sum of these pesticide concentrations in February, March, and November did not exceed the MRV; however, low growth rates were obtained. It is known that mixtures of pesticides can provoke higher than expected impairment (synergism); so that this is one of the hypotheses raised here. Organophosphorus (OP) insecticides, like CPF, have been reported as plant cytochrome P450 monooxigenases inhibitors in terrestrial plants (Munkegaard et al. 2008). Cytochrome P450 monooxigenases are known to play a role in the detoxification processes of xenobiotics and degrading pesticides in higher plants, so it is likely that the degradation of xenobiotics follow similar paths in algae (Munkegaard et al. 2008) and a potential inhibition of P450 by OP insecticides in algae could increase its sensitivity to herbicide exposure.

For the chironomids exposure, growth was expected to be impaired in March and May due to CPF and endosulfan sulfate concentrations, particularly in Alcarreche, because CPF showed a concentration of 0.15µg/L. Previous studies on acute toxicity using *Chironomus tentans* have found values of EC_{50}=0.17µg/L for CPF (Lydy and Austin 2004). Effects of chronic application of CPF on invertebrates were studied in outdoor artificial streams (Ward et al. 1995). Ninety-eight different taxa, including 24 chironomids taxa, were recorded during the study. Both number of taxa and total invertebrate abundance were reduced in response to low (0.1µg/L) and high (5µg/L) CPF concentrations.

Similarly, the same was expected for Odivelas 1 in May where endosulfan sulfate concentration was 0.78µg/L. On the other hand and although CPF and/or endosulfan sulfate were not detected or detected at low concentrations in Montante and Mourão (in March) and Alamos (in May), the main toxicity observed for *C. riparius* might be due to the mixture of the insecticides with atrazine and simazine in May and possibly the mix between metolachlor and terbuthylazine in March. Due to

the fact that pesticides rarely occur in the environment as single compounds, but rather as complex mixtures, a series of recent studies demonstrated that the triazine herbicides promote the effects of OP insecticides (Belden and Lydy 2000, Lydy and Linck 2003, Pape- Lindstrom and Lydy 1997). Pape-Lindstrom and Lydy (1997) demonstrated that atrazine in binary combinations with CPF exhibited greater-than-additive toxicity to the midge *C. tentans*. An increase in toxicity of CPF was also reported in the presence of atrazine in *Hyalella azteca* (Anderson and Lydy 2002). Pape-Lindstrom and Lydy (1997) suggested that atrazine increased biotransformation of OPs, converting them into more toxic O-analog metabolites. OP insecticides require oxidative activation by cytochrome P450 enzymes to become O-analogs, which are much more potent AChE inhibitors than the parent compound. They further suggested that atrazine may be accomplishing this metabolic activation by inducing the cytochrome P450 enzymes responsible for this conversion (Pape-Lindstrom and Lydy 1997).

No toxicity was observed for the water samples exposed to *V. fischeri*. From a study carried out with chemicals usually used in aquacultures (antibiotics, pesticides, therapeutics, herbicides), there was no toxicity of simazine and atrazine to *V. fischeri* at a concentration up to 6 mg/L (Hernando et al. 2007). In addition, the study of Palma et al. (2008) reports that *V. fischeri* was the test organism less sensitive to the main chemicals found at the Alqueva Reservoir, showing EC_{50} values of 69.4 mg atrazine/L, 11.2 mg endosulfan sulfate/L, and 2.84 mg CPF/L, which are much higher than the values found.

2.5. Conclusions

Standardized laboratory bioassays for water quality can be useful tools to assess water quality in aquatic systems and can valuably complement chemical analysis assessments. The results obtained in this study demonstrated that the most sensitive species used in this test battery was the microalgae *P. subcapitata*, possibly due to the high concentrations of herbicides throughout the sampling periods. The growth of *C. riparius* was less affected and this might possibly be due to the fact that this species lives in the sediment and not in the water column, and therefore, several studies have demonstrated that it is usually more resistant to pollutants in the water column. Additionally, insecticide concentrations were low and probably did not affect the larvae.

For a safe use of the aquatic resources of the Alqueva Reservoir, this aquatic system must be monitored frequently, as advised by the Water Framework Directive and the Water Law, using chemical analysis procedures together with ecotoxicological bioassays in addition to a complete evaluation of its faunal and faunistic biodiversity.

2.6. Recommendations and perspectives

With this study, we conclude and recommend the use of test batteries to bio monitor water quality. On their own, chemical analysis is not enough to derive conclusions on the water quality and/or status. Single chemical maximum permissible values and the sum of pesticide concentrations do not take into account possible patterns of synergism, antagonism, dose level dependencies, or even the dominance of several chemicals within a mixture. In addition, several species from different levels in trophic chains are recommended due to differences in species' sensitivities and also chemical compounds that are present.

Acknowledgements

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Chapter 3

Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures

Perez J., Domingues I., Soares A.M.V.M., Loureiro S. (2011) Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures. Ecotoxicology 20(6): 1167-117.

3. Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures

Abstract

Previous work showed the existence of ecotoxicity of water samples from the Alqueva reservoir due to the presence of the herbicides atrazine, simazine, terbuthylazine and metolachlor. In the present study we examine the effects of these herbicides singly and as binary mixtures on the growth rate of the microalgae *Pseudokirchneriella subcapitata*. Usually, the toxicity of mixtures is evaluated in relation to the reference models CA (concentration addiction) and IA (independent action). In this study CA model was selected to evaluate the joint effects of *s*-triazine herbicides on the growth of algae due to their similar mode of action. Moreover, IA reference model was chosen to evaluate the joint toxicity of the chloroacetanilide metolachlor and the *s*-triazine herbicides due to their different mode of action. In this study dose ratio was the common deviation obtained on both reference models. In the binary mixtures between atrazine/simazine and atrazine/terbuthylazine the increase of the mixtures toxicity (synergism) was mainly due to atrazine. Also, in the binary mixture between atrazine and metolachlor, atrazine was responsible for the increase (synergism) of the mixture toxicity. In the cases of the binary mixtures between simazine/metolachlor and terbuthylazine/metolachlor, the increase of the mixtures toxicity (synergism) was detected when metolachlor was the herbicide dominant, and antagonism was detected when simazine and terbuthylazine were dominant in both mixtures. This study represents an important step to understand the interactions among herbicides detected previously in the waters of the Alqueva reservoir.

Keywords: Herbicide mixtures, *Pseudokirchneriella subcapitata*, Concentration addition and Independent action

3.1. Introduction

The constant bio monitoring of aquatic ecosystems is required by the European legislation for the Water Framework Directive (WFD) and can give crucial information on the water quality status and related potential toxicity considering chemical analysis and bioassays. This legislation specifies the protection of water ecosystems as a whole, thus not only based on safe concentrations for individual chemicals (Verro et al. 2008a, b). There are several examples throughout Europe where water quality is not proper for human consumption or for a proper environmental quality and equilibrium (Barata et al. 2007; Damásio et al. 2008; Pérez et al. 2010).

The evaluation of chemical mixtures in the environment can be carried out using the top-down approach (diagnosis) or the bottom-up approach (prognosis). In the first one, a site-specific and retrospective investigation is carried out using bioassays to assess toxicity of (complex) mixtures that occur naturally, and methodologies like the Effect-Directed Analysis (EDA) or Toxicity Identification Evaluation (TIE) can be used to identify (groups of) chemicals responsible for toxicity (Brack 2003). In the second approach, researchers evaluate mixture effects from knowledge on its composition, using the available information on modes of action of individual chemicals. In addition, assumptions on interaction between chemicals are included, using mathematical models to describe interactions after a detailed assumption derived from a test design.

In the study of Pérez et al. (2010), several pesticides were detected in the last years in the waters of the Alqueva reservoir (south of Portugal), showing the existence of water ecotoxicity on samples from the reservoir possibly due to the presence of significant concentrations of *s*-triazine herbicides (e.g. atrazine, simazine and terbuthylazine) and the chloroacetanilide metolachlor. Considering these findings, in the present study a bottom-up approach is carried out and binary mixtures toxicity assessed to derive patterns for toxicity response, considering the available information on the chemical cocktail characterized in this aquatic system and the results from the bioassays.

Few conclusions can be derived concerning specific chemicals interactions in multiple mixtures studies, but binary mixtures studies frequently are conducted with the aim of elucidating the effects of one specific chemical on the biological action of another. To do this, whole dose–response surfaces are created. The dose–response surfaces at different mixtures ratios from both chemicals in the binary mixture describe several dose–response curves, where the projection of the results at specified effect level, mainly the 50% effect concentration (EC_{50}), on the x–y plane is called isobole (Cedergreen et al. 2007a). The shape of the isobole gives information about the joint effect of the two chemicals in the test system and can be explained trough established mathematical models (Jonker et al. 2005).

The two reference models commonly used to derive patterns for joint effects of mixtures are the concentration addition (CA) and the independent action (IA) models (Loewe and Muischnek 1926; Bliss 1939). Both allow the calculation of expected mixture toxicity on the basis of known individual toxicities of the mixture components. The CA model assumes that the mixed chemicals have the same mode of action (MoA), and consequently can be regarded as dilutions of one another. This conceptual model is defined as a summation of the relative toxicities of the individual components in the mixture (Loewe and Muischnek 1926; Groten 2000) and is mathematically defined by:

$$\sum_{n}^{i=1} \frac{C_i}{EC_{xi}} = 1$$

In this equation, C_i are the concentrations of the individual substances present in the mixture with a total effect of x%. EC_{xi} are the equivalent effect concentrations of the single substances, i.e. those concentrations that alone would cause the same quantitative effect x as the mixture. Quotients C_i/EC_{xi} expresses the concentrations of mixture components as fractions of equi-effective individual concentrations and has been termed toxic units (TU):

$$TU = \frac{C}{EC_x}$$

The alternative model of independent action (IA) is based on the idea of a dissimilar action of mixture components (Bliss 1939). Under this presumption, this model is usually used if the question asked is whether the probability of toxicity to one chemical is independent from the probability of toxicity exposure to another chemical (Altenburger et al. 2004; Jonker et al. 2004, 2005). This means that the relative effect of a toxicant remains unchanged in the presence of another chemical. In this case the mathematically formula is expressed as:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(C_i)$$

Where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, q_i (C_i) the probability of non-response, $u_{\max}$ the control response for endpoints and Π the multiplication function.

Deviations from the models can also be tested. Some compounds can interact synergistically becoming more toxic than expected from the toxicity of single compounds or enhancing the probability of effect of one another. Different compounds may also interact antagonistically, defined as situations where the mixture toxicity is lower than expected from the toxicities of single compounds or where the compounds decrease the probability of effect to one another. The interaction between different compounds might also depend on the mixture dose level or on the dose ratio (Jonker et al. 2005). The dose level-dependency means that observed deviations from the reference models are different at low dose levels and at high dose levels. The dose ratio-dependency means that the proportion of the compounds in the mixture affects the deviation from the reference model in an asymmetric fashion, with one of the chemicals being the major responsible for the observed deviation (Jonker et al. 2005).

In the Alqueva aquatic system, pesticide and metallic elements were analysed (Pérez et al. 2010), but the levels found for *s*-triazine herbicides and metolachlor were of the most concern in several time sampling. *s*-Triazines are specific inhibitors of the photosynthetic electron transport. They act by competitive and reversible binding to the same domain of the D1 protein of the photosystem II reaction center, thus displacing the electron acceptor plastoquinone QB from this site (Klaus et al. 1991). Due to this specific mechanism of action, primary producers can be expected to be the most susceptible part of the aquatic community. Therefore, algae are considered to play a decisive role in the regulatory risk assessment of *s*-triazine contaminations (Faust et al. 2001).

On the other hand, the herbicide metolachlor is a chloroacetanilide herbicide that acts by inhibiting protein synthesis. In addition metolachlor has shown to affect the metabolism of fatty acids (Junghans et al. 2003a). Couderche (1998) demonstrated that the inhibition of growth of *Scenedesmus acutus* Pringsh was positively correlated with the intensity of the effect on the fatty acids synthesis in case of alachlor, metolachlor and metazachlor.

Regarding the first results on the Alqueva reservoir and the bottom-up approach that we propose, the principal aim of this study was to derive toxicity patterns for the herbicides present in the aquatic system, singly or as binary mixtures. For that, single and binary combinations of herbicides with similar and dissimilar mode of action, detected previously in the waters of the Alqueva reservoir, were used and the growth of the unicellular algae *Pseudokirchneriella subcapitata* evaluated upon exposure.

3.2. Materials and methods

3.2.1. Test-organism and test-chemicals

The microalgae *P. subcapitata* (formerly *Selenastrum capricornutum*) is currently recommended as a standard species for algal toxicity tests (OECD 2006). It was obtained from nonaxenic batch cultures with Woods Hole Marine Biological Laboratory (MBL) medium (Stein 1973), incubated at 20 ± 2º, under continuous and uniform cool-white light intensity (2000 lux). For the maintenance of the lab cultures, and the start of new cultures, algae were harvested while still in the exponential growth phase (5–7 days old) and inoculated in fresh medium.

The herbicides tested were purchased from Sigma-Aldrich Laboratory in the highest available purity: atrazine with 97.4%, simazine with 99.9%, terbuthylazine with 98.6% and metolachlor with 97.1% of purity.

3.2.2. Tests design

To obtain the EC_{50s} value for each herbicide seven concentrations were tested for atrazine and simazine and six concentrations for terbuthylazine and metolachlor with three replicates each; in addition, a control with MBL was also tested in triplicate.

A ray design was used to obtain the dose response surfaces for the binary herbicide mixtures. The ray design consists of dose response curves of the two individual herbicides tested alone and a number of dose response curves of the herbicides mixed at predefined mixture ratios. The number of mixture ratios was chosen with the aim to obtain a reliable coverage of effect of the two herbicides. Nominal concentrations of the mixtures were calculated based on expected toxic strengths of 0.375 (0.125 + 0.25; 0.25 + 0.125), 0.5 (0.125 + 0.375; 0.25 + 0.25; 0.375 + 0.125), 0.75 (0.125 + 0.625; 0.25 + 0.5; 0.375 + 0.375; 0.5 + 0.25; 0.625 + 0.125), 1 (0.125 + 0.875; 0.25 + 0.75; 0.375 + 0.625; 0.5 + 0.5; 0.625 + 0.375; 0.75 + 0.25; 0.875 + 0.125), 1.5 (0.75 + 0.75; 1 + 0.50;0.50 + 1), 1.75 (1 + 0.75; 0.75 + 1) and 2 (1 + 1) toxic units (TU). The conversion of TU into concentrations for each pesticide was based on the EC_{50s} value obtained on the preceding experiments with the single chemicals. The following herbicide combinations were tested: atrazine and simazine, atrazine and terbuthylazine, simazine and terbuthylazine, atrazine and metolachlor, simazine and metolachlor, and terbuthylazine and metolachlor.

3.2.3. Growth inhibition test with P. subcapitata

The growth inhibition test with the unicellular algae *Pseudokirchneriella subcapitata* was performed in this study to determine the single and joint effects of the herbicides tested. The microalgae was exposed for 72 h to the test toxic medium and the algae growth determined, based on the protocol 201 from OECD (OECD 2006). The artificial medium MBL was used as control.

In the beginning of the single exposure tests, the algae *P. subcapitata* was exposed to 40 ml of test medium in Erlenmeyer flasks with a capacity of 100 ml, and covered with permeable gauze, using three replicates per concentration. Test vials were randomly incubated in an orbital shaker for 72 h, at 21 ± 2ºC and with a constant luminous intensity of 4000 lux cool-white fluorescence.

The initial concentration of algae was of $3x10^4$ cell/ml and after 24, 48 and 72 h the algae concentration was measured by spectrometry (440 nm; Jenway, 6505 uv/vis spectrophotometer), and converted in cells/ml using the equation:

$$C = -17107.5 + ABS \times 7925350$$

Where *C* is the algae concentration (cells/ml); *ABS* is the absorbance obtained at 440 nm.

The average specific growth rate for a specific period was calculated as the logarithmic increase in biomass from the equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i}$$

Where μ_{i-j} is the average specific growth rate from time *i* to *j*; ti is the time for the start of the exposure period; t_j is the time for the end of the exposure period; B_i is the biomass concentration at time *i*; B_j is the biomass concentration at time *j*.

3.2.4. Statistics

The no observed effect concentration (NOEC, i.e. the highest concentration to cause no significant effect on algal growth) and the lowest observed effect concentration (LOEC, i.e. the lowest concentration to cause a significant effect on algal growth) were estimated using the SigmaStat statistical package (Systat 2006) using a One-way ANOVA, followed by a Dunnett's multiple comparisons test to determine which treatments differed significantly from the controls.

The EC_{50s} values for *P. subcapitata* single herbicide exposures at 24, 48 and 72 h were calculated through a three-parametric logistic regression model (Systat 2006).

