Universidade de Aveiro Departamento de Biologia
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Thayres de Sousa Andrade

EFEITOS DOS PARÂMETROS AMBIENTAIS NA TOXICIDADE DE PESTICIDAS PARA EMBRIÕES DE PEIXE-ZEBRA

EFFECTS OF ENVIRONMENTAL FACTORS ON THE TOXICITY OF PESTICIDES TO ZEBRAFISH EMBRYOS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Paula Inês Borralho Domingues, Investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

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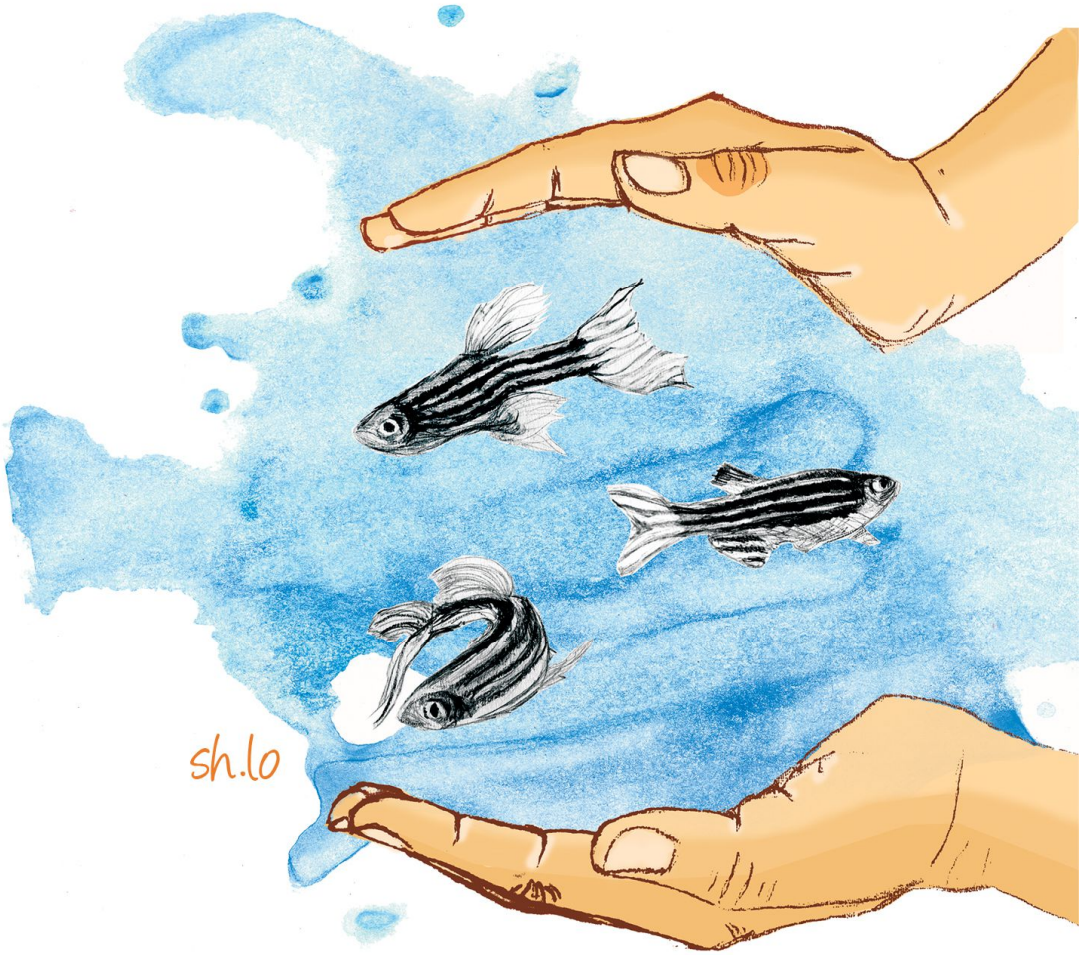

“ O mundo tornou-se perigoso, porque os homens aprenderam a dominar a natureza antes de dominarem a si mesmos”

Schweitzer, Albert

Àquela que foi a minha maior entusiasta, mulher forte e inspiradora que me mostrou que podemos tudo quando perseguimos com fé e amor...àquela que nunca se conformou com a escuridão da falta do conhecimento, mas que o buscou com sempre com muita força. Não estás aqui para me ver galgar mais este degrau do conhecimento, mas não poderia deixar de lhe homenagear. Te amo Vó!

Raimunda Coelho de Sousa
24/04/1932 - 01/06/2003

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Palavras-chave

Ecotoxicologia, peixe-zebra, ecossistemas aquáticos, mudanças climáticas, aquecimento global, toxicidade de misturas, concentração da adição, ação independente, pesticidas, oxigênio dissolvido, pH, radiação ultravioleta

Resumo

Durante o século passado, as temperaturas globais médias têm vindo a aumentar. De acordo com as previsões, a mudança de temperatura deverá ser superior a 1,5ºC neste século e o aquecimento é provável que continue. Os ecossistemas de água doce estão entre os mais sensíveis, principalmente devido às mudanças no ciclo hidrológico e, consequentemente, em diversos parâmetros físico-químicos (ex. pH, oxigénio dissolvido). Alterações nos parâmetros abióticos de ambientes de água doce irão provavelmente afectar a distribuição, morfologia, fisiologia e riqueza de uma ampla gama de espécies levando a mudanças importantes na biodiversidade e funcionamento do ecossistema. Para além disto, eles também podem atuar como co-estressores em ambientes onde os organismos já tem que lidar com contaminação química. Portanto, o objetivo deste trabalho foi avaliar os efeitos de parâmetros ambientais sobre a toxicidade dos pesticidas para embriões de peixe-zebra. Foram estudados os seguintes fatores ambientais: pH (3,0-12,0), nível de oxigénio dissolvido (0-8 mg/L) e radiação UV (0-500 mW/m^2). Os pesticidas estudados foram o inseticida carbamato carbaril e o fungicida benzimidazólico carbendazim. Ambos os estressores (fatores ambientais e pesticidas) foram testados separadamente a fim de obter curvas dose-resposta para estudar mais profundamente os efeitos combinados de estressores ambientais e toxicidade química, aplicando modelos de mistura. A caracterização das respostas do peixe-zebra ao estresse ambiental mostrou que os efeitos do pH foram totalmente estabelecidas após 24 h de exposição e a sobrevivência foi só afetada a valores de pH abaixo de 5 e acima 10. Os níveis reduzidos de oxigénio também afetaram o desenvolvimento dos embriões em concentrações abaixo de 4 mg/L (atraso, redução dos batimentos cardíacos e edema) e em concentrações abaixo de 0.5 mg/L a sobrevivência foi drasticamente reduzida. A exposição contínua a radiações UV mostrou um forte efeito dependente do tempo na sobrevivência dos embriões levando a 100% de mortalidade no final do ensaio. A toxicidade dos pesticidas carbaril e carbendazim foi caracterizada em vários níveis de organização biológica, incluindo desenvolvimento, biomarcadores e comportamental, permitindo uma compreensão mecanicista dos efeitos e destacando a utilidade de respostas comportamentais (locomoção) como um parâmetro sensível em ecotoxicologia.

Uma vez que as curvas dose resposta para cada estressor foram estabelecidas, um estudo de toxicidade combinado foi realizado para avaliar os efeitos do pH sobre a toxicidade do carbaril.

Os resultados mostraram que o pH pode modificar a toxicidade do pesticida carbaryl. O modelo conceitual de adição da concentração permitiu uma previsão precisa da toxicidade dos efeitos conjuntos do pH ácido e carbaril. No entanto, para a condição alcalina ambos os conceitos falharam na previsão dos efeitos. Os desvios ao modelo foram no entanto fáceis de explicar uma vez que os valores de pH elevados favoreceram a hidrólise do carbaril com a consequente formação de um produto de degradação mais tóxico 1-naftol. Embora no presente estudo tal processo explicativo foi fácil de estabelecer, para muitas outras combinações de natureza "interativa" talvez esse processo não seja tão evidente. No contexto das alterações climáticas poucos cenários preveem um aumento tão elevado do pH de sistemas aquáticos, no entanto, esta pode ser considerada uma primeira abordagem focada apenas nos efeitos letais. Numa segunda avaliação, efeitos ao nível sub-letal seriam recomendados uma vez que espera-se que mudanças mais sutis de pH (mais realistas em termos de cenários de mudanças climáticas) possam ter um efeito em níveis fisiológicos e bioquímicos, com possíveis consequências a longo prazo para o fitness das populações.