The mixture data were analysed using the MIXTOX model already described by Jonker et al. (2005). Growth rates from exposures to mixtures with the *s*-triazine herbicides were fit in a first step to the CA model as herbicides that share the same mode of action. For the mixtures with metolachlor and the *s*-triazine herbicides the first fit was done to the IA model. The MIXTOX software framework contains three different deviations from either of the reference models CA and IA: synergy or antagonism (S/A), dose ratio (DR) dependent deviations giving asymmetric isoboles, and dose level (DL) dependent deviations giving variable isoboles depending of the dose level or effect concentration. The S/A deviations are extensions of the CA and IA models and the DR and DL deviations are further extensions of the S/A deviations (please see details in Jonker et al. 2005). These deviations are obtained with the addition of the parameters *a* and *b* forming a nested framework. The extra parameter *a* in the S/A deviations model can become negative or positive, respectively, for both CA and IA. Where $a = 0$, the S/A model reduces to the CA or IA reference models. For dose level dependency, a second parameter b_{DL} is included in addition to *a*, to generate the DL deviation model. In this case *a* value indicates the deviation at low doses (i.e., *a*

< 0=antagonism, and a > 0=synergism) and b_{DL} value indicates at what dose level the deviation changes (i.e., from antagonism to synergism or vice versa). To describe deviations of dose ratio (DR) dependency, again a second parameter b_{DR} is included in addition to a. In this deviation function, the parameter b_{DR} allows the deviation from either reference model to depend on the composition of the mixture. In the case of two substances, antagonism can be observed where the toxicity of the mixture is caused mainly by one of the toxicants, whereas synergism can be observed where the toxicity is caused mainly by the other one. The biological interpretations of these additional parameters are described with more details in Table 3.1.

The nested deviations were compared using the method of maximum likelihood and the best fit chosen using 0.05 as the significance level. In addition, the lowest residual sum of square (*SS*) was preferred when comparing conceptual models and deviations. Full details on the derivation of these deviations functions can be found in more detail in Jonker et al. (2005).

3.3. Results and discussion

3.3.1. Individual toxicity tests

The 72-h growth inhibition tests with the single herbicides showed that all herbicides were highly toxic at highest concentrations to this algae specie, and these results are shown in Figure. 3.1.

The EC_{50} values obtained after the 72 h of exposure were used to calculate the TU values for the mixture experimental setup. EC_{50} for 24, 48, and 72 h are depicted in Table 3.2.

Previous studies demonstrate a notable sensitivity of the microalgae *P. subcapitata* to *s*-triazines like cyanazine and to chloroacetanilide metolachlor (Fairchild et al. 1997). Okamura et al. (2000) found that *P. subcapitata* was sensitive to: atrazine with EC_{50} = 180 µg/l, simazine with EC_{50} = 220 µg/l and terbuthylazine with EC_{50} = 36µg/l, which are values comparable to our 72 h EC_{50s} of 196, 252 and 24 µg/l, respectively, where the toxicity of these herbicides was based on population growth rate.

Fairchild et al. (1998) studied the relative sensitivities of six species of algae to atrazine, metribuzin, alachlor and metolachlor, and *P. subcapitata* was among the most sensitive species tested with 96 h EC_{50} = 117 µg/l to atrazine and 96 h EC_{50} = 84 µg/l to metolachlor.

A study with the green microalgae *Chlorella vulgaris* and the cyanobacterium *Synechococcus elongatus* also recorded an extremely rapid uptake of the *s*-triazine herbicides atrazine and

terbutryn in both species, and consequently the growth rate, biomass, and cell viability were clearly affected by herbicide uptake (González-Barreiro et al. 2006).

3.3.2. Binary mixture toxicity tests

As the theoretical use of independent action and concentration addition is based on a priori knowledge of chemical mode of action, the CA model was selected to evaluate the joint effects of the *s*-triazine herbicides on the growth of the algae *P. subcapitata*.

The fit of the CA model to the binary mixture data of atrazine and simazine exposure obtained an *SS* value of 0.56. However, adding the parameters *a* and *b* the *SS* value decreased significantly (*SS* = 0.43, *p* [x^2] = 0.008, Table 3.3), and a Dose Ratio (DR) dependent deviation from the CA model was concluded. In this herbicide combination, an increase of the mixture toxicity (synergism) was observed and explained mainly when atrazine was the dominant herbicide in the mixture (Figure. 3.2a; Table 3.3). On the other hand, when simazine was dominant in the mixture (i.e. high concentrations of simazine and low of atrazine), the toxicity was lower than expected by the individual toxicity data of both herbicides, presenting an antagonistic pattern.

Table 3. 1. Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from concentration addition (CA) and independent action (IA).

Deviation Pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
synergism/antagonism (S/A)	$a>0$ antagonism		
	$a<0$ synergism		
Dose-ratio dependent (DR)	$a>0$: antagonism except for those mixture ratios where negative *b* value indicate synergism	$b_i>0$: antagonism where the toxicity of the mixture is caused mainly by toxicant i	
	$a<0$: synergism except for those mixture ratios where positive *b* value indicate antagonism	$b_i<0$: synergism where the toxicity of the mixture is caused mainly by toxicant i	
Dose-level dependent (DL)	$a>0$: antagonism low dose level and synergism high dose level	$b_{DL}>1$: change at lower EC_{50} level	$b_{DL}>2$: change at lower EC_{50} level
		$b_{DL}=1$: change at EC_{50} level	$b_{DL}=2$: change at EC_{50} level
	$a<0$: synergism low dose level and antagonism high dose level	$0<b_{DL}<1$: change at higher EC_{50} level	$1<b_{DL}<2$: change at higher EC_{50} level
		$b_{DL}<1$: No change but the magnitude of S/A is DL dependent	$b_{DL}<1$: No change but the magnitude of S/A is effect level dependent

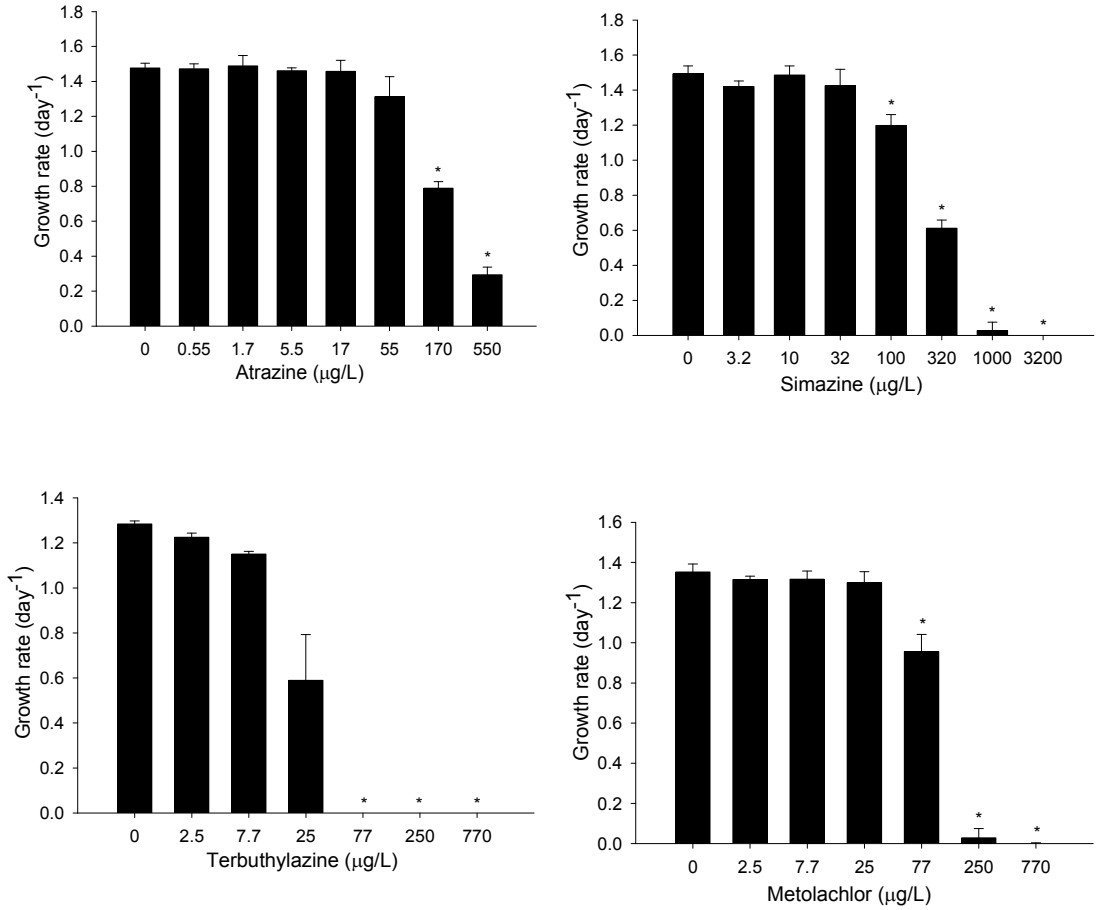

Figure 3. 1. Growth rates of the algae *P. subcapitata* after 72-h of exposure to the single herbicides tested. * Significantly differences from the controls (p<0.05), using One-way ANOVA with Dunnett's test comparisons.

From the application of the same CA model on the binary mixture of atrazine and terbuthylazine we obtained an *SS* value of 1.17, though when we added the parameters *a* and *b* the *SS* decreased significantly (*SS* = 0.47, p [x^2] < 0.05, Table 3.3). In this case a DR deviation was also observed, where the negative parameter *b* indicated an increased toxicity (synergism) when the mixture effect was caused mainly by atrazine and the positive parameter *a* indicated a decreased toxicity (antagonism) when the mixture effect was due mostly to terbuthylazine.

Table 3. 2. EC_{50} values (µg/l) from the individual growth inhibition tests with *Pseudokirchneriella subcapitata* after 24h, 48h and 72h of exposure. Values in brackets refer to Standard errors (SE).

Herbicides	24 h	48 h	72 h
	EC_{50} (SE)	EC_{50} (SE)	EC_{50} (SE)
Atrazine	599 (0.14)	206 (0.09)	196 (0.05)
Simazine	392 (0.21)	241 (0.16)	252 (0.06)
Terbuthylazine	33 (0.07)	20 (0.07)	24 (0.08)
Metolachlor	-	159 (0.06)	98 (0.04)

Values in brackets refer to standard errors (SE)
(-) at this time it was impossible to determinate the EC_{50} value

However, when we apply the same model CA to the mixture between simazine and terbuthylazine no deviation from the model was obtained (p<0.05). This is shown in the isobole diagram of the Figure. 3.2b. Previous studies have demonstrated that the concept of concentration addiction provides a reliable tool for the predictive hazard assessment of multi-component *s*-triazine mixtures, irrespective of the effect level and the concentration ratio of mixture components (Faust et al. 2001). In addition, the CA model has demonstrated a good predictability for the combined effects of similarly acting chloroacetanilide herbicides (Junghans et al. 2003a), and the sulfonylurea herbicides (Junghans et al. 2003b) to the unicellular green alga *S. vacuolatus*.

The present study provided examples where CA is not the best model to explain the data, as shifts for synergism and/or antagonism might occur depending on the dominant chemical present.

Regarding chemicals with different molecular target sites, previous studies with the unicellular green freshwater algae *Scenedemus vacuolatos* demonstrated that the IA conceptual model presented a better prediction when compared to the CA model when testing the mixtures of 16 biocides (Faust et al. 2003). The mixture toxicity of different pollutants with unclear modes of action was also accurately predicted by IA at individual NOECs on the growth of the algae *S. vacuolatus* (Walter et al. 2002).

Table 3. 3. Parameter estimates and tests of fit of the reference models using the MIXTOX model applied to the growth rate of *Pseudokirchneriella subcapitata* exposed for 72 hours to six pesticide mixtures. Equations used to derive these results are detailed in Jonker et al 2005.

Mixture		Concentration addition					Independent action				
		SS_{CA}	r^2	p (x^2)	a	b	SS_{IA}	r^2	p (x^2)	a	b
Atrazine	Reference	0.56	_	_	_	_	_	_	_	_	_
simazine	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_	_	_	_
	DR	0.43	0.96	0.008	0.18	-2.8	_	_	_	_	_
Atrazine	Reference	1.17	_	_	_	_	_	_	_	_	_
terbuthylazine	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_	_	_	_
	DR	0.47	0.95	$4.11x10^{-8}$	0.19	-4.81	_	_	_	_	_
Simazine	Reference	0.49	0.93	$1.12x10^{-17}$	_	_	_	_	_	_	_
terbuthylazine	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_	_	_	_
	DR	_	_	_	_	_	_	_	_	_	_
Atrazine	Reference	_	_	_	_	_	0.57	_	_	_	_
metolachlor	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_	_	_	_
	DR	_	_	_	_	_	0.32	0.94	$2.66x10^{-5}$	5.59	-8.15
Simazine	Reference	_	_	_	_	_	0.59	_	_	_	_
metolachlor	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_			
	DR	_	_	_	_	_	0.46	0.91	0.01	-2.94	4.97
Terbuthylazine	Reference	_	_	_	_	_	2.79	_	_	_	_
metolachlor	S/A	_	_	_	_	_	_	_	_	_	_
	DL	_	_	_	_	_	_	_	_	_	_
	DR	_	_	_	_	_	1.59	0.73	$3.81x10^{-5}$	-1.26	10.03

r^2 is the coefficient of regression; p (x^2) indicates the outcome of the likelihood ratio test (significance level $p<0.05$); *SS* is residuals sum of squares; a and b are the parameters of the deviations; S/A is synergism/antagonism, DR is dose ratio deviations and DL is dose level deviation from the reference models.

The combined effects of pesticides and nickel was also tested on the cladoceran *Daphnia magna* Straus and predicted also by these reference models (Loureiro et al. 2010); in this study the IA model was as adjustable to the data used as CA, and being able to replace the CA model in cases where modes of action are clearly dissimilar or ambiguous.

In addition, Backhaus et al. (2004) employed this IA model to predict the toxicity of six dissimilarly acting substances on the natural algae communities (Backhaus et al. 2004).

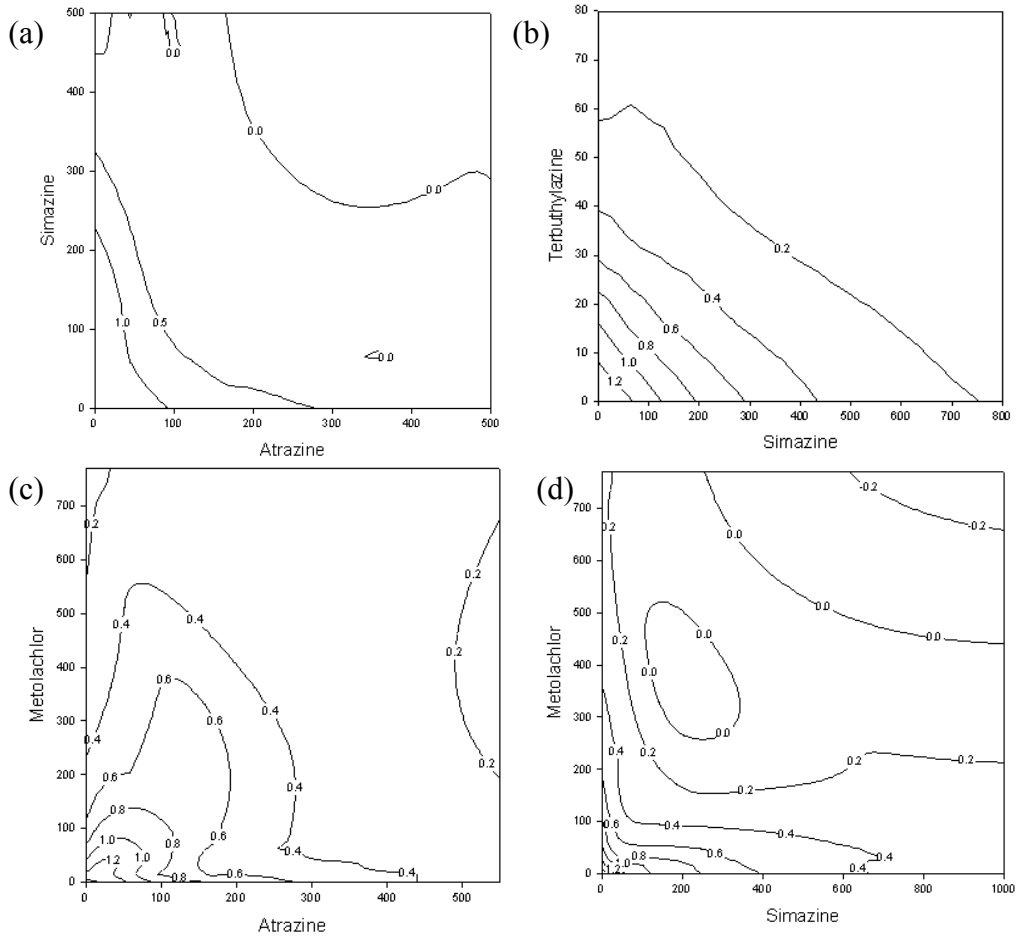

Figure 3. 2. Isobolograms of the herbicide mixtures tested on the growth rate of the algae *P. subcapitata*. **a** dose ratio (DR) deviation of the CA model; **b** CA reference model; **c** dose ratio (DR) deviation of the IA model; **d** dose ratio (DR) deviation of the IA model.

So, in this study the IA reference model was chosen to assess the joint toxicity of the chloroacetanilide metolachlor and the *s*-triazine herbicides due to their different mode of action.

In the case of the mixture of atrazine and metolachlor, the reference model IA originated an *SS* value of 0.57 but with the addition of the parameters *a* and *b* a significant decrease of the *SS* value (*SS* = 0.32, *p* [x^2] <0.05, Table 3.3) was observed and a dose ratio dependent deviation from independent action was concluded, where an increase of the mixture toxicity (synergism) was observed mainly when atrazine was the dominant chemical in the mixture (Figure. 3.2c).

The same deviation for ‘‘dose ratio’’ was obtained after using the independent action model to assess the joint effect between simazine and metolachlor (*SS* = 0.46, *p* [x^2] = 0.01, Table 3.3). In

this case a positive parameter *b* indicated a decrease of the toxicity mixture to what was expected (antagonism) when simazine was the dominant chemical in the mixture; on the other hand an increase of the toxicity mixture (synergism) was obtained at high concentrations of metolachlor (Figure. 3.2d).

In the case of the mixture between terbuthylazine and metolachlor and after using the IA reference model an *SS* value of 2.79 was obtained, which decreased significantly after adding the parameters *a* and *b* (*SS* = 1.59, *p* [x^2]<0.05, Table 3.3). These results show a DR deviation from the IA model, where a positive parameter *b* indicates antagonism when terbuthylazine was the dominant chemical and an increase of the toxicity mixture (synergism) explained when metolachlor was the dominant herbicide.

In this study dose ratio was the common deviation obtained when testing both reference models. In the binary mixtures between atrazine/simazine and atrazine/terbuthylazine, the increase of the mixtures toxicity (synergism) was mainly due to atrazine. Also, in the binary mixture between atrazine and metolachlor, atrazine was responsible for the increase (synergism) of the mixture toxicity, concluding that atrazine accounted for the toxicity of the mixtures where it was involved.

Contrary to our findings, antagonism was the most common type of interaction found in the study of Cedergreen et al. (2007b), where effects of herbicide mixtures on aquatic and terrestrial test systems were assessed. In this study the authors found asymmetric isoboles for the mixture of terbuthylazine and diquat on the terrestrial plant *Stellaria media*, suggesting that only a small amount of terbuthylazine would reduce the effect of diquat dramatically, whereas small amounts of diquat would have only a small effect on the efficacy of terbuthylazine (Cedergreen et al. 2007b).

The present study ratified the susceptibility of the algae *P. subcapitata* to the join toxicity of the herbicides found in the waters of the Alqueva reservoir (Pérez et al. 2010).

Biomonitoring carried out using bioassays in the Alqueva aquatic system showed low algae growth rates even when total pesticide concentration was below 2.5 µg/l and when several pesticide concentrations were above of their individual limits 0.5 µg/l (accordingly to the EU Directive 2008/105/EC and to the Portuguese law DL 236/38 and its annex documents). This supports the inclusion of toxicity bioassays in monitoring processes in aquatic ecosystems, performed in situ but also in the laboratory, providing complementary and sometimes crucial information. In our opinion, the WFD would beneficiate if this kind of approaches were included as they provide extra information compared to the one provided by chemical analysis, biodiversity indexes and hydrological datasets.

Mixture toxicity is presently not included in the risk assessment procedure of the WFD. Nevertheless, this directive establishes that the impact of chemicals in the environment must be sustainable in order to achieve a good ecological quality. Moreover, taking into account the objective of the WFD for the protection of water ecosystems as a whole, and not merely based on safe concentrations for individual chemicals, it would be a great step to consider the mixtures toxicity approach in this legislation since the simultaneous presence of several chemical compounds is typical of environmental exposure and that the effects of chemicals on ecosystems and human health mainly comes from exposures to mixtures rather than from individual chemicals. Syberg et al. (2009) concluded that although most chemical regulation is based on a single substance approach, is scientifically possible and regulatory practicable to integrate a more comprehensive mixture toxicity approach into the European legislations.

The synergies obtained in this study demonstrate once again that the simultaneous presence of several toxic in the aquatic environment may lead to increases in toxicity and therefore cause more devastating effects on ecosystems and human health.

3.4. Conclusion

Our study supports the usefulness of the reference models concentration addition and independent action and their possible deviations to ecological risk assessment of mixtures at low concentrations in real aquatic ecosystems.

Here dose ratio was the common deviation obtained when testing both reference models. In the binary mixtures between atrazine/simazine and atrazine/terbuthylazine, the increase of the mixtures toxicity (synergism) was mainly due to the presence of atrazine. Also, in the binary mixture between atrazine and metolachlor, atrazine was responsible for the increase (synergism) of the mixture toxicity, concluding that atrazine accounted for the toxicity of the mixtures where it was involved. For binary mixtures between simazine/metolachlor and terbuthylazine/metolachlor, the increase of the mixtures toxicity (synergism) was detected when metolachlor was the herbicide dominant, and antagonism was detected when simazine and terbuthylazine were dominants in both mixtures.

In this study atrazine showed to be possibly the responsible for the high inhibition growths of algae obtained while testing the body masses of Alqueva as in our experiments synergistic patterns were always associated with its dominant presence. Although this pattern is not referred elsewhere, atrazine is one of the prioritised pesticides reported in the EU legislations, given even a higher support to our concerns and patterns of algae response.

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Chapter 4

Cholinesterases' characterization in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity

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4. Cholinesterases' characterization in *Chironomus riparius* and the effects of three herbicides on chlorpyrifos toxicity

Abstract

In this study, the toxicity of four pesticides (atrazine, terbuthylazine, metolachlor and chlorpyrifos) previously detected in the Alqueva reservoir/dam (south of Portugal) were evaluated individually and in binary combinations using fourth-instar larvae of the aquatic midge *Chironomus riparius*.

Chlorpyrifos induced toxicity to midges in all the 48 hours toxicity bioassays performed. The swimming behaviour of the larvae was impaired, with EC_{50} values ranging from 0.15 to 0.17 µg/L.

However, neither *s*-triazine (atrazine and terbuthylazine) herbicides nor metolachlor alone at concentrations up to 200 µg/l caused significant toxicity to *C. riparius*. When combined with both *s*-triazine herbicides, chlorpyrifos toxicity was enhanced by approximately 2-fold when tested in a binary mixture experimental setup, at the 50% effective concentration levels.

To evaluate how chlorpyrifos toxicity was being increased, the biochemical characterization of cholinesterases (ChE) was investigated with different substrates and selective inhibitors. The results obtained suggested that the main enzyme present in this species is acetylcholinesterase (AChE) and therefore it was assayed upon *C. riparius* exposures to all pesticides individually and as binary mixtures. Although atrazine and terbuthylazine are not effective inhibitors of AChE, the potentiation of chlorpyrifos toxicity by the two *s*-triazine herbicides was associated with a potentiation in the inhibition of AChE in midges; both *s*-triazine herbicides at 200 µg/l increased the inhibition of the AChE activity by 7 and 8-fold, respectively. A strong correlation was observed between larvae swimming behaviour disturbances and the inhibition of the AChE activity. In contrast, metolachlor did not affect chlorpyrifos toxicity at any of the concentrations tested. Therefore, the herbicides atrazine and terbuthylazine can act as synergists in the presence of chlorpyrifos, increasing the toxicity and consequently underestimating risk based on single chemical levels.

Keywords: Swimming behaviour; Acetylcholinesterase; *Chironomus riparius*; Pesticide mixture; Synergistic ratio

4.1. Introduction

Contaminants in the aquatic environment rarely occur as individual chemicals, but rather as mixtures of different substances at relatively low concentrations. Thus, when evaluating the environmental effects of pesticides, it is important to consider chemical mixtures. Usually a

component-based or bottom-up approach is used in the risk assessment of chemical mixtures. This approach is based on predicting and assessing the toxicity of mixtures of known chemical composition on the basis of the toxicity of the single compounds (Van Gestel et al., 2010). One example of a chemical cocktail previously characterized was the water of the Alqueva reservoir/dam (south of Portugal) (Pérez et al. 2010), showing some level of (eco) toxicity. This study showed that water samples were showing high toxicity levels to algae mainly due to the presence of significant concentrations of several herbicides such as atrazine (ATR), terbuthylazine (TER) and metolachlor (MET). *s*-Triazine herbicides such as ATR and TER are (still) widely used in agricultural areas acting as photosynthesis inhibitors through inhibition of photosystem II in target plants, although atrazine has already been banned. These herbicides are among the most commonly detected pesticides in surface and ground waters as a result of agricultural runoff (Wacksman et al., 2006). On the other hand, the herbicide metolachlor is a chloroacetanilide herbicide that acts by inhibiting protein synthesis in susceptible plants (Jin-Clark et al., 2008). In the Alqueva reservoir the concentrations of these herbicides were above their maximum admissible concentration (MAC) allowed by Portuguese Legislation for surface waters (Decreto-Lei no 236/98, 1998) in some sampling areas/time periods. In addition, the insecticide chlorpyrifos (CPF) was also detected in the water of the Alqueva reservoir. This organophosphate insecticide is commonly used to control crop pest, by inhibiting acetylcholinesterase (AChE) activity and affecting neuromuscular functions of target species. Their toxic effects are caused by the chemical disruption of the normal nervous system function due to an excessive accumulation of acetylcholine in the synapse area, leading to rapid muscular twitching and paralysis in the affected animals; low-level exposures to CPF have been associated in several changes in behavioural and physiological patterns of the coho salmon (Oncorhynchus kisutch) (Sandahl et al., 2005).

Regarding the possible and probable co-existence of several pesticides in aquatic systems, a series of previous investigations demonstrated that ATR, even at lower concentrations considered not ecologically harmful, can increase the expected toxicity of certain organophosphate insecticides to several invertebrate species (Anderson and Lydy, 2002; Belden and Lydy, 2000; Jin-Clark et al., 2002; Pape-Lindstrom and Lydy, 1997). The increase toxicity observed with the addition of ATR is presumed to be due to the induction of the cytochrome P450 activity by the herbicide (Londoño et al., 2004; Miota et al., 2000), which subsequently increases the conversion of the organophosphate to the more potent AChE inhibitor oxon-analogs.

Although cholinesterase activity is widely used as a specific biomarker to monitor the effects of anticholinesterase, its use in a particular species requires the characterization of the enzyme(s) present. Vertebrate cholinesterases are traditionally divided into two classes: AChE and

butyrylcholinesterase or pseudocholinesterase (BChE). The two enzymes may be distinguished functionally, primarily on the basis of substrate specificity and according to their susceptibility to selective inhibitors. ChE characterization is important since distinct ChE may have different sensitivities for anti-cholinesterase agents and thus this may introduce biases when assessing the effects of anti-cholinesterase agents in ecotoxicological studies. *Chironomus riparius* ChEs have been previously assayed by Detra and Collins in 1986 and conclusions were drawn based on acetylthiocholine and aceto-ß-methylthiocholine as the best substrates to use. But no further conclusions were derived.

Therefore, and regarding the possible interactions between pesticides found previously in the water of the Alqueva reservoir, the present study aims at carrying out a bottom-up approach with the following goals: (1) study the toxicity of the four pesticides using as endpoint the chironomids swimming behaviour; (2) characterize biochemically the ChE present in whole body homogenate of *Chironomus riparius* midge (Diptera: Chironomidae), using selective inhibitors and different substrates, and use this parameter and swimming behaviour response to (3) evaluate the combined effects of the herbicides (ATR, TER and MET) and the insecticide CPF. This approach will enable to test the hypothesis that the presence of herbicides (e.g. atrazine, terbuthylazine and metolachlor) will induce no interaction with CPF toxicity.

4.2. Materials and methods

4.2.1. Test-organism and test-chemicals

Chironomus riparius midges were obtained from cultures established at the Department of Biology, University of Aveiro (Portugal). The cultures are maintained in an enclosed transparent acrylic box containing several plastic beakers holding a 2 cm layer of acid-washed and burned commercial sand (<1 mm), and approximately 2.5 L of reconstituted hard water ASTM (ASTM, 2000). A gentle aeration was provided in each beaker. This system permits the occurrence of the whole life cycle of chironomids, by allowing the swarming and copulation of adults (OECD, 2004). The culture was maintained in standard conditions, at 20±2°C and with a 16:8 h light: dark photoperiod. Freshly laid egg masses were transferred to crystallising dishes with culture medium until hatching, and the first instar larvae were used either to start a new culture or a bioassays. Water and sediment were renewed every week and the larvae fed (1mg/animal/day) twice a week with a suspension of ground TetraMin® (Tetrawerke, Germany). Fourth-instar larvae were harvested directly from the cultures and used in all bioassays. The larvae were considered to be fourth instar when the head capsule width 0.63 to 0.71 mm wide and the body length was ≥1 cm.

The reagents used for the ChE characterization namely: Acetylthiocholine iodide (AcSCh), s-butyrylthiocholine iodide (BuSCh), propionylthiocholine iodide (PrSCh), eserine hemisulfate, 1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide (BW284C51), tetraisopropyl pyrophosphoramide (iso-OMPA) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich Laboratory; Bradford reagent was obtained from Bio-Rad (Germany).

All pesticides tested were also purchased from Sigma-Aldrich Laboratory in the highest available purity: atrazine (Chemical Abstracts Service [CAS] no. 1912-24-9) with 97.4%, terbuthylazine (CAS 5915-41-3) with 98.6%, metolachlor (CAS 51218-45-2) with 97.1% and chlorpyrifos (CAS 2921-88-2) with 98% of purity. Some of the physicochemical properties of these pesticides are given in Table 4.1. Pesticide quantification was conducted by Marchwood Scientific Services (Southampton, UK) using liquid chromatography-tandem mass spectrometry LCMS-MS (instrument Agilent 6410 Triple Quad LCMs-MS).

Chemical analyses were performed on the stock solutions, as well as on the lowest and highest concentrations of each pesticide, only at the beginning of the tests. These restrictions on the chemical analysis were due to the short time (48 hours) tests and to the relative stability of these pesticides in water (Table 4.1); therefore, it was assumed that their concentrations were kept constant throughout the tests.

Table 4. 1. Physicochemical properties of ATR, TER, MET and CPF (obtained from FOOTPRINT Pesticide Properties Database).

Pesticides	Atrazine	Terbuthylazine	Metolachlor	Chlorpyrifos
Water solubility	35 mg/l (20˚C)	6.6 mg/l (20˚C)	530 mg/l (20˚C)	1.05 mg/l (20˚C)
Log Kow	2.7 (20˚C, pH 7)	3.4 (20˚C, pH 7)	3.4 (20˚C, pH 7)	4.7 (20˚C, pH 7)
Photolysis rate	2.6 days (pH 7)	Stable	Stable	29.6 days (pH 7)
Hydrolysis rate	86 days (20˚C, pH 7)	Stable, very persistent	Stable, very persistent	25.5 days (20˚C, pH 7)

4.2.2. Cholinesterase characterization and determination

ChE characterization was performed using different substrates and specific inhibitors. *C. riparius* ChE preference of substrate were investigated by determining the enzymatic activity at increasing concentrations (from 0.005 to 20.48 mM) of the substrates AcSCh, BuSCh and PrSCh, in independent experiments. Eserine sulphate, iso-OMPA and BW284C51 were used as specific inhibitors of ChE, BChE and AChE, respectively. For each inhibitor, stock solutions were prepared in ultrapure water or ethanol, as appropriate.

After testing substrate preference, assays were carried out to evaluate ChE inhibitors; for each inhibitor, 5 µl of each stock solution were incubated with 495 µl of homogenate sample extract for 30 min at 25ºC before the addition of the substrate. In these experiments, all enzymatic activities

were determined using AcSCh as substrate, because previously the highest activity was found using AcSCh as substrate. For Eserine and BW284C51, the concentrations tested were 6.25; 12.5; 25; 50; 100 and 200 µM and for iso-OMPA were 0.25; 0.5; 1; 2; 4 and 8 mM. Controls were incubated with 5µl of ultra-pure water and an additional control with ethanol was used in the experiments with iso-OMPA.

The fourth-instar larvae were homogenized (Ystral GmbH D-7801) in ice-cold 0.1 M phosphate buffer (pH 7.2). The homogenates' supernatant obtained after centrifugation (4˚C, 3800 g, and 4 min) were used as an enzyme extract for ChE activity determinations, which were performed using the method described by Ellman et al. (1961), adapted to microplate (Guilhermino et al., 1996). The enzymatic activity was then measured using a Lab system Multiskan EX microplate reader at a wavelength of 414 nm using 0.05 mL of supernatant and 0.25 mL of the reaction solution (1 mL of 10 mM 5.50-dithiobis-2-nitrobenzoic acid (DTNB) solution, 0.2 mL of 0.075 M acetylcholine solution and 30 mL of 0.1 M phosphate buffer).

All enzymatic activities were determined in quadruplicate and expressed as units (U) per mg of protein. A U is a nmol of substrate hydrolyzed per minute.

Protein concentrations before and after each enzymatic analysis were also determined in quadruplicate by the Bradford method (1976), at a wavelength of 595 nm, using γ-globulin as standard.

4.2.3. Toxicity tests with individual pesticides and pesticide mixtures

The toxicity tests were performed using six nominal concentrations of CPF and four nominal concentrations of each herbicide with three replicate each. Forty-eight hour static toxicity tests were conducted using 10 fourth-instar larvae of *C. riparius* per replicate, at 20±2˚C in an experimental chamber using a photoperiod of 16:8 h light: dark. Solvent and negative controls were also tested in triplicate. The experiment was performed using glass beakers contained 500 ml of test solution and 20 g of acid-washed inorganic fine sand (<1 mm) to allow the animals to escape from other midge larvae, due to its cannibalistic nature and to provide substrate for the midges to build cases. Test solutions were prepared by diluting stock solutions in reconstituted water (ASTM, 2000). Stock solutions were prepared using acetone (100 µl/L) as the carrier solvent. The reconstituted water and sand used for testing were the same as used in the cultures. Water parameters (temperature, dissolved oxygen and pH) were monitored at the beginning and end of the test. For individual chemical exposures, the endpoint chosen was based on the ability of larvae to perform three normal figure-eight swimming motions when pinched with a pair of forceps. This

behavioural endpoint has been used in previous studies (Belden and Lydy, 2000; Pape-Lindstrom and Lydy, 1997).