Keywords

Ecotoxicology, zebrafish, aquatic ecosystems, climate change, global warming, mixture toxicity, concentration addition, independent action, pesticides, dissolved oxygen, pH, Ultraviolet radiation

Abstract

During the last century mean global temperatures have been increasing. According to the predictions, the temperature change is expected to exceed 1.5ºC in this century and the warming is likely to continue. Freshwater ecosystems are among the most sensitive mainly due to changes in the hydrologic cycle and consequently changes in several physico-chemical parameters (e.g. pH, dissolved oxygen). Alterations in environmental parameters of freshwater systems are likely to affect distribution, morphology, physiology and richness of a wide range of species leading to important changes in ecosystem biodiversity and function. Moreover, they can also work as co-stressors in environments where organisms have already to cope with chemical contamination (such as pesticides), increasing the environmental risk due to potential interactions. Therefore, the objective of this work was to evaluate the effects of climate change related environmental parameters on the toxicity of pesticides to zebrafish embryos. The following environmental factors were studied: pH (3.0-12.0), dissolved oxygen level (0-8 mg/L) and UV radiation (0-500 mW/m^2). The pesticides studied were the carbamate insecticide carbaryl and the benzimidazole fungicide carbendazim. Stressors were firstly tested separately in order to derive concentration- or intensity-response curves to further study the effects of binary combinations (environmental factors x pesticides) by applying mixture models. Characterization of zebrafish embryos response to environmental stress revealed that pH effects were fully established after 24 h of exposure and survival was only affected at pH values below 5 and above 10. Low oxygen levels also affected embryos development at concentrations below 4 mg/L (delay, heart rate decrease and edema), and at concentrations below 0.5 mg/L the survival was drastically reduced. Continuous exposure to UV radiation showed a strong time-dependent impact on embryos survival leading to 100% of mortality after 72 hours of exposure. The toxicity of pesticides carbaryl and carbendazim was characterized at several levels of biological organization including developmental, biochemical and behavioural allowing a mechanistic understanding of the effects and highlighting the usefulness of behavioural responses (locomotion) as a sensitive endpoint in ecotoxicology.

Once the individual concentration response relationship of each stressor was established, a combined toxicity study was conducted to evaluate the effects of pH on the toxicity of carbaryl.

We have shown that pH can modify the toxicity of the pesticide carbaryl. The conceptual model concentration addition allowed a precise prediction of the toxicity of the joint-effects of acid pH and carbaryl. Nevertheless, for alkaline condition both concepts failed in predicting the effects. Deviations to the model were however easy to explain as high pH values favour the hydrolysis of carbaryl with the consequent formation of the more toxic degradation product 1- naphtol. Although in the present study such explanatory process was easy to establish, for many other combinations the “interactive” nature is not so evident. In the context of the climate change few scenarios predict such increase in the pH of aquatic systems, however this was a first approach focused in the lethal effects only. In a second tier assessment effects at sublethal level would be sought and it is expectable that more subtle pH changes (more realistic in terms of climate changes scenarios) may have an effect at physiological and biochemical levels with possible long term consequences for the population fitness.

INDEX

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Figures

Chapter 1

Chapter 3

Chapter 5

rate after exposure to a range (0 – 256 mM) of MES, MOPS and TRIS concentrations. b) Concentration response curve for zebrafish embryos survival at 96h after exposure to a range (0 – 445 mM) of CHES (closed circles) and CAPS (open circles) concentrations. All the assays were conducted at pH around 8.0.

Figure S2 – Supplementary data – Predicted and observed mixture toxicity increasing buffers concentrations. a) mixture ratio for acidic conditions using a buffer (MES) concentration of 40 mM; b) mixture toxicity for alkaline conditions using a buffer (CAPS) concentration of 220 mM. ——— , prediction according to concentration addition; – – – -, prediction according to independent action.

Figure S3 – Supplementary data – Concentration response curve for zebrafish embryos exposed to 1-naphthol at 96 hpf.

Chapter 7

Figure 1 – Framework to investigate multiple stressors effects.

Chapter 1

General Introduction

1. General Introduction

1.1 Contextualization

Strong scientific evidences have demonstrated that global climate is changing and in some extent responsibilities can be attributed to human activities. The warming observed since the mid-20th century is mainly due to anthropogenic greenhouse gas emissions (GHG), such as carbon dioxide and methane. Despite the effort to reduce emissions, GHG have largely increased between 2000 and 2010 (IPCC, 2014). The most recent assessment report by the Intergovernmental Panel on Climate Change (IPCC, 2014) indicated that the last three decades were the warmest at the Earth's surface since 1850. Moreover, the warming is predicted to continue beyond the 21st century in all scenarios and the temperature increase may reach 4.8 ºC. Changes in climate have caused impacts on human and natural systems in recent decades with strongest impacts on natural systems. Fig.1 summarizes the impacts of climate change on natural and human systems based on available scientific publication from 2001 to 2010. As can be observed, the impacts of climate change are strongest and most comprehensive for natural systems. Changes in precipitation or melting snow and ice for instance may affect quantity and quality of water resources (IPCC, 2014).

Aquatic ecosystems are particularly susceptible to climate change because of their strong dependence on precipitation and hydrologic cycles (Allan et al., 2005). These processes affect not only the quantity of water but also its quality. Indeed, recent studies emphasized the relationship of climatic and hydrological parameters to surface water quality. For instance, a negative correlation between climate related parameters such as precipitation, evaporation, air temperatures, water level and discharge flow and modifications in Dissolved Oxygen (DO), pH and conductivity was found in the Mekong River (Prathumratana et al., 2008). In addition, a clear relationship between temperature increase and water quality changes, namely nutrient loadings (soluble reactive phosphorus, nitrate among others) was reported in semi-arid streams in Spain (Benítez-Gilabert et al., 2010). These changes in physicochemical characteristics of freshwater environments affect directly and indirectly the biodiversity of ecosystems, with changes in distribution,

morphology, physiology and richness of many species (Mezcua et al., 2004; C. M. O. Reilly et al., 2003).

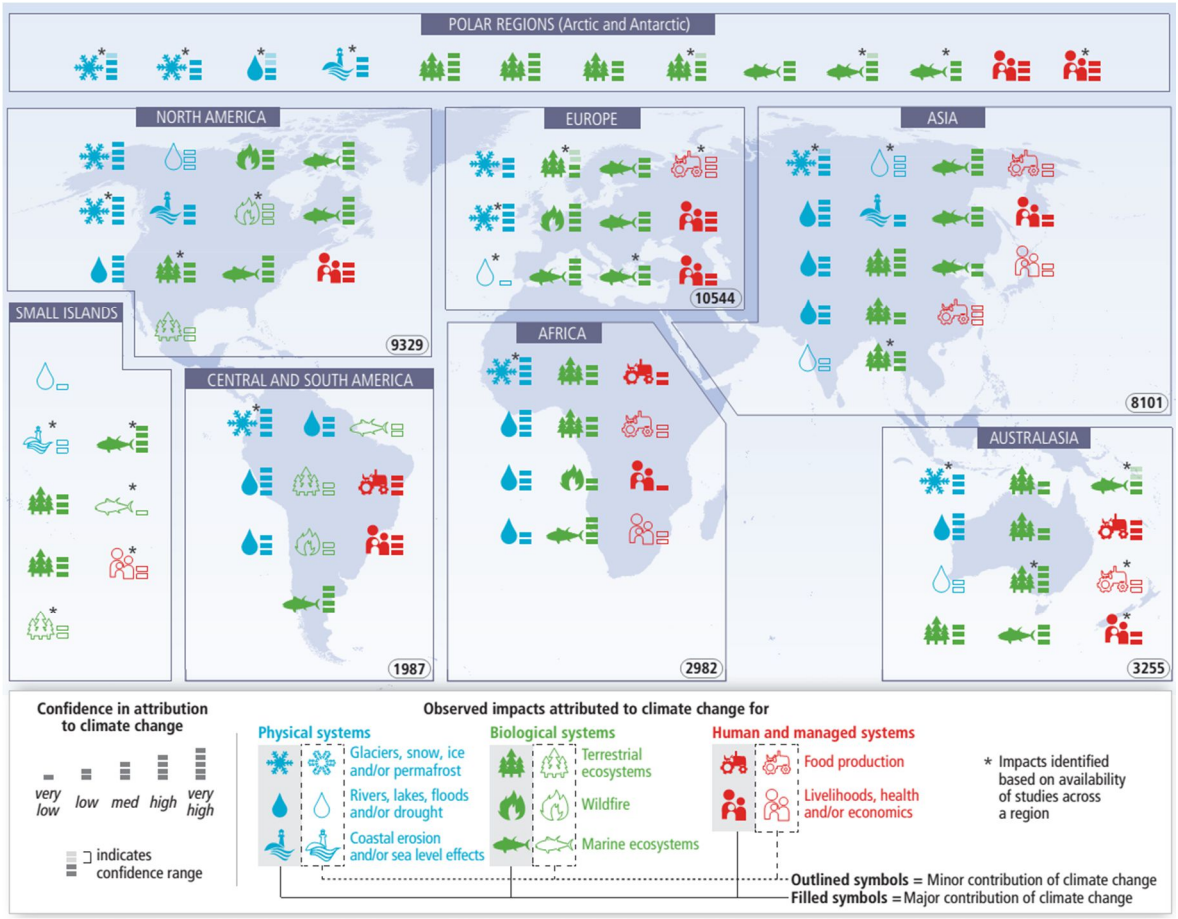

Figure 1: Widespread impacts attributed to climate change based on the available scientific literature since 2000. Symbols indicate categories of attributed impacts, the relative contribution of climate change to the observed impact and confidence in attribution. Number in ovals indicates regional totals of climate change publications from 2001 to 2010 (IPCC Synthesis report, 2014).

The immediate effects of global climate change in hydrologic patterns may indirectly cause alterations in the physicochemical water properties with temperature being a determinant factor affecting almost all physicochemical parameters and biological processes. pH is an important environmental factor conditioning survival of many freshwater species. Water pH depends on a complexity of factors including temperature. For example, during a drought period in the Meuse river van Vliet & Zwolsman (2008) observed an increase in the pH reflecting a decrease in CO_2 concentration due to proliferation of algae blooms elicited by higher water temperature and nutrients concentrations. Fluctuations in pH may compromise physiological fitness of aquatic life

and also influence the speed of chemical reactions, degradations and bioavailability of contaminants. The DO in water can vary temporally and spatially and these changes are directly related to atmospheric gas exchange, temperature and may also be related to eutrophication phenomena (van Vliet and Zwolsman, 2008). A temperature increase, for example, can cause a decrease in oxygen concentration due to the lower water capacity to carry oxygen (Kundzewicz and Krysanova, 2010). DO has also an important role in the physiology of aquatic organisms and at low levels has been shown to affect survival and development of fish and invertebrate organisms (Ferreira et al., 2008; Küster and Altenburger, 2008; Padilla and Roth, 2001). Other important environmental factor conditioning survival of aquatic organisms is ultraviolet (UV) radiation. In the case of UV radiation, the depletion of the stratospheric ozone along with changes in dissolved organic matter (DOM) (as changes in climate may lead to a reduction of DOM in water), may lead to an enhanced exposure of aquatic life to UV-B radiation which can also compromise survival and development of aquatic organisms as demonstrated by many studies (Charron et al., 2000; Häkkinen et al., 2002; Oromi et al., 2008). The risks of fluctuations in the above mentioned environmental parameters are exacerbated as they may be able to modify the toxicity of chemical compounds and/or act as an additional stress to aquatic life.

Changes in climate will also affect the development and geographical distribution of many agricultural pests, diseases and weeds, caused mainly by alterations in temperature, precipitation and wind patterns (Patterson et al., 1999). In particular, climate change will cause the spreading of insect-borne diseases and pests (Koleva and Schneider, 2009; Porter et al., 1991) as well as the increased incidence of weeds (Coakley, 1999). For instance, 1º C increase in temperature will influence the development rate and distribution of the European Corn Borer inducing a northward extension (up to 1200 km) of this pest, as predicted by Porter, Parry et al. (1991). The increased incidence of existing pests, diseases and weeds predicted under climate change scenarios may imply an extensive and more frequent application of pesticides (Koleva and Schneider, 2009; J. Reilly et al., 2003). Chen and McCarl (2001) studied the relationship between climate change and pesticide usage in US agriculture by employing projected climate change scenarios. Their results suggest that climate change will considerably increase pesticide use/costs in US agriculture. This will imply that more pesticides residues will reach the aquatic environment by runoff or leaching from agricultural fields which may increase their

concentrations and bioavailability to aquatic biota and, thus, affecting the survival of the most sensitive species.

Thus, in some climate changes scenarios species have to cope not only with variations of natural environmental parameters such as pH, DO and UV radiation that may fallout of their optimal range but also with an increased load of pollutants as it is the case of pesticides. Moreover, and with particular relevance for this thesis, chemical, physical or biological interactions between these two types of stressors (environmental X chemical) may occur, exacerbating ecological risk for aquatic systems. However, these possible interactions are not usually taken into account in the toxicity evaluation and in the risk assessment of chemicals. The toxicity evaluation is carried out by exposing organisms in "standard conditions" where temperature, oxygen and pH are held constant. These conditions do not always reflect the heterogeneity and the multiple stress factors that natural populations experience in the wild and, more importantly, do not take into account the upcoming changes resulting from global alterations leading to inaccurate ecological risk assessment. Therefore, in order to improve risk assessment, new methodologies need to be designed, considering the evaluation of combined stress of environmental factors and chemicals.

A model organism that could be suitable to address this issue should have particular characteristics including: i) relatively well understood growth and development; ii) relatively easy to grow and maintain in laboratory; iii) relatively short generation time; iv) closely resemble others organisms or systems; v) be compliant with ethical legislation/requirements/issues and vi) provide a set of easily quantifiable functional parameters allowing mode of action analysis among other features. This last is an essential requirement in a model organism and encompasses a good knowledge on the species biology and biochemical processes. The development of adequate technology is also important in order to enable precise measurements of responses and high throughput analyses. The zebrafish (*Danio rerio*) shows to fulfill these requirements, being an excellent model to unravel mechanisms of combined toxicity in the aim of this thesis as explained below. Zebrafish is a small tropical fish indigenous to South Asia and India. Recently, it has become a popular model in many fields of science including human disease (Bakkers, 2011), genetics (Haffter and Nüsslein-Volhard, 1996), pharmacology (Redfern et al., 2008) and (eco)toxicology (Braunbeck et al., 2014, 2005; Lammer et al.,

2009; Scholz et al., 2008). A number of unique attributes have contributed to its rise in popularity as a model organism:

(1) Zebrafish are easy and economic to maintain;
(2) They have a small size;
(3) They present a high fecundity producing a large number of embryos;
(4) Embryos develop rapid and outside the mother;
(5) Zebrafish development has been well characterized including morphological and physiological information at all stages of early development (Kimmel et al., 1995);
(6) The transparency of embryos allows unobstructed observation of the main morphological changes during earlier developmental stages;
(7) Zebrafish genome is completely characterized.

In the process of risk assessment of chemicals (plant protection products, pesticides, pharmaceuticals, effluents, etc.), a set of ecotoxicological tests is required for chemical toxicity assessment including the acute toxicity test with vertebrates (Scholz et al., 2013). The implementation of the new European Union regulation for the Registration, Evaluation and Authorization of Chemicals (REACH) is certainly increasing dramatically the animal testing (Piersma, 2006). Moreover, the global production of chemicals is increasing with tons of new substances released in the market every year. However, within the current animal welfare legislation in Europe which demands the incorporation of the 3Rs principles (replacement, reduction, refinement), testing with vertebrate animals should be reduced or even replaced by alternative methods (EU, 2010). In this context, the zebrafish embryo toxicity test has emerged as a true alternative – or at least a refinement – for the acute fish toxicity test (Braunbeck et al., 2014, 2005; Scholz et al., 2013, 2008; Schulte and Nagel, 1994). The approval of the OECD testing guideline nº 236 has consolidated the fish embryo test (FET) with zebrafish as a test to assess toxicity of the embryonic forms of fish and a full alternative for the use of vertebrates (OECD, 2013).

The use of embryonic forms of fish has numerous advantages. Particularly, early life stages of fish (characterized by lack of independent feeding) are considered as non-protected life stages complying with the ethical framework of the 3Rs (EU, 2010). In the case of zebrafish, besides all the advantages mentioned before, numerous studies have reported the good accordance of zebrafish embryos assays with the acute adult test

(Belanger et al., 2013; Lammer et al., 2009) and in vivo results in mammals (Selderslaghs et al., 2009). In addition, the FET test enables the monitoring of a whole organism from early embryogenesis (1.5 hpf – hours post fertilization), until hatching (48-72 hpf) and beyond (96-120 hpf). Furthermore, the FET is less time consuming and requires very low volumes of test solution.

Briefly, the zebrafish embryos toxicity test consists in exposing newly fertilized eggs (≤1.5 hpf) to the test chemical for a total period of 96 h (Fig 2). During this period, the embryos are evaluated daily and four observations are performed as indicators of lethality: coagulation of embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat. In addition, hatching is also recorded daily starting form 48 hpf until the end of the test.