In order to assess the combined effects of the herbicides (ATR, TER and MET) and the insecticide CPF, fifteen fourth-instar larvae per replicates were exposed to the EC_{50} level of CPF (predetermined from the single toxicity test) alone and in combination with four levels of each herbicides (25, 50, 100 and 200 µg/l). Negative controls, solvent controls, and herbicide-only controls were conducted in all tests. Toxicant exposure methods were the same as those previously described for the single pesticides exposures. In addition to the mobility parameter used in the single exposures, AChE activity was also measured. For that, at the end of the tests the live larvae were collected and clusters of five were frozen in ice-cold 0.1 M phosphate buffer (pH 7.2) and immediately stored at -80°C until AChE activity analyses.

4.2.4. Acetylcholinesterase activity bioassays

All larvae samples (clusters of five larvae per replicate) were homogenized in ice-cold potassium phosphate buffer (0.1 M, pH 7.2). The supernatant obtained after centrifugation (4˚C, 3800 g, and 4 min) was used as enzyme extract for AChE activity determinations, which were performed as described in 4.2.2.

4.2.5. Data analysis

Data of ChE characterization were analysed using a one-way Analysis of Variance (ANOVA) using Sigma-Stat statistical package (Systat, 2006) in order to determine significant differences between substrates or inhibitors and the respective control ($p<0.05$), followed by a Dunnet method to individualise differences. Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on ranks was performed, followed by a Dunn's method when significant differences were found. Enzyme kinetic parameters Vmax (maximum rate of hydrolysis reached when the enzyme is saturated with substrate) and Km (substrate affinity-concentration needed to reach one-half of the maximum velocity) were estimated by fitting experimental data to the Michaelis-Menten equation.

Log-probit analysis (Minitab-15., 2007) was used to estimate the EC_{50} values for the swimming behaviour response for each pesticide in the single experiments. Statistical analysis of the mixture data were also conducted using One-Way ANOVA. Comparisons among treatment classes were carried out by Tukey's test when significant differences were found. The null hypothesis was that the herbicide concentration had no effect on CPF toxicity. Thus, a significant F statistic ($p<0.05$)

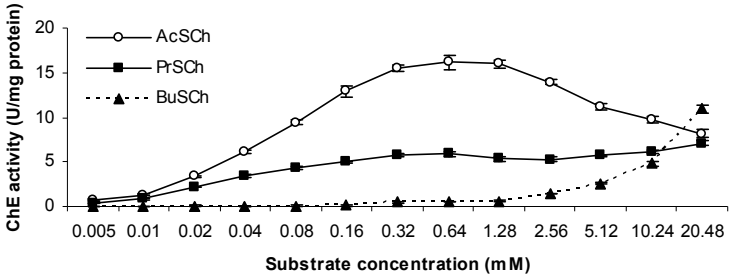
for the herbicide treatments indicated greater or less toxicity than expected in the test. In addition, the EC_{50} value was estimated for each herbicide and CPF treatment using log-probit analysis for the swimming behaviour data and by a three-parameter Logistic regression curve for the AChE activity data. Using these values, synergistic ratios were calculated as the ratio between the EC_{50} value for CPF (without herbicide) and the EC_{50} values for each of the herbicide and CPF treatments. Synergistic ratios of 1.0 indicate no effects of the herbicides on CPF toxicity (or an additivity of responses), whereas values >1.0 and <1.0 indicate greater and weaker effects than expected, respectively. The significant differences between the EC_{50} values of the CPF and its binary combinations with the herbicides were based on the non-overlapping of their 95 % confidence intervals.

4.3. Results

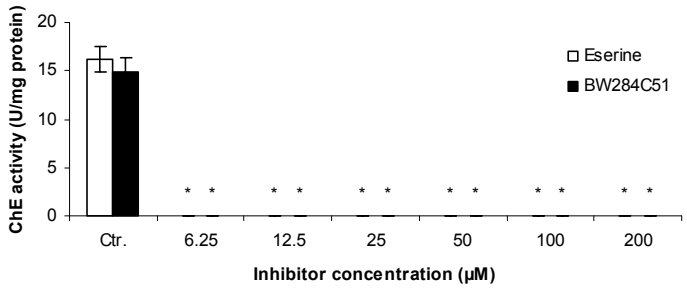
4.3.1. Cholinesterase characterization

ChE activity of *C. riparius* as a function of increasing concentrations of AcSCh, PrSCh and BuSCh is showed in Fig. 4.1a. The highest activity of ChE was obtained with AcSCh (16.15 ± 0.82 U/mg protein at 0.64 mM). Lower activities were observed when PrSCh (7.06 ± 0.25 U/mg protein at 20.48 mM) and BuSCh (10.99 ± 0.45 U/mg protein at 20.48 mM) were used as substrates. The apparent Km and Vmax values for the AcSCh substrate were 0.14 mM and 19.57 U/mg proteins, respectively. Regarding eserine sulphate, general inhibitor of ChE, and BW284C51, selective inhibitor of AChE, they completely inhibited the activity of *C. riparius* ChE (Kruskal-Wallis: H = 19.860, p = 0.003) even at the lowest concentration tested (6.25 µM) (Fig. 4.1b). No significant effects were observed regarding the use of the BuChE inhibitor, iso-OMPA, on ChE activity when compared to the control (Fig. 4.1c).

a)



b)



c)

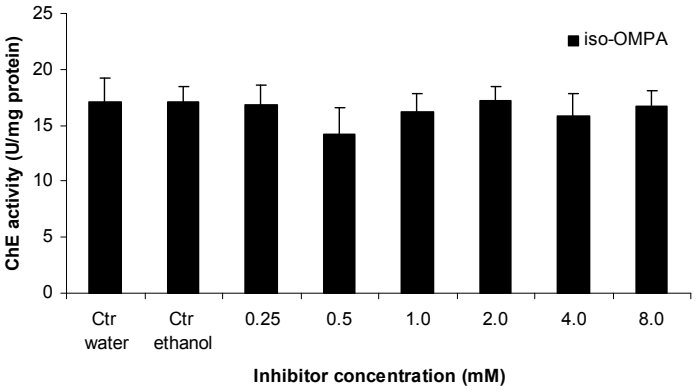


Figure 4. 1. Cholinesterase characterization assay for *Chironomus riparius*: (a) effect of substrate concentration on ChE activity; (b) effect of eserine and BW284C51 on ChE activity with substrate AcSCh; (c) effect of iso-OMPA on ChE activity with substrate AcSCh. Results are expressed as the mean ± standard error.* Significant difference from control (p<0.05).

4.3.2. Chemical analysis and water parameters

To assess dosing accuracy at the beginning of all tests, ATR, TER, MET and CPF quantifications were made and results showed that measured concentrations varied generally less than 20 % from the nominal concentrations. So, all calculations were based on nominal concentrations.

Water parameters monitored at the beginning and end of the bioassays were similar for all tests in this study. Temperature was maintained at 20±2˚C. Dissolved oxygen levels were > 80% and pH was maintained in the range of 7.3 to 7.8.

4.3.3. Individual toxicity tests

Chlorpyrifos was toxic to midges in all single tests performed. The EC_{50} values were calculated for 1) the swimming behaviour for the single experimental set up and for the single exposures carried out under the mixture experimental setup; and for 2) the AChE activity from single exposures carried out simultaneously within the mixture experiments. The values obtained were consistent and ranged from 0.15 to 0.17 µg/l and from 0.10 to 0.13 µg/l for the respective endpoints (Table 4.2). On the other hand, herbicides were not toxic regarding the concentrations tested in the present study. Although there were no significant effects caused by the herbicides on larvae swimming behaviour and the AChE activity, this enzyme was inhibited by ATR, TER and MET highest concentrations at 27 ± 8.6%, 39 ± 2.2% and 17 ± 0.8% levels, respectively (Fig. 4.2 a, b and c).

4.3.4. Effects of the pesticide combinations on the swimming behaviour and the AChE activity

ATR and TER up to 200 µg/l did not show any effect to the swimming behaviour of the larvae when tested alone. However, combined exposure of 200 µg ATR/L with the calculated EC_{50} of CPF (0.17 µg/l) significantly increased the percentage of larvae affected when compared to CPF alone at a EC_{50} level, with an effect from 73 ± 6.7% to 100% in their ability to swim ($F_{5,12} = 168$, $p<0.05$) (Fig. 4.2a). Moreover, TER concentrations of 50, 100 and 200 µg/l in combination with CPF also increased significantly the percentage of midges affected as compared with this insecticide alone ($F_{5,12} = 156$, $p<0.05$) (Fig. 4.2b). The effects of these *s*-triazine herbicides were also supported by the synergistic ratios calculated from the EC_{50} values and their 95% confident intervals (Table 4.3). ATR had the largest magnitude of effects on the toxicity of CPF with significant SR values of 1.88, 1.67 and 1.88 at the concentrations of 50, 100 and 200µg/L, respectively. In addition, TER had significant SR values of 1.33 and 1.67 at the concentrations of 100 and 200µg/L, respectively. In contrast, MET in binary mixture with CPF did not significantly affect the swimming behaviour of larvae compared with CPF alone at any of the concentration tested ($F_{5,12} = 21$, $p > 0.05$) (Fig. 4.2c). This was also confirmed by the synergistic ratio around 1 obtained for the mixtures of CPF and MET, showing no changes on the EC_{50} values for CPF (Table 4.3).

Table 4.2. EC_{50} values (µg/l) for the swimming behaviour response and AChE activity of *Chironomus riparius* after 48 hours of exposure. Values were calculated from single exposure tests and from single exposure sets run simultaneously with mixture exposures. Values in brackets refer to standard errors (SE).

Pesticides	Swimming behaviour	AChE activity	Experiments
Chlorpyrifos	0.17 (0.01)	-	Single exposure
	0.15 (0.02)	0.10 (0.01)	Mixture exposure with atrazine
	0.16 (0.01)	0.12 (0.01)	Mixture exposure with terbuthylazine
	0.15 (0.01)	0.13 (0.01)	Mixture exposure with metolachlor
Atrazine	>200	>200	Mixture exposure with chlorpyrifos
Terbuthylazine	>200	>200	Mixture exposure with chlorpyrifos
Metolachlor	>200	>200	Mixture exposure with chlorpyrifos

(-) not applicable

Table 4.3. EC_{50} (± 95% CIs) values (µg/l) and synergistic ratio for the swimming behaviour of *Chironomus riparius* calculated for chlorpyrifos when co-occurring with each concentration of herbicide in the mixture experiments.

	Concentrations of herbicides (µg/L)				
Herbicides	0	25	50	100	200
Atrazine					
EC_{50}	0.15 (0.12-0.18)	0.12 (0.11-0.13)	0.08 (0.07-0.11)	0.09 (0.07-0.12)	0.08 (0.07-0.11)
SR		1.25	1.88*	1.67*	1.88*
Terbuthylazine					
EC_{50}	0.16 (0.15-0.18)	0.16 (0.15-0.18)	0.14 (0.13-0.16)	0.12 (0.10-0.13)	0.10 (0.08-0.11)
SR		1.00	1.14	1.33*	1.67*
Metolachlor					
EC_{50}	0.15 (0.14-0.17)	0.17 (0.15-0.20)	0.16 (0.15-0.17)	0.14 (0.12-0.15)	0.14 (0.12-0.15)
SR		0.88	0.94	1.07	1.07

SR = EC_{50} (CPF without herbicide) / EC_{50} (CPF and herbicide treatments). The asterisk next to SR indicates a significant difference between the EC_{50s} of the CPF and its binary combinations with the herbicides based on the non-overlapping 95% CIs of the EC_{50} values.

The AChE activity of the larvae exposed to the EC_{50} value of CPF alone and in combination with each herbicide treatments of 25, 50, 100 and 200 µg/l are also shown in Fig. 4.2. All ATR concentrations in combination with CPF at 0.17 µg/l caused a significant reduction in AChE activity when compared with CPF alone ($F_{6,14}$ = 97, p<0.05) (Fig. 4.2a), this observation was sustained by the significant SR values shown in Table 4.4. TER at 100 and 200 µg/l in combination with 0.17 µg CPF/L also inhibited significantly the AChE activity of the midges ($F_{6,14}$ = 96, p<0.05) when compared with CPF alone (Fig. 4.2b), which was also supported by the significant SR values calculated (Table 4.4). In contrast, MET in binary mixture with CPF did not change the response pattern of the enzyme activity when exposed to CPF alone ($F_{6,14}$ = 20 p > 0.05) (Fig. 4.2c) and this observation was also supported by SR around 1 (Table 4.4).

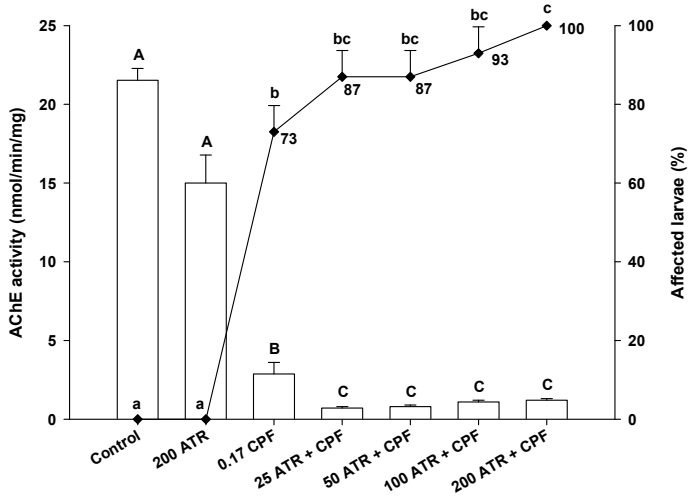
Table 4.4. EC_{50} (± 95% CIs) values (µg/l) and synergistic ratio for the AChE activity of *Chironomus riparius* calculated for chlorpyrifos when co-occurring with each concentration of herbicide in the mixture experiments.

	Concentrations of herbicides (µg/L)				
Herbicides	0	25	50	100	200
Atrazine					
EC_{50}	0.10 (0.08-.11)	0.11 (0.10-0.11)	0.09 (0.06-0.10)	0.02 (0.003-0.06)	0.01 (0.02-0.05)
SR		0.90	1.13	4.79*	6.77*
Terbuthylazine					
EC_{50}	0.12 (0.10-.13)	0.12 (011-0.12)	0.06 (0.03-0.09)	0.04 (0.01-0.08)	0.01 (0.02-0.04)
SR		0.98	2.11*	2.92*	8.31*
Metolachlor					
EC_{50}	0.13 (0.12-.14)	0.13 (0.10-0.14)	0.13 (0.10-0.14)	0.12 (0.10-0.13)	0.13 (0.11-0.14)
SR		1.05	1.04	1.09	1.07

SR = EC_{50} (CPF without herbicide) / EC_{50} (CPF and herbicide treatments). The asterisk next to SR indicates a significant difference between the EC_{50s} of the CPF and its binary combinations with the herbicides based on the non-overlapping 95% CIs of the EC_{50} values.

In this study there was a very high negative correlation between the swimming ability and the AChE activity of the larvae for all mixtures tested. The Pearson correlation coefficient (r) was -0.93 (p<0.05) for the mixture of ATR and CPF, -0.91 (p<0.05) for the mixture of TER and CPF and -0.90 (p<0.05) for the mixture of MET and CPF.

a)



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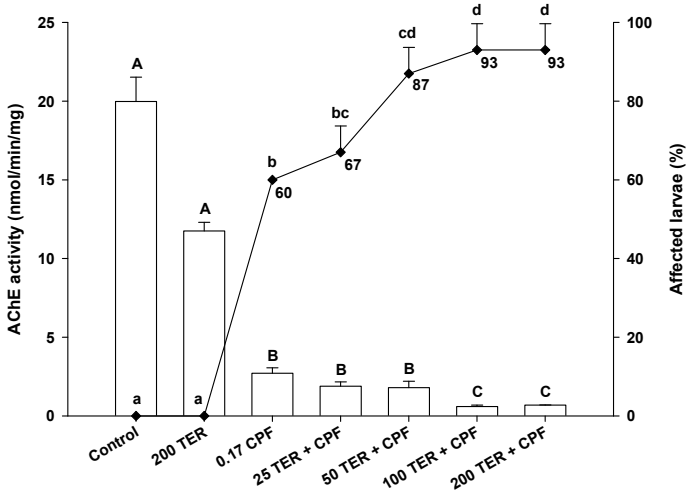

c)

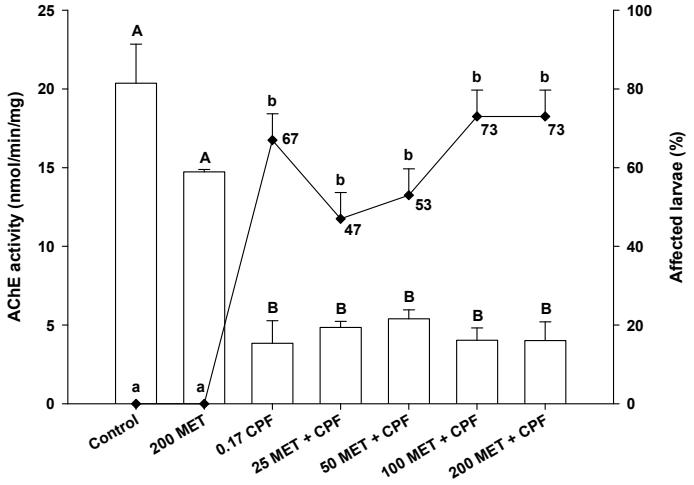

Figure 4. 2. Effects of single and combined exposures of chlorpyrifos and atrazine (a), terbuthylazine (b) or metolachlor (c) on the swimming behaviour (lines) and AChE activity (bars) of fourth-instar *C. riparius* (mean ±standard error). Values correspond to the percentage of larvae with swimming behaviour affected. Different letters on the treatments indicate significantly differences among treatments ($p < 0.05$), using Tukey's test comparisons.