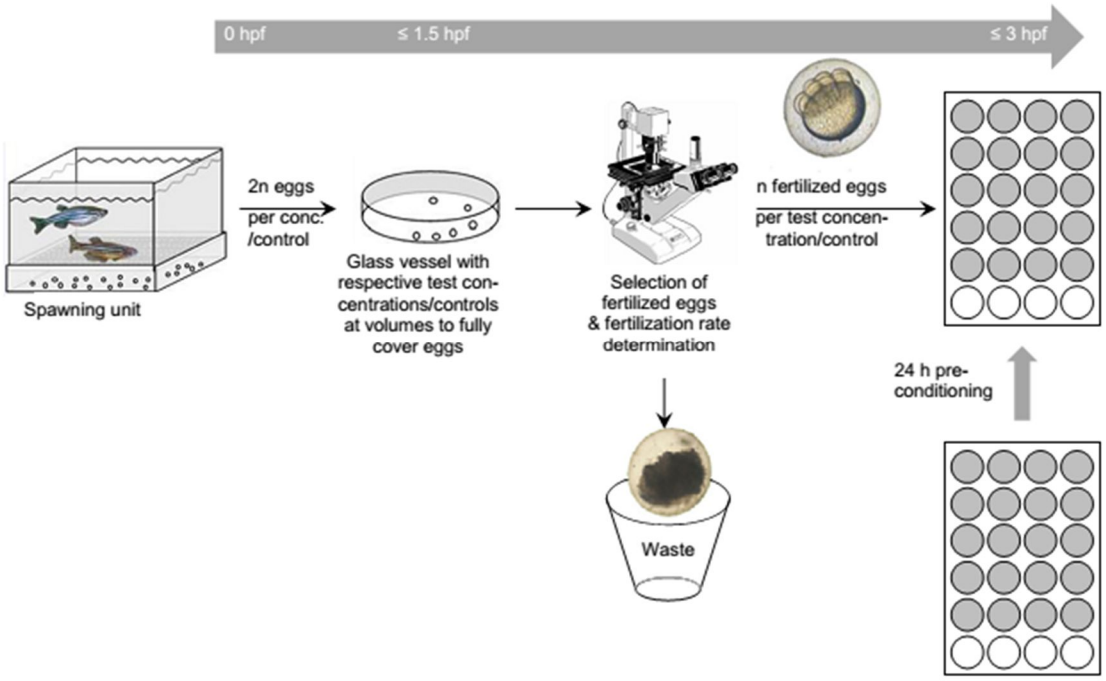

Fig 2: Schematic overview of the fish embryo acute toxicity test procedure. Braunbeck et al (2014).

The assessment of sub-lethal endpoints in the FET assay such as changes in heart beat, presence of edema and deformities, changes in length, altered behaviour among others makes this test a powerful tool for the detection of effects at several levels and may provide information on the mode of action and, finally, indicate long-term effects of chemicals (Scholz et al., 2008).

1.2 Aim and outline

Regarding the several direct and indirect impacts of climate change on aquatic environments and, at the same time, recognizing the complexity of environmental risk assessment for the combination of two or more stressors, this thesis aims to contribute to the discussion concerning the influence of climate change related environmental factors (pH, DO and Ultraviolet radiation) on the toxicity of pesticides. Therefore, the main goal of this thesis is to evaluate the toxicity of binary combinations of environmental parameters and pesticides using zebrafish (*Danio rerio*) embryos as a model. In order to accomplish this goal, both stressors (environmental factors and pesticides) were evaluated separately and in combination and thus 3 specific objectives were established:

1) To evaluate the effects of the environmental factors pH, DO and UV radiation on the development of zebrafish embryos in order to establish clear concentration-response relationships to be further used in combined experiments with chemical compounds;
2) To study the toxicity of the selected pesticides (the carbamate insecticide carbaryl and the benzimidazole fungicide carbendazim) combining lethal (survival) and sublethal (developmental, biochemical and behaviour parameters) endpoints to zebrafish embryos in order to better understand their toxic effects by expanding the sensitivity of the test and also to establish concentration response relationships to be posteriorly used in the combined experiments.
3) Ultimately, to study the influence of environmental factors on the toxicity of pesticides by testing the binary combination of pH (acidic and alkaline) and carbaryl based on the previously established individual toxicity of each stressor.

This thesis is composed by seven chapters. The chapter 1 consists of this contextualization and a brief introduction with an overview of the climate change process and its subsequent effects on aquatic environments. Chapters 2 to 6 are structured as scientific papers (two submitted and the others in preparation to be submitted in international peer reviewed scientific journals) and present all results of effects assessment of environmental factors (pH acid and alkaline, low DO levels and ultraviolet radiation)

and pesticides (carbaryl and carbendazim) as well as the influence of environmental factors (acid and alkaline pH) on the toxicity of the pesticide carbaryl in combination. The description of each chapter is summarized below:

Chapter 1: General Introduction. Contains a contextualization of the climate change process and the consequences to aquatic environments.

Chapter 2: Pesticide contamination in a changing environment: the role of pH, UV radiation and oxygen depletion in the modulation of toxicity. A careful and massive review of the literature was conducted concerning the climate change process and its consequent impact on water quality through alterations in environmental factors and on pesticides fate and behaviour. Global warming, along with higher pesticide exposure in natural ecosystems may pose an increased risk in terms of quality of water resources and wildlife conservation. Climate change will produce changes in various abiotic parameters such as pH, UV radiation and dissolved oxygen in which we focused in this review and in this thesis. The combination of these abiotic factors with pesticides may be deleterious to aquatic species as they may interact in many ways producing higher toxicity and therefore affecting their development and long term survival.

Chapter 3: Zebrafish embryo tolerance to environmental stress factors – concentration/dose response analysis of oxygen limitation, pH and UV-light irradiation. In this study we evaluated the tolerance of zebrafish (*Danio rerio*) embryos to variations in three climate change related environmental stress parameters: pH, DO and UV light intensity. All stressors showed clear concentration- or intensity-dependent effects, on zebrafish embryo survival and development allowing the application of established models and determination of half-maximal effect concentrations (LC_{50}, EC_{50}). These data provide an important source to study the interaction of environmental stress factors with contaminants in the zebrafish embryo model in the context of climate change scenarios.

Chapter 4: Effects of carbaryl in zebrafish embryos development, biochemical makers and locomotion. In this work the acute toxicity of the carbamate insecticide carbaryl was evaluated in zebrafish embryos using a battery of lethal and sub-lethal

endpoints. Embryos survival, development, biochemical markers and behaviour was studied. Our results showed that carbaryl is moderately toxic to zebrafish embryos with a 96 h-LC_{50} of 14.9 mg/L; however, low concentrations of carbaryl also demonstrated to impact embryos development. At the sub-lethal level, carbaryl significantly decreased heart rate and body length and increased malformations (edemas, red blood cell clutch, deformities such as axis curvature and tail deformity, etc.) and also resulted in significant alterations in enzymatic activities and behaviour. This study highlights the importance of considering the sub-lethal effects of environmental contaminants in risk assessment in order to better estimate their effects to aquatic biota.

Chapter 5: Carbendazim exposure induced physiological, biochemical and behaviour disturbance in zebrafish embryos. The toxicity of the benzimidazole fungicide carbendazim was studied by performing a fish embryos toxicity test with zebrafish using several endpoints. The survival, development, biomarkers and behaviour of embryos was affected after exposure to carbendazim. The behaviour proved to be a very sensitive endpoint to evaluate the effects of pesticides to zebrafish embryos.

Chapter 6: Influence of pH on the toxicity of carbaryl to zebrafish early life stages. In this study, the combined effect of pH and carbaryl was studied. We analysed and compared the predictive power of the concepts of concentration addition and independent action. Results show that observed mixture toxicity was rather well predicted by concentration addition. In the case of acid range the median lethal concentration (LC_{50}) of the mixture is predicted with an error of only 8%. Considering the alkaline range, both concepts underestimate the LC_{50} (by a factor of 3 for concentration addition and by a factor of 4.6 for independent action). The use of concentration addition to evaluate the combined effects of pH and chemical stress seems to be suitable at least for the case of carbaryl and acid conditions. In the case of alkaline level, high pH values played a crucial role by modifying the toxicity of carbaryl through hydrolysis and consequently increasing its toxicity to zebrafish embryos. More research need to be conducted to evaluate mixture toxicity composed by chemical and non-chemical stressors in order to improve our understanding of these type of combinations and to improve the risk assessment of mixtures composed of chemical and non-chemical stressors.

Chapter 7: Discussion and Final Remarks. This chapter provides a general discussion on the results obtained in Chapters 2 to 6 and gives a short overview with the main highlights of the thesis.

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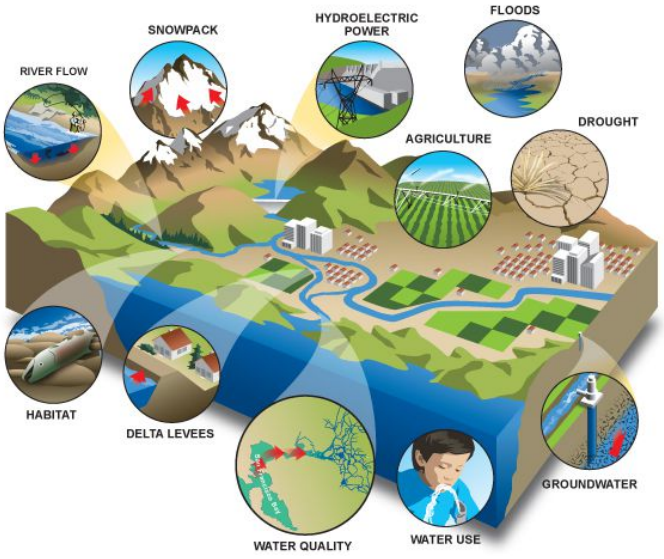
Chapter 2

Pesticide contamination in a changing environment: the role of pH, UV radiation and oxygen depletion in the modulation of toxicity



Credit image: California Department of water resoures, US

Pesticide contamination in a changing environment: the role of pH, UV radiation and oxygen depletion in the modulation of toxicity

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Abstract

Environmental pollution and global warming are two of the topics of most concern worldwide. With increasing anthropogenic pollution and rising temperatures, quality of water ecosystems is becoming seriously compromised. Climate change will also induce proliferation of many invasive pests as conditions tend to be favourable to their life cycles. In this context, pesticide use will surely increase as a measure to safeguard crop productivity. Global warming, along with higher pesticide exposure of natural ecosystems may pose and increase risk in terms of quality of water resources and wildlife conservation. Climate change will produce changes in various abiotic parameters such as pH, ultraviolet radiation and dissolved oxygen in which we focus in this review paper. We compiled data from 28 studies on the effects of the above mentioned environmental factors on the toxicity of pesticides (insecticides, fungicides, herbicides etc.) to different model organisms. The evaluation revealed that in 71% cases the environmental factors significantly increased the toxicity of pesticides mainly by changing the bioavailability and consequently the uptake of the chemicals by the organisms. However, gaps concerning the study of the effects of environmental parameters on the toxicity of pesticides were also identified, especially concerning dissolved oxygen depletion with only 3 studies available. This review highlights the need of further research in what concerns pesticide toxicity in the climate change context in order to improve the risk assessment of these chemicals and adequately protect aquatic biota. Understanding the mechanisms underlying these effects is a pressing need and essential to build better predictive models and therefore implementing better policies for climate change mitigation.

Keywords: temperature, pH, dissolved oxygen, UV radiation, pesticides, aquatic ecosystems

1. Introduction

Climate change, particularly global warming is one of the major topics of concern worldwide and has been highlighted for its influence in natural and human systems. Assessment reports by the Intergovernmental panel on climate change (IPCC, 2013), indicate that global mean surface temperature has increased since the late 19th century further referring that the 2000's were the warmest among all the previous recorded. This temperature change is expected to exceed 1.5ºC and the warming is likely to continue beyond 2100 in almost all scenarios. Apart from the direct effect that temperature has on organisms, it can also impact water quality affecting physicochemical parameters such as pH and dissolved oxygen (DO).

Freshwater ecosystems are naturally the most sensitive ecosystems due to changes in the hydrologic cycle promoted by climate change (Allan et al., 2005). Several natural systems have been already described as impacted by recent climate change such as temperate lakes (Benítez-Gilabert et al., 2010; Fenoglio et al., 2010; Mooij et al., 2005), catchments, streams and lakes. Analysis of extensive time sequences of these ecosystems have shown that climate change affects water quality parameters such as nutrient loading (Benítez-Gilabert et al., 2010; George et al., 2004; Komatsu et al., 2007), DO concentrations (Mimikou et al., 2000; Prathumratana et al., 2008) and pH (Bates et al., 2008; Vliet and Zwolsman, 2008). Furthermore, environmental parameters such as DO, pH and conductivity have been shown to be negatively correlated with hydrological parameters such as the mean level and discharge flow which are certainly associated with climatic processes (evaporation and precipitation).

A case-study in Spain, in a semi-arid stream, has shown a clear relationship between temperature increase and organic matter content and nutrient (nitrate and phosphorus) concentrations (Benítez-Gilabert et al., 2010), being these important factors to consider when assessing the quality of water resources. Another case-study in the UK highlighted that important hydrological changes will certainly occur, such as frequent droughts in summer as well as flash flooding, leading to uncontrolled discharges from urban areas to water courses and estuaries (Whitehead et al., 2009). Also, other scenarios predict lower flows, reduced velocities and consequently higher water stagnation in rivers and lakes will enhance the potential for toxic algal blooms and DO depletion (Whitehead

et al., 2009).

Besides all these important effects referring to the direct physical alterations in what concerns climate change, other indirect effects are previewed that should not be neglected. Changes in climate are likely to result on spreading of some insect-borne diseases and agricultural pests (Koleva and Schneider, 2009; Porter et al., 1991), in addition to the increased incidence of weeds (Coakley, 1999) caused mainly by alterations in temperature, precipitations and wind patterns. Several pests, diseases and weeds are already spreading or moving such as the Western corn rootworm, the Colorado beetle in potato, grass weeds in maize and Barley Yellow Dwarf virus in cereals (Parry, 1990). Thus, it is expected, in the future, an increase in pesticide use and therefore increased bioavailability for non-target aquatic organisms.

1.1 Effect of climate change related factors on pesticide toxicity

Recently, special attention of the scientific community has been directed towards the combined effects of multiple stressors instead of a only chemical toxicity evaluation, especially considering the Global warming scenario. Standard chemical toxicity evaluation usually means exposing organisms in "standard conditions" where temperature, moisture, oxygen and pH are held constant and in controlled situations. However, these conditions do not always reflect the heterogeneity and the multiple stress factors that natural populations experience in the wild but more importantly in the worldwide context, do not take into account the upcoming changes resulting from global alterations and do not lead into an accurate ecological risk assessment.

Some recent reviews which focus on the influence of abiotic/environmental parameters - highlighted as impacted by climate change - in the biological effects of contaminants such as Holmstrup et al., (2010) reporting interactions involving heat stress, freezing temperatures, desiccation, oxygen depletion, starvation and pathogens and a huge roll of chemicals including metals, pesticides and other types of chemicals such as ammonia or phenol. Similarly, Laskowski et al., (2010) emphasizes the need to incorporate natural environmental conditions, especially adjusted to different geographical areas, in ecological risk assessment. Further they refer that not only toxicity of single chemicals is affected but also that combinations of different chemicals may be modulated by various environmental conditions. Therefore, a pressing need arise to

design new methodologies to improve ecological risk assessment and environmental safety analysis.

However, the mechanisms by which variations of environmental parameters increase the toxicity of chemical pollutants, in freshwater systems, are not fully understood. These may act simply by inducting metabolic changes which can be a likely factor for increased toxicity as referred many times in the literature (Heugens et al., 2001; Holmstrup et al., 2010; Lydy et al., 1999; Noyes et al., 2009) or can interact chemically or biologically in more complex ways. Thus, scientific community is only starting to understand how multiple stressors such as salinity, temperature, hypoxia or Ultraviolet radiation (UVR) interact with chemical pollution and in which way are these affecting the biota in all kinds of ecosystems (Schiedek et al., 2007).

1.2 Mixture toxicity

In what concerns aquatic toxicology, two different concepts are broadly used to describe general relationships between the effects of single substances and the corresponding mixtures of stressors with similar and different modes of action (MoA) therefore defined as concentration addition (CA) and independent action (IA). These models allow the calculation of expected mixture's toxicity on the basis of known toxicities of the mixture's individual components (Barata et al., 2007).

These concepts have now been applied to environmental factors as well being used in a similar way to evaluate existing interactions. However, these terms must be used with caution as environmental factors, contrary to chemical pollutants do not possess a specific MoA that can be used to integrate the stressor into IA or CA models. Although, these models have already been used to investigate such interactions, none of the models obtained relates to biochemical and physiological processes which are the key to understanding those environmental-chemical interactions (Laskowski et al., 2010). Therefore, hereby, synergism or antagonism is referring to the degree by which the effects are either higher (synergism) or lower (antagonism) than the expected results in standard situations.