4.4. Discussion

4.4.1. Cholinesterase characterization

The measurement of ChE inhibition is widely used to assess exposure and/or the effects of pesticides in aquatic invertebrates. However, distinct enzymatic isoforms with different sensitivities towards anticholinergic contaminants may exist (Xuereb et al., 2007), and it should be accurately evaluated. In this study, a full inhibition (100%) of ChE by eserine hemisulfate, even at the lower concentration tested, was found indicating that the measured activity is predominantly from ChE and not from other esterases. The highest ChE activity of *C. riparius* was obtained with AcSCh, as also reported by Detra et al. (1986) an optimum concentration for this substrate of 0.64 mM was obtained for acetylthiocholine. Furthermore, in the present study a full inhibition (100%) occurred with BW284C51 while no significant inhibition was observed with iso-OMPA. Thus, it seems that the main form present in this species is AChE. The level of AChE activity obtained for this Chironomidae species (16.15 ± 0.82 U/mg protein at 0.64 mM) was similar to those reported in the literature for several aquatic invertebrates (between 9 and 20 U/mg protein) (Callaghan et al., 2002; Diamantino et al., 2003; Guilhermino et al., 2000). Similar AChE activities values have been also reported for other invertebrates, like 20-45 U/mg protein for annelids Eisenia andrei (Caselli et al., 2006), Nereis diversicolor (Scaps and Borot, 2000). The Km value for AcSCh in this study was 0.14 mM, which is similar to the one found for E. andrei (0.18 mM) (Caselli et al., 2006). In addition, low Km values represent high ChE affinity to the substrate used. Therefore, the ChE activity for *C. riparius* presented in this study shows that there is a high affinity for this enzyme to the substrate AcSCh.

4.4.2. Toxicity tests with individual pesticides and pesticide mixtures

The herbicides used in this study showed no toxicity to *C. riparius* when tested alone at concentrations up to 200 µg/l. However, mixtures of CPF and ATR at 50, 100, 200 µg/l resulted in approximately a two-fold increase in toxicity (SR= 1.88, 1.67 and 1.88, respectively) compared with CPF alone. In addition, in the mixture between CPF and TER at the concentrations of 100 and 200µg/L the toxicity of the insecticide was significantly increased by 1.33 and 1.67-fold respectively. Several previous studies have demonstrated that ATR caused also a two to seven-fold increase in organophosphate insecticides (OPs) toxicity to selected aquatic invertebrates including chironomids (Anderson and Lydy, 2002; Anderson and Zhu, 2004; Belden and Lydy, 2000; Jin-Clark et al., 2002; Lydy and Linck, 2003; Pape-Lindstrom and Lydy, 1997). Our results supported these previous studies. Moreover, our study is the first reporting the potentiation of CPF toxicity by TER *s*-triazine herbicide in *C. riparius*.

The mechanism by which *s*-triazine herbicides increase the toxicity of the OPs in midges is speculated to be due to the induction of cytochrome P450 activity by the herbicide (Londoño et al., 2004; Miota et al., 2000), which subsequently increases conversion of the OP to the more potent AChE inhibitor oxon-analogs. Several researches have linked the induction of the P450 system to increased biotransformation of OPs in turn leading to increased concentrations of the more potent AChE inhibitor oxon metabolite. For example, Belden and Lydy (2000) showed increased biotransformation of chlorpyrifos to the toxic oxon form in the presence of atrazine by the midge *Chironomus tentans*. Similarly, Jin-Clark et al. (2002) demonstrated that both atrazine and cyanazine herbicides caused significant inhibition of AChE in a dose-dependent manner in the presence of CPF. Anderson and Lydy (2002) determined that AChE activity was around 40% less at the LC_1 concentrations of chlorpyrifos and diazinon in the presence of atrazine compared with the OPs alone. Moreover, Schuler et al. (2005) found that the *s*-triazine herbicides (atrazine, simazine, cyanazine, and hexazinone) were also capable of potentiating the toxicity of chlorpyrifos and diazinon.

Since CPF is a potent AChE inhibitor, in this study the quantification of AChE inhibition was used as a bioindicator of exposure. Previous studies on the midge *C. tentans* indicated that ATR alone does not significantly alter AChE activity. However, when larvae were exposed to a binary mixture of ATR and CPF, AChE levels were significantly lower than for those organisms exposed to CPF alone (Belden and Lydy, 2001). In our study the inhibition of the AChE activity by the herbicides alone was also not significant. However, similar results to the previous studies were obtained when CPF was mixed with ATR and TER. ATR significantly increased in 4.79 and 6.77-fold the toxicity of CPF at the concentrations of 100 and 200µg/L, respectively. And TER at the concentrations of 50, 100 and 200 µg/L also increased significantly the toxicity the CPF in 2.11, 2.92 and 8.31-fold, respectively.

Based on the results obtained in this study and also on previous research, we can hypothesise that ATR and TER can be inducing cytochrome P450 enzymes responsible for oxidative activation of CPF to CPF-oxon. Because CPF-oxon is a much more potent AChE inhibitor than its parent compound, the accelerated activation process for CPF-oxon could finally result in the increase of the inhibition of AChE, thereby causing greater toxicity to midges than expected based on their individual toxicity, as observed in the current study.

In addition to *s*-triazine herbicides tested in this study, the herbicide MET was also examined in combination with CPF, but in this case there were no significant effects observed on the swimming behaviour and AChE activity at any concentrations tested. Contrary to this result, potentiation of

CPF toxicity was obtained in the study of Jin-Clark et al. (2008) where it was suggested that the potentiation was caused and associated with the decreased protein synthesis in MET-treated midges which led to the reduction of specific detoxification enzymes such as cytochrome P450 and GST, and consequently impeding the metabolic detoxification of CPF and increasing the susceptibility of the midges to this insecticide. Therefore, based on this previous research, we can assume that in our study MET also decreased the synthesis of detoxifying enzymes thereby preventing the oxidative activation of CPF to CPF-oxon. Hence, in this case the toxicity was mainly induced by CPF and not due to CPF-oxon as occurs with the *s*-triazine-treated midges.

In our study there was a very high negative correlation between the increase of the swimming disturbance and the decrease of the AChE activity of the larvae in all mixture combinations tested. This pattern and correlation were also observed for *Danio rerio* larvae exposed to CPF and the *s*-triazine herbicides ATR and TER (Pérez et al., 2013). These significant correlations supported the relationship that exists between the swimming disturbances of the larvae as a consequence of the inhibition of the AChE activity. Continuous stimulation of the nervous system by xenobiotics produces uncontrollable muscle tremors which reduce locomotion and therefore survival in natural environments since larvae would be more vulnerable to capture by predators, which in turn would cause significant impacts on the population and community level (Dell'Omo, 2002).

These results can explain biomonitoring results already published for aquatic systems where low levels of insecticides and herbicides induce toxicity to model test organisms (Pérez et al., 2010). Although the concentrations used in the present study are higher than those that can be found in the environment, one cannot disregard that this are model studies, to evaluate patterns and that safety factors have to be applied in order to extrapolate to real scenarios. In addition, in the field several other factors are to take into account as the water characteristics or the surrounding factors (e.g. temperature, dissolved oxygen, dissolved organic matter) can interfere with organisms sensitivity and therefore increase toxicity.

4.5. Conclusions

In conclusion, our results indicate that the predominant ChE form in *C. riparius* midges is AChE. Moreover, this work supports the previous studies where potentiation of the CPF toxicity was observed in mixtures with *s*-triazine herbicides. Here, the herbicides did not cause significant toxic effects on *C. riparius* when tested alone. However, the co-exposure of CPF and the herbicides ATR and TER caused an increase of CPF toxicity. The results of this study show that TER can also potentiate the toxicity of CPF indicating that this is not only an effect associated to the ATR

herbicide. But on the other hand it is not a general pattern for herbicides as MET did not potentiate the inhibition of AChE in chironomids.

These results highlights once again that the simultaneous presence of several chemicals in the aquatic environment (even with different mechanisms of action) may lead to increases in toxicity, causing more disturbing effects on the aquatic ecosystems than expected, regarding their single toxicity.

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Chapter 5

Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early life stages of the zebrafish *Danio rerio*

Pérez J., Domingues I., Monteiro M., Soares A.M.V.M., Loureiro S. (2013) Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early life stages of the zebrafish *Danio rerio*. Environmental Science and Pollution Research 20(7): 4671-4680.

5. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early life stages of the zebrafish *Danio rerio*

Abstract

This study examined the effects of three widely used pesticides that have been previously detected in aquatic systems neighbouring agricultural fields on the early life stages of the zebrafish *Danio rerio*. Tests involving single exposures and binary combinations of the *s*-triazine herbicides (atrazine and terbuthylazine) and the organophosphate insecticide chlorpyrifos were performed. Several endpoints, such as swimming behaviour, morphological abnormalities and mortality were studied. In addition, the inhibition of acetylcholinesterase (AChE) activity was investigated in order to evaluate the mode of action and toxicity of chlorpyrifos in the presence of these herbicides. Results indicate that both binary mixtures elicited synergistic responses on the swimming behaviour of zebrafish larvae. Moreover, although the herbicides were not effective inhibitors of the AChE on their own, a synergistic inhibition of the enzyme activity was obtained by exposure to mixtures with chlorpyrifos. We observed a correlation between impairment of swimming behaviour of the larvae and inhibition of AChE activity. This study supports previous studies concerning the risk assessment of mixtures since the toxicity may be underestimated when looking only at the single toxicants and not their mixtures.

Keywords: Swimming behaviour; Acetylcholinesterase; *Danio rerio*; Pesticide mixture; Synergistic ratio; MIXTOX model

5.1. Introduction

In the natural environment, organisms are usually exposed to mixtures of chemicals rather than to a single contaminant. Consequently their simultaneous presence in the aquatic environment may lead to increases in toxicity, therefore cause greater effects on ecosystems and human health than expected.

The risk assessment of complex mixtures can be carried out using two approaches: (1) a “whole-mixture” or “top-down” approach, in which the toxicity of environmental samples containing complex mixtures is assessed using bioassays, alongside an additional study to analyses of which individual compounds drive the observed total toxicity of the sample; and (2) a “component-based” or “bottom-up” approach, based on predicting and assessing the toxicity of mixtures of known chemical composition on the basis of the toxicity of the single compounds, often used to decipher the mechanism of mixture interactions (Van Gestel et al., 2010).

In both such approaches, it is essential to address two concepts of mixture toxicity: the Concentration Addition (CA) and the Independent Action (IA) (Bliss, 1939; Loewe and Muischnek, 1926). The CA model assumes a similarity of the mode of action of chemicals in the mixture, and it is defined as a sum of the relative toxicities of the individual components. On the other hand, IA presupposes a dissimilar mode of action of the chemicals as the starting point (Bliss, 1939). This means that the relative effect of a toxicant remains unchanged in the presence of another chemical. The theoretical assumption behind both models is that chemicals in a mixture do not interact physically, chemically, or biologically, thus assuming that they act independently of each other (Greco et al., 1995). But toxicokinetic and toxicodynamic interactions can occur among chemicals in a mixture, causing deviations from both models and consequently, synergistic or antagonistic behaviour can occur (Andersen and Dennison, 2004).

s-Triazine herbicides such as atrazine (ATR) and terbuthylazine (TER) are widely used in agricultural areas acting as inhibitors of photosynthesis by inhibition of photosystem II in target plants. These herbicides are among the most commonly detected pesticides in surface and ground waters as a result of agricultural runoff (Wacksman et al., 2006). In the Alqueva reservoir, concentrations of these herbicides were above their maximum admissible concentration (MAC) as set by the Portuguese Legislation for surface waters (Decreto-Lei no 236/98, 1998) at certain sampling periods. The insecticide chlorpyrifos (CPF), which was also detected in water of the Alqueva reservoir, is an organophosphate insecticide commonly used to control crop pests. Organophosphate insecticides are known as acetylcholinesterase (AChE) inhibitors. Their toxic effects are caused by the chemical disruption of the normal nervous system function due to an excessive accumulation of acetylcholine in the synapse area. Symptoms of exposure to organophosphate insecticides include muscle twitching, hyperactivity, paralysis, loss of equilibrium and eventually death (Sandahl et al., 2005).

The zebrafish *Danio rerio* has been widely used as a model organism in various ecotoxicology studies (e.g. Domingues et al., 2010; Kienle et al., 2009; Scheil and Köhler, 2009). The use of this species holds several advantages in chemical toxicity evaluation, such as its rapid development, easy maintenance in the laboratory, large number of offspring, transparency of embryos and access to experimental manipulation (Scholz et al., 2008). Nagel (2002) proposed the embryo test with *D. rerio* as a substitute test for the acute test using adult fish. In addition, the use of early life stages in toxicity tests has been recommended as an alternative for the acute tests performed with juveniles or adults, in order to overcome the ethical issues of using large numbers of free-feeding larva, juvenile or adult animals (Lammer et al., 2009).

Results from previous work in the Alqueva reservoir/dam (south of Portugal) showed that looking only at single chemical concentrations will lead us to a completely different assumption on water quality when compared to the (eco)toxicity results obtained (Pérez et al., 2010). Therefore, several questions arose based on the possible interactions among the chemicals present and a bottom-up approach was setup to assess the toxicity effects of binary mixtures of pesticides, using as test model the zebrafish *Danio rerio* (Hamilton-Buchanan 1822) in early life stages (embryos and larvae).

The primary objective of this study was to evaluate the impact of the *s*-triazine herbicides (ATR and TER) on CPF toxicity in early life stages of the *D. rerio*. For that we have first studied the individual effects of the three pesticides and derive the most sensitive endpoint, to be used on the mixture toxicity approach. Additionally, and as the most sensitive endpoint showed to be swimming behaviour, we aimed at examining the variation in AChE activity when organisms were exposed to CPF in combination with the herbicides. Results of this bottom-up study are expected to provide information on the interactions of pesticide mixtures found previously in the Alqueva reservoir, thereby improving the general assessment of pesticide effects on aquatic systems.

5.2. Materials and methods

5.2.1 Test organism and eggs acquisition

The zebrafish (*D. rerio*) research facility established at the Department of Biology, University of Aveiro (Portugal) provided all organisms (zebrafish eggs) used in this study. In the zebrafish facility, organisms were kept in carbon-filtered water supplemented with salt "Instant Ocean Synthetic Sea Salt", at 27.0±1ºC under a 16:8 h light/dark photoperiod cycle. Conductivity was maintained at 750±50 µS/cm, pH at 7.5±0.5 and dissolved oxygen at 95% saturation. This water was monitored daily to ensure quality and it was used as dilution water in the preparation of the test solutions in all tests performed. Fish were fed twice per day with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

Zebrafish eggs were collected with spawn traps (marbles) placed at the bottom of each aquarium the evening before spawning, which were then removed from the aquaria in the morning. Eggs were collected 30 min after spawning, and were then transferred to Petri dishes containing culture water and examined under a stereomicroscope (Stereoscopic Zoom Microscope—SMZ 1500, Nikon) in order to select viable eggs. Unfertilised or damaged eggs were discarded. All tests were initiated as soon as possible after fertilization of the eggs and not later than 3 h post-fertilization (128-cell stage).

5.2.2 Test chemicals

Test pesticides were purchased from Sigma Aldrich, St. Louis, USA with the highest available purity: atrazine (Chemical Abstracts Service [CAS] no. 1912-24-9) with 97.4%, terbuthylazine (CAS 5915-41-3) with 98.6% and chlorpyrifos (CAS 2921-88-2) with 98% of purity.

Pesticide quantification was conducted by Marchwood Scientific Services (Southampton, United Kingdom) using liquid chromatography-tandem mass spectrometry (LSMS-MS). Representative samples (300-500 ml of water) of the stock solutions and exposure water of both herbicides were extracted with 2-3 portions of dichloromethane, and carefully evaporated to dryness. The samples were then reconstituted with methanol/water and analysed by LCMS-MS with quantification using reference standards. Representative samples of the stock solution and exposure water of CPF were extracted with 20 ml of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LCMS-MS system (instrument Agilent 6410 Triple Quad LCMs-MS) without any clean-up. Standards were prepared in solvents at seven concentrations with recoveries in the range of 70-120%.

Chemical analyses were performed on the stock solutions, as well as on the lowest and highest concentrations of each pesticide, at the beginning of the test. Since CPF is known to have a high degradation rate, chemical analysis of the CPF preparation was also performed after 96h. In contrast, herbicide quantification was not performed after 96 hours due to their relative stability in water, therefore, assuming that their concentrations were kept constant throughout the tests.