Taking into account all the information that was reported above, the main objective of this review is to highlight the state of the art of current research on interactions regarding environmental factors and pesticides and to identify data gaps that need to be fulfilled The environmental parameters to be addressed in this review will be the DO depletion, pH and UVR. Selection was made according to current state of the art and gaps in the literature concerning pesticides and climate change.

2. Combined effects of environmental factors and pesticides

2.1 Effect of acid and alkaline pH

The pH is pointed out as one of the most important factors conditioning survival and fitness of many freshwater species since it affects many chemical and biological processes. The pH of freshwater systems (rivers and lakes) depends on a complexity of factors and can be indirectly affected by changes in climatic variables including higher temperatures, increased summer drought, intense rainfall among others (Wright and Jenkins, 2001; Wright and Schindler, 1995; Wright, 2008). For instance, a temperature increase may be indirectly related to a pH increase reflecting a decrease in dissolved CO_2 concentrations due to proliferation of algae blooms as reported for European river (Meuse) after a severe drought period (van Vliet and Zwolsman, 2008). In remote locations where acid deposition is absent such as alpine lakes (Psenner and Schmidt, 1992; Sommaruga-Wögrath et al., 1997) a strong positive correlation is observed between pH and mean air temperature. In this case, factors such as a nitrogen deposition and/or biological activity are indicated as possible significant variables for this variation.

As one of the most determinant parameter for any chemical and biochemical effect, pH alone has been extensively explored in the past. Variations in pH showed to have a significant impact on survival, hatching success, reproduction, pigmentation, swimming performance behavior and body chemistry of both fish species and invertebrate aquatic species (Fromm, 1980; Haines, 1981; Havas and Rosseland, 1995; Ikuta et al., 2000; Jordahl and Benson, 1987; Lechleitner et al., 1985; Okland and Okland, 1986; Ye and Randall, 1991), with early life stages of development being more sensitive to pH

variations. Effects of this environmental parameter are not restricted to the organismal level but also affect the community level, being responsible for changes in the structure of populations and for decreasing species diversity of aquatic organisms (Havas and Rosseland, 1995; Jordahl and Benson, 1987; Okland and Okland, 1986). Therefore, in the current and future climate change scenarios it is crucial to understand the consequences of this pH variations, especially in ecosystems where organisms are exposed to other chemical stressors being forced to cope simultaneously with multiple stress factors.

The majority of studies considering the influence of pH on the toxicity of environmental pollutants have focused on the effects of pH mainly on metals and phenols toxicity (Bervoets and Blust, 2000; Dave, 1985; Dietrich and Schlatter, 1989; Grosell et al., 2006; Reader et al., 1989; Stouthart et al., 1996). Gerhardt (1993) reviewed the effects of pH on the toxicity of metals, concluding that metals toxicity depends on pH, because it affects sorption, complexation and solubility of metals. In general, it seems that decreases in pH implies increased metal uptake, consequently, increasing their toxicity. However, the effects vary among species, life stage and duration of exposure. The influence of pH on the toxicity of environmental pollutants has been studied for more than 50 years; nevertheless, few studies have dedicated to understand its influence on pesticides toxicity.

In **Table 1** we summarize 18 studies found in the literature addressing the effects of pH on the toxicity of pesticides. For almost all the pesticides, toxicity is correlated with changes in pH. For organhosphates and carbamate pesticides, hydrolysis seems to be an important factor as decreases in toxicity were related to rapid hydrolysis of pesticides, especially under alkaline conditions. However, the generation of more toxic hydrolysis products was correlated to an increase in the toxicity in higher pH values. In the specific case of trichlorofon, its toxicity increases at higher pH values due to formation of a more toxic form (Howe et al., 1994). Similarly, the herbicide mexacarbate zectran was found to be 38 times more toxic at pH 9.5. In contrast two authors (Kar and Singh, 1978; Rath and Adhikary, 1996) reported a reduction in the toxicity of carbaryl and carbofuran (Furadan) to cyanobacteria and algae at high pH. In both cases, toxicity decreases as consequence of a hydrolysis process, being these two more toxic at low pH (4.0-6.0).

Table 1- Summary of studies concerning interaction between acid and alkaline pH and pesticides

Pesticide	Type	pH range	Test organism	Species	Life stage	Endpoint	Interaction	Reference
Glyphosate (Roundup)	Herbicide	6.5-9.5	Fish	*Salmo gairdneri (rainbow trout)*	Embryo/ Larvae	Mortality	Synergism (neutral pH)	(Folmar et al., 1979)
Glyphosate (Roundup)	Herbicide	6.5-9.5	Fish	*Lepomis macrochirus (bluegills)*	Embryo/ Larvae	Mortality	Synergism (neutral pH)	(Folmar et al., 1979)
Pentachlorophenol	Organochlorine Pesticide	5.5-10	Fish	*Carassius auratus (goldfish)*	Adult	Mortality/ Accumulation	Synergism (low pH)	(Kishino and Kobayashi, 1995)
Pentachlorophenol	Organochlorine Pesticide	4.0-9.0	Fish	*Danio rerio*	Embryo	Mortality	Synergism (low pH)	(Dave, 1984)
2,4-Dichlorophenol	Organochlorine compound	7.3-9.1	Fish	*Fathead minnow*	Juvenile	Survival	Synergism (pH around 7.0)	(Holcombe et al., 1980)
Deltamethrin	Insecticide (Pyrethroid)	6.9-9.0	Fish	*Cyprinus capio*	Larvae	Mortality	Synergism (high pH)	(Ghillebaert et al., 1996)
Trichlorfon	Insecticide Organophosphate	6.5-9.5	Fish	*Oncorhynchus mykiss*	Not stated	Mortality	Synergism (high pH)	(Howe et al., 1994)
2,4-Dinitrophenol	Herbicide	6.5-9.5	Fish	*Oncorhynchus mykiss*	Not stated	Mortality	Synergism (low pH)	(Howe et al., 1994)
Carbendazim	Fungicide Benzimidazole	6.5- 7.5	Fish	*Salmo gairdneri*	Embryo/ Larvae	Survival	Synergism (high pH)	(Palawski and Knowles, 1986)
Dimethrin	Insecticide Pyrethroid	6.5-9.5	Fish	*Lepomis macrochirus*	Adult	Survival	None	(Mauck et al., 1976)
d-trans Allethrin	Insecticide Pyrethroid	6.5-9.5	Fish	*Lepomis macrochirus*	Adult	Survival	None	(Mauck et al., 1976)

Pesticide	Type	pH range	Test organism	Species	Life stage	Endpoint	Interaction	Reference
RU-11679 [1R, trans]-ethanomethrin	Insecticide Pyrethroid	6.5-9.5	Fish	*Lepomis macrochirus*	Adult	Survival	None	(Mauck et al., 1976)
S-bioallethrin	Insecticide Pyrethroid	6.5-9.5	Fish	*Lepomis macrochirus*	Adult	Survival	None	(Mauck et al., 1976)
Resmethrin	Insecticide Pyrethroid	6.5-9.5	Fish	*Lepomis macrochirus*	Adult	Survival	None	(Mauck et al., 1976)
Vision (glyphosate)	Herbicide	5.5-7.5	Amphibian	*R. pipiens*	Larvae	Survival	Synergism (neutral pH)	(Chen et al., 2004)
Carbaryl	Insecticide Carbamate	6.0-8.0	Amphibian	*Rana catesbeiana*	Larvae	Survival/ growth	None	(Relyea, 2006)
Carbaryl	Insecticide Carbamate	6.0-8.0	Amphibian	*Rana clamitans*	Larvae	Survival/ growth	None	(Relyea, 2006)
Glyphosate (Roundup)	Herbicide	6.0-9.0	Waterflea	*Ceriodaphnia dubia*	Adult	Mortality	Synergism (at high pH)	(Tsui and Chu, 2003)
Vision (glyphosate)	Herbicide	5.5-7.5	Waterflea	*Simocephalus vetulus*	Juvenile/ Adult	Survival/ reproduction	Synergism (neutral pH)	(Chen et al., 2004)
Pentachlorophenol	Organochlorine Pesticide	6.5 -9.0	Waterflea	*Daphnia magna*	Adult	Immobilization	Synergism (low pH)	(Xing et al., 2012)
2,4-Dichlorophenol	Organochlorine	6.5 -9.0	Waterflea	*Daphnia magna*	Adult	Immobilization	Synergism (low pH)	(Xing et al., 2012)
2,4,6-Trichlorophenol	Organochlorine compound	6.5 -9.0	Waterflea	*Daphnia magna*	Adult	Immobilization	Synergism (low pH)	(Xing et al., 2012)
Aldicarb	Insecticide (carbamate)	4.0-8.0	Midge	*Chironomus riparius*	Larvae	Locomotion	None	(Fisher, 1991)
Benzene hexachloride	Fungicide	4.0-8.0	Midge	*Chironomus riparius*	Larvae	Locomotion	Synergism (low pH)	(Fisher, 1991)
Carbaryl	Insecticide Carbamate	4.0-8.0	Midge	*Chironomus riparius*	Larvae	Locomotion	Synergism (low pH)	(Lohner and Warwick Fisher, 1990)