5.2.3 Early life stages assay: single experimental setup

The assays were based on the OECD draft guideline on Fish Embryo Toxicity (FET) test (OECD, 2006). For the single exposure experiments, 30 fertilized eggs per treatment (3 replicates with 10 eggs each) were selected and distributed onto 24-well microplates. Individual eggs were placed in each well with 2 mL of test solution. A range-finding test was carried out to derive the final and definitive concentrations to be tested. Five nominal concentrations per pesticide were tested at three replicates: 0.5, 1, 1.5, 2 and 2.5 mg/L for CPF; 20, 25, 30, 35 and 40 mg/L for ATR and 3.2, 6.5, 9.7, 13 and 16.2 mg/L for TER; in addition, a negative control and a solvent control were also tested in triplicate. Test solutions were prepared by dilution of stock solution in water. Stock solutions were prepared by dissolving the pesticides in dimethyl sulfoxide (DMSO). Solvent controls consisted of clean water containing DMSO at the highest volume used in the bioassays, (in the majority of cases around 100 µl/L and never exceeding 200 µl/L). The exposures to the

respective test solutions were performed through a semi-static test design during 96 hours, with replacement of the whole water volume after 48 hours to minimize the effects of possible degradation of the chemicals. This indication was given by results from chemical analysis performed on CPF after 96h depicted in the results' section. All tests were conducted in an environmental chamber using a photoperiod of 16:8 h light:dark at a temperature of 27±2ºC. Water parameters (temperature, dissolved oxygen and pH) were monitored at the beginning and end of the tests. These restrictions were because the tests were performed in microplates so it was impossible to measure the parameters during exposures.

Embryos and larvae were monitored daily using a stereomicroscope for morphological anomalies. In the embryo phase the following parameters were evaluated: egg coagulation, detachment of the tail-bud from the yolk sac, otolith and somite formation, eye and body pigmentation, heartbeat, tail circulation and hatching. After hatching, larval heartbeat, oedemas, tail malformations, mortality and abnormalities on swimming behaviour were observed and reported. The swimming behaviour response consisted of avoidance of a probe, while simultaneously maintaining equilibrium. Probing was repeated three times, whereas an organism was considered affected if it did not respond positively on all three occasions. Then, the effective concentration values (EC_{50}) of each chemical for the most sensitive endpoint were calculated and used later in the mixture exposures design.

5.2.4 Early life stages assay: mixture experimental set up

After analysing the results from single exposures and decide upon the most sensitive endpoint, two combinations for the pesticides used were tested: CPF and ATR, and CPF and TER. The binary mixture assays were also based on the OECD draft guideline on Fish Embryo Toxicity (FET) test (OECD, 2006), with adaptations. For the mixture exposures, viable eggs were distributed onto several Petri dishes containing test water (15 eggs per Petri dishes and treatments). Petri dishes were covered with lids to avoid evaporation. Single pesticide concentrations, as well as the negative and solvent controls were also run simultaneously, without replication, in each mixture test. Toxicant exposure methods and test parameters were the same as those previously described.

Mixture concentrations were based on the EC_{50s} value for the most sensitive parameter obtained in the single chemical tests. In this study, the swimming behaviour response of post hatch larvae was the most sensitive parameter. This endpoint was also chosen over mortality after preliminary single exposures indicated that sub-lethal swimming behavioural impacts would likely limit survival in natural environments (i.e., organisms that swam erratically with a visible loss of equilibrium were considered affected by the pesticides). AChE activity was also measured based on the hypothesis

that the impairment on swimming ability could be due to effects on the central nervous system (please see details in section 5.2.5).

A ray design was used to obtain the dose-response surfaces for the binary pesticide mixtures. The ray design consisted on exposure to a set of single-doses for the two test pesticides individually at the same concentrations used in preliminary tests (with exception for the highest treatment concentrations of CPF which were eliminated due to previously having resulted on high mortality rates), as well as to a number of binary mixture doses at predefined mixture ratios. The number of mixture ratios to be tested was chosen with the aim of obtaining a reliable coverage of effects of the two pesticides. Nominal concentrations of both mixtures were calculated based on expected toxic strengths of 0.375 (0.125+0.25; 0.25+0.125), 0.5 (0.125+0.375; 0.25+0.25; 0.375+0.125), 0.75 (0.125+0.625; 0.25+0.5; 0.375+0.375; 0.5+0.25; 0.625+0.125), 1 (0.125+0.875; 0.25+0.75; 0.375+0.625; 0.5+0.5; 0.625+0.375; 0.75+0.25; 0.875+0.125), 1.5 (0.75+0.75; 1+0.50; 0.50+1), 1.75 (1+0.75; 0.75+1) and 2 (1+1) toxic units (TUChemical-1+TUChemical-2). For each pesticide, the conversion of TU into concentration was based on the EC_{50s} value obtained on the preceding experiments using the chemicals individually. TUs are quotients (C_i/EC_{xi}) that express the concentrations of the mixture components as fractions of their equi-effective individual concentrations, thus quantifying the relative contribution of chemical i to the overall toxicity, in a mixture of n chemicals (Jonker et al., 2005).

In the mixture exposures, the number of replicates per combination was reduced to one, so that more combinations could be tested and thus a wider set of points along the response surface could be obtained (Ferreira et al., 2008). As a consequence, the power of the analysis increased since the analysis performed focused on regression models and differences calculated between the data obtained and the modelled values (Jonker et al., 2005).

The embryos and larvae, including the swimming behaviour responses, were also monitored and recorded daily, as described above. At the end of the tests, larvae were collected and clusters of five larvae were frozen in microtubes containing 0.25 mL of the adequate buffer and immediately stored at -80°C until AChE activity analysis.

5.2.5 Acetylcholinesterase activity analysis

The AChE activity of the *D. rerio* larvae was measured using the method described by Ellman et al. (1961), adapted to microplate by Guilhermino et al. (1996). On the day of enzymatic analysis, all collected samples (3 replicates with a pool of five larvae each) were homogenized (Ystral GmbH D-7801) in an ice-cold 0.1 M phosphate buffer (pH 7.2). The supernatant obtained after the

centrifugation of the homogenate (4˚C, 3800 g, 4 min) was removed and used as enzyme extract for determining AChE activity. The AChE activity was measured using a Lab System Multiskan EX microplate reader at 414 nm using 0.05 mL of supernatant and 0.25 mL of the reaction solution (1 mL of 10 mM 5.50-dithiobis-2-nitrobenzoic acid (DTNB) solution, 0.2mL of 0.075 M acetylcholine solution and 30 mL of 0.1 M phosphate buffer).

AChE activity was determined in quadruplicate and expressed as nmol/ min/ mg protein. Protein concentration in the samples was also determined in quadruplicate by the Bradford method (1976), at 595 nm, using γ-globulin as standard.

5.2.6 Data analysis

One-way ANOVA was performed for comparison between treatments. When significant differences were found, the post-hoc multiple comparisons Dunnett's method was used to evaluate differences between tested concentrations and controls. Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on ranks was performed, followed by a Dunn's method when significant differences were found. The EC_{50} values were calculated using the best-fit regression model. A three-parameter Logistic regression model and a four-parameter Weibull regression model were used in this study (Systat, 2006). The lethal concentration (LC_{50}) for CPF was calculated using Minitab (Minitab-15., 2007). Correlations between measured parameters (swimming behaviour and AChE activity) were analysed by a Pearson correlation (p=0.05).

For the mixtures, data were analysed using the MIXTOX model previously described by Jonker et al. (2005). The MIXTOX model is used to fit data to both reference models "CA" and "IA", allowing comparison between observed and expected toxicity of the mixture, and enabling estimating possible deviations from the two reference models. These deviations may be for synergism (greater effect than expected) or antagonism (less effect than expected). Deviations are obtained with the addition of the parameters a and b forming a nested framework. If the parameter is below zero, this means that a greater effect than expected (synergism) has been observed, whereas for values greater than zero, a weaker effect than expected (antagonism) has occurred. The nested deviations were compared using the method of maximum likelihood and the best-fit chosen using 0.05 as the significance level. In addition, the lowest residual sum of squares (SS) was preferred when comparing deviations. A detailed description on the way the respective functions and parameters can be derived is described by Jonker et al. (2005). In this study, the independent action (IA) model was used exclusively as the mixture components chosen are expected to display different modes of action.

In addition, synergistic ratios (SR) were calculated in order to estimate the magnitude of the synergistic inhibition of AChE, exerted by the chemical mixtures. This was done because it was previously observed that the range of concentrations used for the herbicide exposure did not inhibit AChE activity, thus not allowing for a dose-response curve to be obtained (needed for the IA modelling approach). In order to calculate SR, the EC_{50} values were firstly estimated for each CPF and herbicide treatments using, where feasible, the same dose-response regression curve used within the MIXTOX model, namely a three-parameter Logistic regression curve. Using such values, synergistic ratios were then calculated as the quotient between the EC_{50} value for CPF (without herbicide) and the EC_{50} values for each of the herbicide and CPF treatments. Synergistic ratios of 1.0 indicate no effects of the herbicides on CPF toxicity (or an additivity of responses), whereas values >1.0 and <1.0 indicate greater and weaker effects than expected, respectively.

5.3. Results and discussion

5.3.1 Chemical analysis and water parameters

To assess dosing accuracy at the beginning of all tests, ATR, TER and CPF quantifications were made and results showed that measured concentrations varied generally less than 20% from the nominal concentrations. In addition, previous CPF quantification had shown that approximately 50% of the compound had been already degraded within 96h and therefore, changes in exposure media were made every 48h. So, all calculations were based on nominal concentrations.

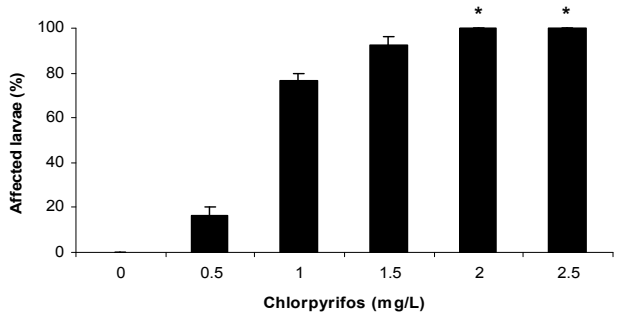
Water parameters monitored at the beginning and end of the bioassays were not found to deviate from standard experimental conditions. Temperature was maintained at 27±2˚C. Dissolved oxygen levels were always > 80% and pH was maintained in the range between 7.5 and 7.8.

5.3.2 Single chemical exposures

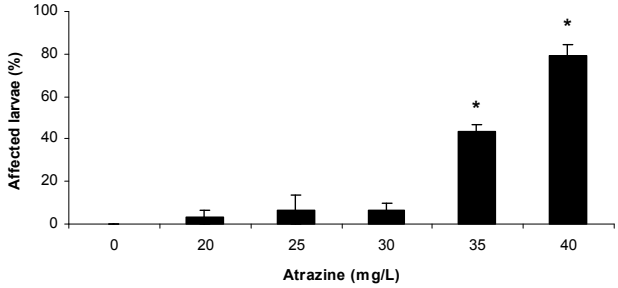
D. rerio in the control groups had normal embryonic and larval development as described by Kimmel et al. (1995). Pesticides were not toxic to the embryos while inside the egg, with no effects observed on the embryo development parameters (detachment of the tail-bud from the yolk sac, otolith and somite formation, eye and body pigmentation, heart-beat, tail circulation,). In contrast, larvae were affected by the pesticides after hatching. Differences in the effects of a chemical between the pre- and post-hatching period may be attributed to the chorion, which acts as a barrier for certain chemicals (lipophilic compounds in particular) (Braunbeck et al., 2005), and thus, can protect the embryo.

On the other hand, no delay was observed for time to hatching regardless of treatment. Embryos started to hatch at 48 hours and 100% of the embryos had hatched after 72 hours in all treatments. The swimming behaviour response of post hatched larvae was the most sensitive endpoint in these single exposure experiments, where larvae showed significant impairment of the swimming motion and loss of equilibrium as a response to the exposure with CPF, ATR and TER (Dunnett or Dunn's test, $p \leq 0.05$; Figure. 5.1 A, B and C). These responses were only observed at the two greatest test concentrations for each of the pesticides. It is possible that in these tests the exposure time of larvae contributed to the sensitivity to pesticides, as under the larval stage they were exposed to the toxic only for 24 hours, after hatching from the egg. The EC_{50s} values of the swimming behaviour endpoint calculated in the single experiments and in the single pesticide exposures executed simultaneously within the mixture experiments were considered similar (Table 5.1, Figure. 5.3SD, Figure. 5.4SD and Figure. 5.5SD).

A



B



C

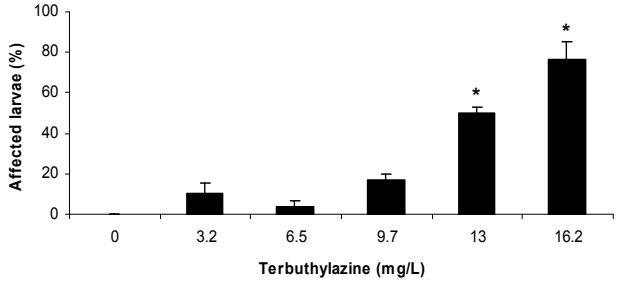


Figure 5. 1. Percentage of larvae with swimming behaviour abnormalities (mean values and standard error) upon exposure to CPF (A), ATR (B) and TER (C), for 96 h. * indicate significant differences from the controls (Dunnett's or Dunn's test, $p \leq 0.05$)

In addition, *D. rerio* larvae exposed to CPF showed a significant increase in the percentage of organisms with morphological deformations, with effects having been more pronounced at a concentration of 1.5 mg/L. Larvae suffered abnormal bending of the spine (Kruskal-Wallis H=16.890, p<0.05, Figure. 5.2C, Figure. 5.6SD) and cardiac oedema (Kruskal-Wallis H=16.886, p<0.05, Figure. 5.2D) compared to the controls; in contrast, none of the herbicides did induce morphological malformations. Mortality increased significantly at the highest concentrations of

CPF, with an LC_{50} of 1.6 mg/L. In the case of the herbicides, mortality was not observed at any of the concentrations tested.

Table 5. 1. EC_{50} values (in milligrams per litre) for the swimming behaviour response and AChE activity of *Danio rerio* after 96 h of exposure. Values were calculated from single exposure tests and from single exposure sets run simultaneously with mixture exposures. Values in brackets refer to standard errors (SE).

Pesticides	Swimming behaviour	AChE activity	Experiments
Chlorpyrifos	0.75 (0.02)	_	Single exposure
	1.87 (1.65)	0.71 (0.06)	Mixture exposure with atrazine
	0.53 (0.002)	0.61 (0.14)	Mixture exposure with terbuthylazine
Atrazine	35 (1.07)	_	Single exposure
	32 (0.05)	_	Mixture exposure with chlorpyrifos
Terbuthylazine	13 (1.81)	_	Single exposure
	13 (0.001)	_	Mixture exposure with chlorpyrifos

(-) not applicable

A clear concentration-response relationship for AChE activity was obtained for CPF in the single exposure experiment with this insecticide executed simultaneously in mixtures with the herbicides (Table 5.1, Figure. 5.5SD). The swimming behaviour of the hatched larvae exposed to CPF was likely a result of AChE inhibition induced by this insecticide. Both endpoints had similar EC_{50} values for CPF, which suggests a link between impairment of the swimming behaviour of the larvae and the inhibition of the AChE activity.

On the other hand, the two herbicides did not induce a full dose-response relationship for AChE activity. Only an 18% and 14% decrease of AChE activity occurred at the highest concentrations of ATR and TER, respectively, when compared to the controls in the single exposure. For this reason it was impossible to calculate valid EC_{50} values for this endpoint for herbicide exposures, and further analysis in the MIXTOX model had to be undertaken with fixed EC_{50} and slope parameters, by underestimation of these values. In the case of the ATR herbicide the EC_{50} was fixed to 54 mg/L and the slope parameter β was fixed to 2.81 and for the TER herbicide the EC_{50} was fixed to 75 and β was fixed to 0.85. This alternative was previously successfully implemented in a study by Loureiro et al. (2010), where it was also impossible to calculate a valid EC_{50} value for feeding rates of *Daphnia magna* exposed to CPF.