Pesticide	Type	pH range	Test organism	Species	Life stage	Endpoint	Interaction	Reference
Parathion	Insecticide Organophosphate	4.0- 8.0	Midge	*Chironomus riparius*	Larvae	Locomotion	Low effect	(Lydy et al., 1990)
Pentachlorophenol	Organochlorine Pesticide	4.0-8.0	Midge	*Chironomus riparius*	Larvae	Locomotion	Synergism (low pH)	(Fisher, 1991)
Terbufos	Insecticide Organophosphate	6.5-9.5	Amphipod	*Grammarus pseudolimnaes*	Not stated	Mortality	None	(Howe et al., 1994)
Trichlorfon	Insecticide Organophosphate	6.5-9.5	Crustacean	*Grammarus pseudolimnaeus*	Not stated	Mortality	Synergism (high pH)	(Howe et al., 1994)
Atrazine	Herbicide	7.5-8.6	Alga	*Selenastrum capricornutum*	-	Growth rate	None	(Mayer et al., 1998)
Pentachlorophenol	Organochlorine Pesticide	6.5 -9.0	Alga	*Scenedesmus obliquus*	-	cell growth	Synergism (low pH)	(Xing et al., 2012)
2,4-Dichlorophenol	Organochlorine compound	6.5 -9.0	Alga	*Scenedesmus obliquus*	Adult	Immobilization/ cell growth	Synergism (low pH)	(Xing et al., 2012)
2,4,6-Trichlorophenol	Organochlorine compound	6.5 -9.0	Alga	*Scenedesmus obliquus*	Adult	Cell growth	Synergism (low pH)	(Xing et al., 2012)
Chlorsulfuron	Herbicide Sulfonylurea	5.0- 6.5	Alga	*Chlorella fusca*	-	Cell growth/ reproduction	Synergism (low pH)	(Fahl et al., 1995)
Carborfuran	Insecticide Carbamate	5.0-10.0	Alga	*Nostoc muscorum*	-	Growth	Synergism (low pH)	(Kar and Singh 1978)
Carbofuran	Insecticide Carbamate	5.0-10.0	Alga	*Anabaena fertilissima*	-	Growth and chlorophyll (Chl)	Synergism (low pH)	(Rath and Adhikary 1996)
Carbofuran	Insecticide Carbamate	5.0-10.0	Alga	*Anabaena variabilis*	-	Growth and chlorophyll (Chl)	Synergism (low pH)	(Rath and Adhikary 1996)

Regarding pyrethroids, the pH effects on toxicity of these compounds were investigated in the three studies and no significant changes in the toxicity were found (Ghillebaert et al., 1996; Howe et al., 1994; Mauck et al., 1976). The greater molecular stability of these compounds across the studied pH range (6 to 9.5) may explain the low or no influence of pH in the toxicity of this class/group of pesticides. However, tests under acidic conditions were absent in these studies.

The toxicity of all the herbicides studied seem to be dependent on pH conditions except for the banned herbicide atrazine referred in Mayer et al., (1998). In the extreme case of chlorsulfuron, toxicity is enhanced by 25-fold when pH is lowered from 6.0 to 5.0. In this particular case, a pH-dependent sorption and bioconcentration was observed, suggesting that this herbicide primarily crosses the cell membrane in undissociated lipophilic form and accumulate inside the cell by ion trapping.

As for the ubiquitous organochlorine herbicides 2,4 – dichlorophenol, 2,4,6 – trichlorophenol and pentachlorophenol, four studies (Dave and Garside, 1980; Fisher, 1991; Kishino and Kobayashi, 1995; Xing et al., 2012) account for a strong correlation between an increase in toxicity and low pH with severe effects on the survival and growth of the exposed organisms. On the other hand, the toxicity of the broad spectrum herbicide glyphosate increases only at higher pH values (7.5 – 9.0) (Chen et al., 2004; Folmar et al., 1979) being this also observed by Tsui and Chu (2003) which reported a higher toxicity of this herbicide at high pH (9.0). Further, it was concluded that glyphosate became non-ionic under alkaline pH resulting in a greater toxicity to this species through a non-specific membrane disruption mechanism.

As can be seen from these studies, pH can strongly affect the toxicity of pesticides. From the 18 studies reviewed, in the majority of cases (66%), it is reported an increase in the toxicity due to changes in pH. The influence of pH on the bioavailability and bioaccumulation of pesticides was the main explanation to the observed effects at different pH levels mainly through transformation or degradation originating more toxic metabolites (e.g. Mayer et al 1998; Tsui and Chu 2003; Howe, Marking et al 1994). In fact, it is well recognized that pH can act directly on the physicochemical properties of chemical compounds leading to drastic changes in the bioavailability and also affecting the uptake by the organisms (Rendal et al., 2011a, 2011b). Understanding the influence of pH on bioavailability, uptake and also on the

sensitivity of organisms is extremely important to understand the combined effects between pesticides and pH changes. The data sets available in the literature relating pH to interactions with pesticides are very small comprising only 18 pesticides evaluated. In addition, half the studies investigate the pH effects only in the range of 6 to 9 which may not adequately reflect the actual toxicity of pesticides in cases in which pH falls outside the natural levels, such as acidic lakes (Psenner and Schmidt 1992). Therefore, more research is needed to better predict the impact of fluctuations of pH on the toxicity of pesticides in order to protect aquatic biota in the current and future climate change scenarios.

2.2 Effect of low dissolved oxygen levels (DO)

No environmental parameter of such ecological importance to coastal and estuarine environments has changed as fast as DO (Diaz, 2001). Hypoxia is defined by concentrations of DO below 2 mg O_2/L, (Diaz and Rosenberg, 1995; Shang and Wu, 2004; Wu, 2002). Hypoxia has been referred as a problem regarding mainly marine environments affecting thousands of Km^2 of marine water all over the world and has been responsible for mass mortality in various groups of organisms (Diaz, 2001). Notwithstanding, this phenomenon also occurs in a wide range of aquatic ecosystems (e.g. streams, lakes, etc.) and varies temporarily and seasonally depending on many factors like atmospheric gas exchanges and temperature. While occurring naturally, hypoxia has been enhanced by anthropogenic activities related to organic and nutrient enrichment with special relevance on lakes and coastal areas which tend to be highly sensitive to nutrient enrichment (Diaz and Breitburg, 2009) in a phenomenon known as eutrophication.

Due to rapid human growth and global warming, the problem of hypoxia is likely to worsen in the upcoming years. An increasing temperature will lead to a considerable reduction in oxygen solubility, therefore leading to a more anoxic environment. Hypoxia due to anthropogenic factors may be a result of excessive input of nutrients and organic matter into water bodies with poor water circulation. Singly, it is referred in Bagatto (2005) that hypoxia has profound effects on the onset of all cardiovascular responses but also shifted the onset relative to the developmental

programme. Hanazato & Dodson (1995) refers that oxygen depletion may lead to lower growth rates in daphnids neonates due to an extra investment in haemoglobin production to compensate lower oxygen availability which will reduce energy needed to carbaryl detoxification. In this case, a synergism occurred mainly by changing patterns of energy allocation.

Synergistic interactions between oxygen depletion and environmental contaminants have already been reported, mainly focusing on heavy metals (e.g. Cd). Very few authors such as Ferreira et al. (2008), Hanazato and Dodson (1995), Van der Geest (2002), studied interactions concerning pesticide toxicity and DO depletion (Table 2). Interaction between these two parameters may not always be of synergistic nature as influenced by the specific mode of action of each pesticide. While Ferreira et al. (2008) states that oxygen depletion and carbendazim interact synergistically, Van der Geest (2002) described that oxygen depletion had no effect on the toxicity of pesticide diazinon. In another experiment, the synergism detected for low DO and high carbendazim concentrations in daphnids was attributed to the production of reactive oxygen species by hypoxic conditions and consequently a loss of detoxification capability at the cellular levels (Ferreira et al., 2008). This synergism was further confirmed by LC_{50} calculation at different DO levels.