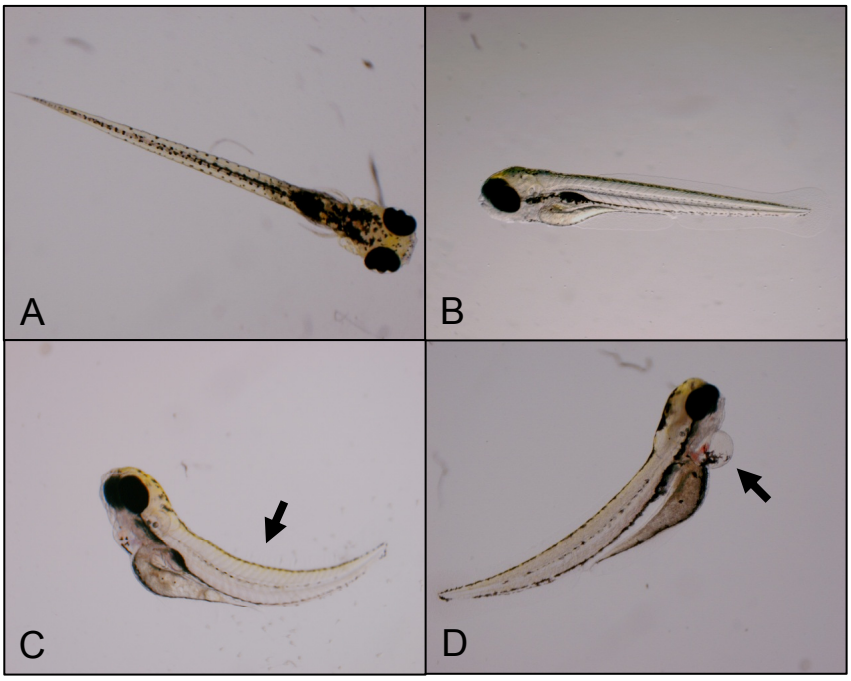


Figure 5. 2. Observed morphological malformations: A, B Four-day-old control larvae of *D. rerio* with well-developed tail and normal body structure. C Four-day-old larva exposed to CPF with deformations of the spine. D Four-day-old larva exposed to CPF with cardiac oedema

The signs and symptoms described above are consistent with the general neurotoxic mode of action of CPF and corroborate results obtained in previous studies where relationships between AChE inhibition, behavioural responses and morphological deformations were observed (Kienle et al., 2009; Sandahl et al., 2005). Scheil et al. (2009) observed higher activity and uncontrolled convulsions in hatched larvae of zebrafish exposed to 0.6 and 1 mg/L of CPF. In addition, Levin et al. (2003; 2004) observed that exposure to concentrations of 100 ng/L CPF during early development of *D. rerio* could impair the swimming behaviour of older larvae, while 10 to 100 µg/l of CPF could cause significant spatial discrimination learning impairments in adult zebrafish, like spatial escape or avoidance discrimination learning.

The inhibition of cholinesterases by pesticides is accepted as a biomarker of exposure, and has been successfully applied in previous studies (Küster, 2005; Küster and Altenburger, 2006; 2007). In these studies, zebrafish embryos were exposed to the organophosphate paraoxon-methyl, and carbamates such as aldicarb and aldicarb-sulfoxide resulting in a significant and concentration-dependent inhibition of AChE activity in these animals. A different study reported inhibition of AChE and behavioural impairment in 4-5 month old coho salmon (Oncorhynchus kisutch) exposed to CPF, and directly linked AChE inhibition with behavioural measurements (Sandahl et al., 2005). The authors described a concentration-dependent inhibition of the AChE activity in brain and

muscle tissue, as well as the inhibition of the behavioural patterns investigated. The results lead the authors to conclude on a possible close relationship between the degree of AChE inhibition and behavioural impairment.

Swimming behaviour of zebrafish larvae was only affected at very great concentrations of ATR and TER (Figure. 5.1). This is likely to be explained by the fact that ATR and TER are *s*-triazine herbicides, whose mechanisms of action are mainly oriented to affect plants. Despite the fact that *s*-triazine levels selected for use in this study are not considered environmentally relevant, further investigation is required to assess environmentally relevant mixture, as potential synergistic effects at low concentrations cannot be excluded. Therefore, robust dose-response curves are necessary to adequately evaluate the overall toxicity of the mixture, considering the theory for the application of the Independent Action model, where full dose-response curves should be available. The EC_{50} values of these herbicides related with the swimming behaviour endpoint of the larvae were mainly used to calculate the TU values for the mixture experimental setup.

5.3.3 Mixture exposures

The setup for the mixture exposures was based on the EC_{50} values for the most sensitive parameter obtained in the tests for the individual chemicals. In this study, the swimming behaviour response of post-hatched larvae was the most sensitive parameter. This endpoint was exclusively chosen after preliminary single exposure tests suggested that sub-lethal swimming behavioural impairment would likely restrict survival in natural environments, since fish larvae would be more vulnerable to be captured by predators. Furthermore, food-searching behaviour could also be affected due to the diminished activity with subsequent negative impacts on their growth and development. At the individual level, behaviour is considered an early warning tool in ecotoxicology since it is one of the most sensitive indicators of chemical stress. In addition, assessing behaviour alterations allows also the integration of individual physiological processes and mechanisms that were impaired upon chemical exposure (Dell'Omo, 2002). On the other hand, one cannot discard the possible about the subjectivity of this type of endpoint, which can cause by itself some uncertainty.

After fitting the IA model to data from the two binary combinations synergistic interactions between chemicals were observed in the binary mixtures. The fitting of the IA model to the binary data of ATR and CPF exposure obtained an *SS* value of 112. However, adding parameter a caused the SS value to decrease significantly ($SS = 62$, $p[x^2] < 0.05$, Table 5.2), while the negative value ($a = -11.5$) indicated a synergistic interaction (more details on the equation used and data modelling can be found in Jonker et al. 2005).

Similar synergistic deviations were observed when data from the TER and CPF exposure were fitted to the IA model. In this case an *SS* value of 308 was obtained. Again, adding the parameter a resulted in the *SS* value to decrease significantly (*SS* = 298, $p[x^2]$ = 0.002, Table 5.2), and a negative parameter (*a* = -2.4) indicated again synergism. Despite the different mechanisms of action of these chemicals, synergism was the deviation function from the IA model obtained in this study for both mixtures. Synergistic deviations from the conceptual models of mixtures have been frequently found in previous studies with invertebrates, showing that there may be an interaction between chemicals rather than an additive or independent response. Species such as *Chironomus tentans*, *Hyalella azteca*, and *Ceriodaphnia dubia* exposed to ATR and organophosphate insecticide mixtures have shown greater than additive toxicity (Anderson and Lydy, 2002; Banks et al., 2005; Belden and Lydy, 2000; Schuler et al., 2005). Wacksman et al. (2006) examined the interactions between ATR and CPF in four aquatic vertebrate species, and the presence of ATR at 1000 µg/l resulted in a significant increase in the acute toxicity of CPF in the African clawed frog (*Xenopus laevis*). Also, a lack of a clear toxicity pattern was observed in the fish *Pimephales promelas*, with certain data showing greater than additive toxicity, and other, an additive response depending on the development stage of the fish (Wacksman et al., 2006).

In addition, the present study examined the variation of the AChE activity as a function of exposure to the investigated pesticide mixtures to help explaining the swimming behaviour effects as a result of enzymatic inhibition. In the mixture exposure experiments, herbicides did not describe a clear dose-response relationship for the AChE activity. It was therefore not possible to calculate valid EC_{50} values for this endpoint, and the analysis in the MIXTOX model were carried out with fixed EC_{50} and slope parameters, as explained before. Therefore, when the IA model was fitted to AChE activity data, synergism was also obtained in combinations of both chemicals. In the case of the binary data for ATR and CPF, it was initially obtained an *SS* value of 705, after data fitting to the IA model. By adding the parameter *a*, the *SS* value decreased significantly (*SS* = 584, $p[x^2]$ = 0.02, Table 5.2), and a negative parameter (*a* = -1.23) indicating that synergism was achieved. The same deviation was obtained in the binary combination between TER and CPF with an initial *SS* value of 1343, having decreased following insertion of parameter *a* (*SS* = 1157, $p[x^2]$ = 0.02, *a* = -1.56, Table 5.2).

Table 5. 2. Summary of the analysis done for the effects on the swimming behaviour response and the AChE activity of *Danio rerio* larvae exposed for 96h to the two pesticide mixtures. Equations used to derive these results are detailed in Jonker et al. (2005)

Mixture		Independent action model							
		Swimming behaviour				AChE activity			
		SS	r^2	$p(x^2)$	*a*	*SS*	r^2	$p(x^2)$	*a*
Atrazine and chlorpyrifos	IA	112	_	_	_	705	_	_	_
	Synergism	62	0.78	$1.8x10^{-12}$	-11.5	584	0.87	0.02	-1.23
Terbuthylazine and chlorpyrifos	IA	308	_	_	_	1343	_	_	_
	Synergism	298	0.47	0.002	-2.4	1157	0.71	0.02	-1.56

r^2 coefficient of regression, $p(x^2)$ outcome of the likelihood ratio test (significance level $p<0.05$), *SS* residuals sum of squares, *a* is the parameter of the deviations

Despite that none of the herbicides has been found to significantly inhibit AChE activity in the single exposure experiments, their presence in a mixture with CPF has significantly increased the inhibition of AChE activity. This indicates a synergistic interaction between the tested pesticides. For this reason and in order to estimate the magnitude of the synergistic inhibition of the AChE activity as a result of the herbicide mixture with CPF, synergistic ratios were calculated for each *s*-triazine concentration tested. Synergistic ratios > 1.0 indicated greater effect than expected. Both *s*-triazine herbicides had considerably higher synergistic ratios for CPF at all exposure levels (Table 5.3). Higher concentrations of triazine resulted in higher synergistic ratios, indicating that the effect is likely to follow a traditional dose–response curve.

The lowest herbicide concentrations tested had SR values ≥ 1.5 (namely ATR had a SR value of 3.1 at 9 mg/L and TER had a SR value of 1.5 at 3 mg/L), showing that the toxicity of CPF is potentiated. Although these concentrations are not considered ecologically relevant, but considering the safety factors usually used in risk assessment, this can indicate that the magnitude of these synergistic ratios is important for evaluating the potentiating effect in terms of increased risk of the chemical mixtures to the environment since even low toxic concentrations may sum up toxic effects when occurring in mixtures.

Table 5. 3. EC_{50} values (in milligram per litre) and synergistic ratio calculated for the AChE activity estimated for chlorpyrifos when co-occurring with each concentration of herbicide in the mixture experiments

Atrazine (mg/L)	0	9	18	26	35
EC_{50}	0.71	0.23	0.12	0.03	0.06
SR		3.1	5.9	23.6	11.8
Terbuthylazine (mg/L)	0	3	7	10	13
EC_{50}	0.61	0.41	0.29	0.02	0.05
SR		1.5	2.1	30.5	12.2

SR=EC_{50} (CPF without herbicide) /EC_{50} (CPF and herbicide treatments)

In our study there was a significant negative correlation between both endpoints, where the swimming disturbance increased while AChE activity decreased for both mixtures (including data from chemical mixture but also the individual exposures carried out simultaneously). The Pearson correlation coefficient (r) for both mixtures was equal to -0.50. Although correlations with this r value are classified as moderate, they were statistically significant in both mixture experiments ($r = -0.50$, $p<0.05$).

The insecticide CPF exerts its toxicity mainly after metabolic transformation of the original compound to chlorpyrifos-oxon, which is a more potent inhibitor of the enzyme AChE (Tyler Mehler et al., 2008). On the other hand, some *s*-triazine herbicides such as ATR and cyanazine have been hypothesized to induce the cytochrome P450 enzyme system, accelerating such a conversion of CPF in invertebrates (Jin-Clark et al., 2002; Pape-Lindstrom and Lydy, 1997). Our findings of enhanced inhibition of AChE in *D. rerio* larvae exposed to ATR or TER and CPF mixtures may also support this hypothesis. The inhibition of AChE activity was a sensitive indicator of the toxicity of the binary mixtures tested here. Likely, our synergistic results were related to the biotransformation of CPF into its more toxic oxon metabolite due to the induction metabolic provoked by the herbicides ATR and TER, thereby causing greater toxicity to larvae than expected considering their single toxicity responses.

Our results conclusively show that simultaneous exposure to several chemicals (even when holding different mechanisms of action) may lead to increases in toxicity, causing more disturbing effects than expected. Moreover, this study supports the utility of the component-based approach to predict the toxic effects of mixtures of known composition in the aquatic environment and not only the single chemical evaluation approach, following legislation maximum admissible concentrations for single components.

5.4. Conclusions

Atrazine and terbuthylazine potentiated the chlorpyrifos toxicity to *D. rerio* in early life stages. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when zebrafish larvae were exposed to CPF mixtures containing ATR and TER. Possibly the presence of these herbicides accelerated the transformation of CPF in its oxon form, increasing therefore toxicity by inhibiting AChE activity. These results highlight once again the possible interactions between pesticides when present as cocktails in aquatic systems.

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5.5. Supplementary data

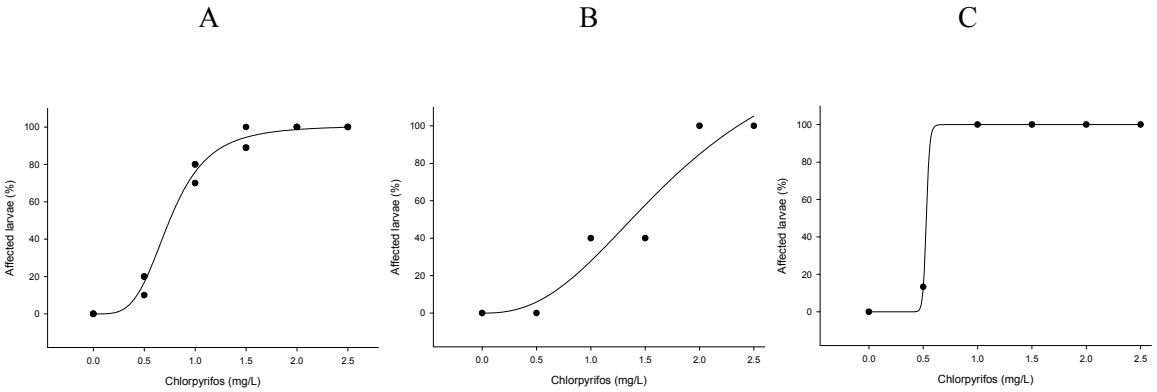

Figure 5. 3SD Dose-response curves for the swimming behaviour response of *Danio rerio* larvae when exposed singly to chlorpyrifos under the experimental set up of: (A) single exposure, (B) mixture exposure set up with atrazine and (C) mixture exposure set up with terbuthylazine (C).

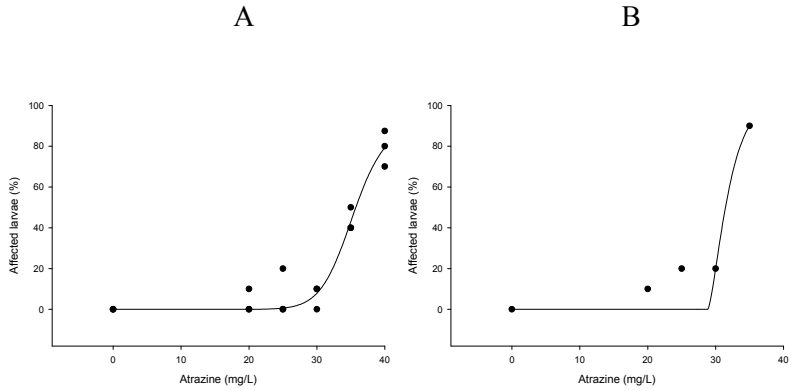

Figure 5. 4 SD Dose-response curves for the swimming behaviour response of *Danio rerio* larvae when exposed to atrazine single exposure (A) and atrazine single exposure run simultaneously in the mixture experimental set up with chlorpyrifos (B).

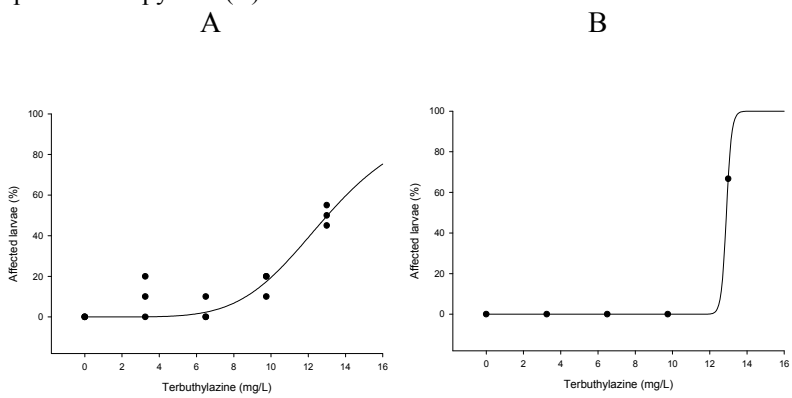

Figure 5. 5 SD Dose-response curves for the swimming behaviour response of *Danio rerio* larvae when exposed to terbuthylazine single exposure (A) and terbuthylazine single exposure run simultaneously in the mixture experimental set up with chlorpyrifos (B).

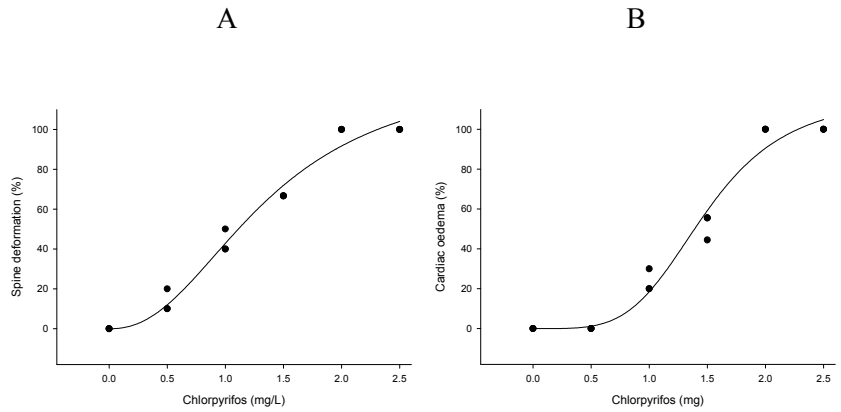

Figure 5. 6 SD Dose-response curves for the morphological malformations of *Danio rerio* larvae when exposed to chlorpyrifos single exposure, percentage of larvae with spinal deformity (A) and percent of larvae with cardiac oedema (B).

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Chapter 6

Final considerations and conclusions

6. Final considerations and conclusions

The fundamental aim of this work focused on the environmental risk Assessment of pesticides found in the water of Alqueva reservoir and their binary mixtures. A chemical cocktail was characterized in this aquatic system (Chapter 2) and several pesticides were above of the environmental quality standard, according to the European directive and the Portuguese legislation in some of the sampling spots of the reservoir. However, in addition, there were several sampling points of the dam where ecotoxicity was observed despite the presence of contaminants at low concentrations. It is known that the joint toxicity of chemicals is often greater or less than their individual toxic effects. Particularly, individual chemicals at nontoxic effect concentrations could result in significant toxicity, if they coexist with others in a chemical mixture. For this reason it is important to consider chemical mixtures and their effects in the environmental risk assessment of chemicals.

The growth inhibition test with the microalgae *Pseudokirchneriella subcapitata* showed to be the most sensitive bioassay when testing the water samples in this study (Chapter 2). Algae growth inhibition was strongly related to the joint presence of high and low concentrations of herbicides such as atrazine, simazine, terbuthylazine and metolachlor, present in this aquatic system. These previous results suggested that the increased toxicity was due to the herbicide mixtures. So, considering this fact, the objectives of this study were firstly to estimate the environmental risk of pesticides previously found in the water of the Alqueva reservoir and secondly estimate the environmental risk of herbicide mixtures found simultaneously in three different locations of the reservoir. To perform these risk characterizations we used data obtained from the different assays and that are presented in the chapters of this thesis. Specifically, the predicted environmental concentration values (PECs) were obtained from chapter 2 and as predicted no effect concentrations (PNECs), we used the results of toxicity tests performed on chapter 3, 4 and 5 and additional data from the literature.

As described previously the environmental risk of pesticides in aquatic ecosystem is usually calculated as a PEC/PNEC ratio, which is used as an indicator of risk and is called risk quotient (RQ). A chemical is seen as potentially inducing no harm to the environment if the PNEC is higher than the PEC, which is the concentration one expects to find in the environment.

In this study the PEC values were based on the maximum concentrations found in the water of the Alqueva reservoir as the contamination was due to diffuse sources from agricultural runoff and not to point sources or discharges. The PNEC values were calculated from the EC_{50} values obtained from preliminary studies described in the chapters 3, 4 and 5 and additional data coming from

literature (Table 6.1), as data on NOEC (no observed effect concentration) values are not so available and it will be also hard to use under a mixture risk approach. To these PNEC parameters it was applied the abovementioned Assessment Factor (AF), which in our case was established as 1000 since our data come from short-term toxicity tests. The PNEC values calculated by selecting the most sensitive test (representing the most sensitive trophic level), the PEC values and the calculated risk quotients are displayed on Table 6.2.

Table 6. 1. Toxicity level of pesticides to *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Chironomus riparius*, *Danio rerio* and in a general way to fish.

Pesticides	EC_{50}(72h) *P.subcapitata* µg/l	EC_{50}(48h) *D.magna* µg/l	LC_{50}(96h) Fish µg/l	EC_{50}(48h) *C.riparius* µg/l	EC_{50}(96h) *D.rerio* µg/l	LC_{50}(96h) *D.rerio* µg/l
Atrazine	196[a]	35500[b]	4500[c]	>200[d]	35000[e]	>35000[e]
Terbuthylazine	24[a]	21200[c]	2200[c]	>200[d]	13000[e]	>13000[e]
Simazine	252[a]	1100[c]	90000[c]	-	-	-
Metolachlor	98[a]	23500[c]	3900[c]	>200[d]	-	-
Chlorpyrifos	480[c]	0.74[b]	1.3[c]	0.17[d]	750[e]	1600[e]

- No data
[a] Data reported in Pérez et al. (2011) Chapter 3
[b] Data reported in Palma et al. (2008)
[c] FOOTPRINT Pesticide Database
[d] Data reported in Chapter 4
[e] Data reported in Pérez et al. (2013) Chapter 5

Table 6. 2. Lowest EC_{50} (µg/l) values obtained for atrazine, terbuthylazine, simazine, metolachlor and chlorpyrifos, and their correspondent PNEC (AF=1000), the PEC and respective Risk Quotient (RQ)

Pesticides	Acute toxicity (EC_{50})	PNEC[c]	PEC[d]	RQ[e]
Atrazine	196[a]	0.196	2.60	13.27
Terbuthylazine	24[a]	0.024	0.67	27.92
Simazine	252[a]	0.252	1.80	7.14
Metolachlor	98[a]	0.098	0.22	2.25
Chlorpyrifos	0.17[b]	0.17[b]	0.18	1.05

Assessment factor (AF) = 1000.
[a] Data reported in Pérez et al. (2011) Chapter 3
[b] Data reported in Chapter 4
[c] PNEC: EC_{50}/1000 (µg/l)
[d] PEC: maximum pesticide concentrations measured in the Alqueva aquatic systems (µg/l) (Pérez et al., 2010) Chapter 2
[e] RQ : PEC/PNEC

In this risk characterization (Table 6.2), the calculated risk quotients for the herbicides atrazine, terbuthylazine, simazine and metolachlor were higher than 1, meaning that these herbicides presented a high risk for the Alqueva ecosystem. Here, the microalgae *P. subcapitata* was the most sensitive specie to the herbicides. However, these herbicides pose no or low risk to other aquatic

organisms tested in this study, with EC_{50} values much higher than the concentrations found in this aquatic ecosystem (Table 6.1).

The RQ of chlorpyrifos was equal to 1.05; here the PEC value was higher than the EC_{50} of *C. riparius*, representing some risk for this specie (Table 6.2).

In this study, considering the previous risk characterization of individual pesticides found in the Alqueva reservoir, it was also estimated the environmental risk of herbicide mixtures found simultaneously in three different locations in the dam. To perform the predictive ecotoxicological risk assessment of chemical mixtures it was implemented the approach proposed by Backhaus et al. (2012).

The stepwise approach proposed by these authors for the calculation of an "ecosystem risk quotient" for mixtures (ratio between the exposure and the hazard of the mixture) is based on a set of single chemical ecotoxicity data from different trophic levels (primary producers, primary and secondary consumers) and may be estimated in two different ways:

1. The PNEC is calculated individually for each mixture component. Then, the PEC/PNEC ratios of all mixture components are summed up to a final Risk Quotient (termed $RQ_{PEC/PNEC}$), of the mixture. The equation is:

$$RQ_{PEC/PNEC} = \sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} = \sum_{i=1}^{n} \frac{PEC_i}{\min\left(EC50_{algae}, EC50_{daphnids}, EC50_{fish}\right)_i \times \left(1/AF_i\right)}$$

2. The Sum of Toxic Units (∑TU) is calculated for each trophic level. Then, the ∑TU for the trophic level with the highest predicted sensitivity to the mixture (maximum ∑TU of all analysed trophic levels) is multiplied with the Assessment Factor (AF) for estimating the risk quotient of the mixtures (termed RQ_{STU}):

$$RQ_{STU} = \max\left(\sum TU_{algae}, \sum TU_{daphnid}, \sum TU_{fish}\right) \times AF$$

$$= \max\left(\sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,algae}}, \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,daphnids}}, \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,fish}}, \right) \times AF$$

However, the authors argue that for a stepwise application of both approaches one should start with the calculation of $RQ_{PEC/PNEC}$ and only continue with RQ_{STU} if the data indicate a possible reason of

concern that is; if $RQ_{PEC/PNEC}$ is above 1 then RQ_{STU} can be calculated in a next step. More details on this tiered approach can be found in Backhaus et al. (2012).

In our study the environmental risk assessment of chemical mixtures was limited only to herbicide mixtures found simultaneously in three different locations of the Alqueva dam; namely: Álamos (upstream), Mourão (middle) and Moinho das Barcas (downstream). The herbicide mixtures selected were due to the evident risk of these herbicides, previously detected, for the reservoir. The herbicide concentrations found in these locations were used as PEC values in the mixture risk characterizations and are displayed on Table 6.3. In addition, the PNEC values were also calculated from the EC_{50} values obtained from preliminary studies described in chapters 3, 4 and 5 and additional data coming from literature (Table 6.1).

Table 6. 3. Herbicide concentrations in water samples from three different stations of the Alqueva aquatic systems

Herbicides (µg/l)	Stations		
	Álamos (upstream)	Mourão (middle)	Moinho das Barcas (downstream)
Atrazine	0.50	1.30	0.11
Terbuthylazine	0.50	0.02	0.52
Simazine	1.80	0.32	0.98
Metolachlor	0.19	0.00	0.18

The risk quotients derived from the application of the two approaches for the assessment of the herbicide mixtures present in the selected locations are given on Tables 6.4, 6.5 and 6.6. In all three cases the RQs were higher than 1, meaning a high risk of these mixtures for the locals selected within the reservoir. Moreover, in the three cases both methods produce the same risk estimates, which is due to the similar toxicity profiles of the herbicides (Backhaus and Faust, 2012).

Table 6. 4. Environmental risk characterization of herbicides mixture simultaneously found in Álamos, using the tiered approach proposed by Backhaus et al. (2012)

	Atrazine (µg/l)	Terbuthylazine (µg/l)	Simazine (µg/l)	Metolachlor (µg/l)
EC_{50} (algae)	196	24	252	98
EC_{50} (daphnids, acute)	35500	21200	1100	23500
EC_{50} (fish, acute)	4500	2200	90000	3900
Resulting PNEC (AF=1000)	0.196	0.024	0.252	0.098
PEC	0.50	0.50	1.80	0.19
$RQ_{PEC/PNEC}$ (based of sum of PEC/PNECs)	$RQ_{PEC/PNEC} = \frac{PEC_{ATR}}{PNEC_{ATR}} + \frac{PEC_{TER}}{PNEC_{TER}} + \frac{PEC_{SIM}}{PNEC_{SIM}} + \frac{PEC_{MET}}{PNEC_{MET}} = 32.47$			
RQ_{STU} (based of sum of Toxic Units) Algae	$= \frac{0.50\mu g/L}{196\mu g/L} + \frac{0.50\mu g/L}{24\mu g/L} + \frac{1.80\mu g/L}{252\mu g/L} + \frac{0.19\mu g/L}{98\mu g/L} = 0.03247$ $sum\ of\ TUs \times AF = 32.47\ \ (STU\ A\lg ae)$			
Daphnids	$= \frac{0.50\mu g/L}{35500\mu g/L} + \frac{0.50\mu g/L}{21200\mu g/L} + \frac{1.80\mu g/L}{1100\mu g/L} + \frac{0.19\mu g/L}{23500\mu g/L} = 0.00169$ $sum\ of\ TUs \times AF = 1.69\ \ (STU\ Daphnids)$			
Fish	$= \frac{0.50\mu g/L}{4500\mu g/L} + \frac{0.50\mu g/L}{2200\mu g/L} + \frac{1.80\mu g/L}{90000\mu g/L} + \frac{0.19\mu g/L}{3900\mu g/L} = 4.07102 \times 10^{-4}$ $sum\ of\ TUs \times AF = 0.407102\ \ (STU\ Fish)$			
Final RQ_{STU}	$RQ_{STU} = \max(STU_{algae}, STU_{crustaceans}, STU_{fish}) = 32.47$			

Table 6. 5. Environmental risk characterization of herbicides mixture simultaneously found in Mourão, using the tiered approach proposed by Backhaus et al. (2012)

	Atrazine (µg/l)	Terbuthylazine (µg/l)	Simazine (µg/l)	Metolachlor (µg/l)
EC_{50} (algae)	196	24	252	98
EC_{50} (daphnids, acute)	35500	21200	1100	23500
EC_{50} (fish, acute)	4500	2200	90000	3900
Resulting PNEC (AF=1000)	0.196	0.024	0.252	0.098
PEC	1.30	0.02	0.32	0.00
$RQ_{PEC/PNEC}$ (based of sum of PEC/PNECs)	$RQ_{PEC/PNEC} = \frac{PEC_{ATR}}{PNEC_{ATR}} + \frac{PEC_{TER}}{PNEC_{TER}} + \frac{PEC_{SIM}}{PNEC_{SIM}} + \frac{PEC_{MET}}{PNEC_{MET}} = 8.74$			
RQ_{STU} (based of sum of Toxic Units) Algae	$= \frac{1.30\mu g/L}{196\mu g/L} + \frac{0.02\mu g/L}{24\mu g/L} + \frac{0.32\mu g/L}{252\mu g/L} + \frac{0.00\mu g/L}{98\mu g/L} = 0.00874$ $sum\ of\ TUs \times AF = 8.74\ (STU\ Algae)$			
Daphnids	$= \frac{1.30\mu g/L}{35500\mu g/L} + \frac{0.02\mu g/L}{21200\mu g/L} + \frac{0.32\mu g/L}{1100\mu g/L} + \frac{0.00\mu g/L}{23500\mu g/L} = 3.29 \times 10^{-4}$ $sum\ of\ TUs \times AF = 0.329\ (STU\ Daphnids)$			
Fish	$= \frac{1.30\mu g/L}{4500\mu g/L} + \frac{0.02\mu g/L}{2200\mu g/L} + \frac{0.32\mu g/L}{90000\mu g/L} + \frac{0.00\mu g/L}{3900\mu g/L} = 3.02 \times 10^{-4}$ $sum\ of\ TUs \times AF = 0.302\ (STU\ Fish)$			
Final RQ_{STU}	$RQ_{STU} = \max(STU_{algae}, STU_{crustaceans}, STU_{fish}) = 8.74$			

Table 6. 6. Environmental risk characterization of herbicides mixture simultaneously found in Moinho das Barcas, using the tiered approach proposed by Backhaus et al. (2012)

	Atrazine (µg/l)	Terbuthylazine (µg/l)	Simazine (µg/l)	Metolachlor (µg/l)
EC_{50} (algae)	196	24	252	98
EC_{50} (daphnids, acute)	35500	21200	1100	23500
EC_{50} (fish, acute)	4500	2200	90000	3900
Resulting PNEC (AF=1000)	0.196	0.024	0.252	0.098
PEC	0.11	0.52	0.98	0.18
$RQ_{PEC/PNEC}$ (based of sum of PEC/PNECs)	$RQ_{PEC/PNEC} = \frac{PEC_{ATR}}{PNEC_{ATR}} + \frac{PEC_{TER}}{PNEC_{TER}} + \frac{PEC_{SIM}}{PNEC_{SIM}} + \frac{PEC_{MET}}{PNEC_{MET}} = 27.95$			
RQ_{STU} (based of sum of Toxic Units) Algae	$= \frac{0.11\mu g/L}{196\mu g/L} + \frac{0.52\mu g/L}{24\mu g/L} + \frac{0.98\mu g/L}{252\mu g/L} + \frac{0.18\mu g/L}{98\mu g/L} = 0.02795$ $sum\ of\ TUs \times AF = 27.95\ (STU\ Algae)$			
Daphnids	$= \frac{0.11\mu g/L}{35500\mu g/L} + \frac{0.52\mu g/L}{21200\mu g/L} + \frac{0.98\mu g/L}{1100\mu g/L} + \frac{0.18\mu g/L}{23500\mu g/L} = 9.26 \times 10^{-4}$ $sum\ of\ TUs \times AF = 0.926\ (STU\ Daphnids)$			
Fish	$= \frac{0.11\mu g/L}{4500\mu g/L} + \frac{0.52\mu g/L}{2200\mu g/L} + \frac{0.98\mu g/L}{90000\mu g/L} + \frac{0.18\mu g/L}{3900\mu g/L} = 3.18 \times 10^{-4}$ $sum\ of\ TUs \times AF = 0.318\ (STU\ Fish)$			
Final RQ_{STU}	$RQ_{STU} = \max(STU_{algae}, STU_{crustaceans}, STU_{fish}) = 27.95$			

As result of this risk characterization we can conclude that the herbicides and their mixtures were found to be the main risk to the ecosystem of Alqueva. In this case, and as expected due to herbicides targets and modes of action, the most sensitive species was the microalgae *Pseudokirchneriella subcapitata* implying a greater risk to the ecosystem. This is an important species within aquatic trophic chains as primary producer and also as food for invertebrates and fish. Therefore, assuming that when protecting the most sensitive trophic level all other organism groups are protected as well, and that protecting the structure of an ecosystem also protect ecosystem functions, thus future actions are required in order to mitigate the adverse effects to this important ecosystem. Moreover, in our opinion, it is necessary to continue with a pesticide monitoring program and the implementation of ecotoxicological testing with local species, experimental studies in the field, multispecies and mesocosms in order to obtain more accurate information for the environmental risk assessment of chemicals and their mixtures in this aquatic ecosystem.

In addition, while *s*-triazine herbicides pose no or low risk to other aquatic organisms in this study, when tested alone, with EC_{50} values much higher than the concentrations found in this aquatic ecosystem (Table 6.1), they are able to increase the toxic effects of chlorpyrifos when they are tested in binary mixtures. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when *Danio rerio* (Chapter 5) and *Chironomus riparius* (Chapter 4) larvae were exposed to chlorpyrifos mixtures containing atrazine and terbuthylazine. Possibly the presence of these herbicides accelerated the transformation of chlorpyrifos in its oxon form, increasing therefore toxicity by inhibiting AChE activity. These results highlights once again that the simultaneous presence of several chemicals in the aquatic environment may lead to increases in toxicity, causing more disturbing effects on the aquatic ecosystems than expected, regarding their single toxicity.

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