Table 2 – Summary of studies concerning interactions between depletion of dissolved oxygen and pesticides

Pesticide	Type/Chemical class	Test organism	Species	Life stage	Endpoint	Interaction	Reference
Carbendazim	benzimidazole fungicide	Daphnid	*Daphnia magna*	Neonate	Mortality	Synergism	(Ferreira et al. 2008)
Carbendazim	benzimidazole fungicide	Daphnid	*Daphnia magna*	Fourth instar	Feeding rate	Antagonism	(Ferreira et al. 2008)
Carbaryl	Carbamate insecticide	Daphnid	*Daphnia pulex*	All stages	Growth, reproduction, development	Synergism	(Hanazato and Dodson 1995)
Diazinon	Carbamate insecticide	Insect	*Ephoron virgo*	Larvae	Mortality	None	(van der Geest et al 2002)

Despite referring to possible hypothesis and mechanisms by which synergism may occur, in none of this studies is taken a molecular approach in order to properly assess the nature of this synergism. Although one paradigm remains, the synergistic interactions resulting from lower oxygen concentrations should always be related, in some sort of way, to the energy allocation which is used to acclimate and therefore not available for detoxification of these contaminants.

The data available among the bibliography surely lacks for the combined effects of oxygen depletion and pesticides in vertebrate species as well as a convincing explanation and description of the mechanisms involved in these synergistic interactions. In this way, toxicodynamics and toxicokinetics studies should provide a better understanding on the effects of oxygen depletion in pesticide toxicity, particularly if these approaches are integrated into the respiratory chain and in antioxidant defence such as (e.g.) superoxide dismutase (SOD) or catalase (CAT) but also physiological indicators such as haemoglobin counts as referred by Hanazato & Dodson (1995). However, it is noteworthy the lack of bibliography in this matter.

2.3 Effect of UV radiation

In the last years, there is a growing awareness on the effects of UVR in terrestrial ecosystems mainly due to an increasing concern related to human-induced depletion of the stratospheric ozone layer. Despite the attention given and the efforts to mitigate this depletion, projections point out that baseline levels pre-1980 will not be accomplished in the next decades (Weatherhead and Andersen, 2006), however, the most recent and promising report shows that ozone is no longer decreasing and is expected to return to normal levels before the mid of the 21st century (WMO, 2011).

Nevertheless, many other factors can cause alterations in UVR. Besides the direct consequence of changes in the ozone layer, changes in aerosols, clouds, or surface reflectance may also affect UVR as reviewed by McKenzie and co-authors (McKenzie et al., 2011). However, future projections on UVR changes are uncertain due to the complexity in the projection of clouds and aerosol changes and also due to the complex interaction between climate change and ozone depletion (McKenzie et al., 2011). Other important variable conditioning UVR effects in natural waters is the dissolved organic matter (DOM), which has a strong effect on the attenuation of UV-B radiation in

aquatic environments (Helbling and Zagarese, 2003). Changes in quantity and quality of DOM directly influence the exposure of aquatic organisms to UV-B radiation (Clements et al., 2008). Moreover, climate change may affect the production, transport and cycling of DOM leading, in some scenarios, to its reduction in aquatic ecosystems (Young et al., 2005, 2004). These changes in the amount of DOM, accompanied by increasing UV radiation, may exacerbate UV effects to aquatic biota.

Ultraviolet (UV) is the shortest wavelength (100-400 nm) that reaches the surface of earth and is divided into three wavelength bands: UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280nm) (Helbling and Zagarese, 2003; WHO, 2002; WMO, 2011). Furthermore, the amount of UV radiation reaching the earth's surface is mainly composed of UV-A and a small quantity of UV-B as UV-C radiation is almost all absorbed by stratospheric ozone. As almost no UV-C reaches the earth surface, UV-B remains as the most hazardous to organisms (Häder, 2000; Preston et al., 1999; Williamson et al., 2001).

Recently, the direct and indirect effects of UV-B radiation in aquatic ecosystems have received attention. Studies confirmed that solar UV-B radiation have harmful effects on aquatic organisms (Häder et al., 2007; Helbling and Zagarese, 2003). UV-B radiation demonstrated to inhibit photosynthesis (Bracher and Wiencke, 2000), to cause DNA damage (Zeng et al., 2009), to alter behavior (Blaustein et al., 2000), growth and reproduction (Huovinen et al., 2001a), to reduce body size and hatching success (Dethlefsen et al., 2001) and increase deformities and mortality (Blaustein et al., 1997, 2003).

Nonetheless, it is important to note that there is high inter-specific variability regarding the sensitivity to UV-B radiation and thus, some species are not so susceptible (Häkkinen et al., 2002; Oromi et al., 2008). These aspects may pose difficulties to assess the UV effects in natural waters (Häder et al., 2007). Furthermore, the co-exposure of organisms to UV radiation and a variety of environmental pollutants may induce interactive effects (antagonistic or synergistic).

UV-B radiation has been reported to modify the toxicity of many stressors, such as metals (Prasad and Zeeshan, 2005; Preston et al., 1999; Rai et al., 1998), polycyclic aromatic hydrocarbons (PAHs) (Huovinen et al., 2001b; Nikkilä et al., 1999), antibiotics (J. Kim et al., 2009; J.-W. Kim et al., 2009) among others. Arfsten et al., (1996) reviewing the effects of UVR on the toxic effects of PAHs in aquatic organisms

found that apparently the UV seems to increase PAHs toxicity. According to their results, exposure to UVR and PAH in concert induced a lethal phototoxic effect, where lethality may be a result of a rapid accumulation of cellular damage or death in higher aquatic organisms. Moreover, they found evidences that PAHs enhance carcinogenic properties of UV light. Another study investigating the effects of combined UVR and toxic chemicals associated changes on amphibian populations to synergistic interactions of UVR and contaminants (Blaustein et al., 2003). Although, a large amount of studies have tried to address interaction between environmental pollutants (mainly metals and PAHs) and UV-B radiation, few research have focused on the determination of interaction between UV-B radiation and the fate and effects of pesticides and the few studies addressing this issue are quite recent, dating from 1998 (14 years ago). Table 3 summarizes all the studies conducted on interactions effects of UV-B and pesticides on aquatic organisms. In the majority of studies explicit synergistic effects were found. Nevertheless, the mechanisms through how UV-B radiation enhances the toxicity of pesticides are heterogeneous and in some cases not clear.

Three studies assessed the combined effects of UV-B and insecticides. Beketov et al., (2011) investigated the UV-B related toxicity of pesticides fenoxycarb, pirimicarb and tebufenpyrad on the survival, reproduction and population growth rate of daphnids. The combination of sublethal pesticides concentrations and UV doses produced a synergistic effect on both cumulative reproduction and population growth rate for fenoxycarb and pirimicarb, but a less-than-additive effect for tebufenpyrad. The authors stated two different processes that may explain the observed synergistic effects: (1) UV radiation possibly activates the compounds inside the tested organisms after exposure to chemicals, or (2) sensitivity of tested organisms is probably increased by UV radiation. They concluded that the synergistic effects are mainly due to the combined effects of both stressors (pesticides and UV) on daphnid physiology.

Table 3 – Summary of studies concerning interactions between UV and pesticides

Pesticide	Type/Chemical class	Test organism	Species	Life stage	Endpoint	Interaction	Reference
Carbaryl	Insecticide Carbamate	Amphibian	*Rana sphenocephala*	Tadpoles	Survival/Body length	None	(Bridges and Boone 2003)
Carbaryl	Insecticide Carbamate	Amphibian	*Hyla versicolor*	Embryos/Tadpoles	Survival/Locomotory activity	Synergism	(Zaga et al., 1998)
Carbaryl	Insecticide Carbamate	Frog	*Xenopus laevis*	Embryos/Tadpoles	Survival/Locomotory activity	Synergism	(Zaga et al., 1998)
Fenoxycarb	Insecticide Carbamate	Waterflea	*Daphnia magna*	Adults	Reproduction/Population growth rate	Synergism	(Beketov et al., 2011)
Pirimicarb	Insecticide Carbamate	Waterflea	*Daphnia magna*	Adults	Reproduction/Population growth rate	Synergism	(Beketov et al., 2011)
Tebufenpyrad	Insecticide Pyrazole	Waterflea	*Daphnia magna*	Adults	Reproduction/Population growth rate	Synergism	(Beketov et al., 2011)
Carbendazim	Fungicide Benzimidazole	Waterflea	*Daphnia magna*	Adults/Neonates	Reproduction/feed inhibition	Synergism /Antagonism	(Ribeiro et al., 2011)
Acifluorfen	Herbicide (diphenyl ether)	Waterflea	*Daphnia magna*	Adults	Locomotory Activity/Immobilization	Synergism	(Scrano et al., 2002)
Triazine	Herbicide Triazine	Alga	*Scenedesmus gutwinskii*	-	Photosynthetic rate	Synergism	(Kasai and Arts, 1997)
Atrazine	Herbicide Triazine	Bacterium	*Vibrio fischeri*	-	Bioluminescence	Antagonism	(Lin et al., 1999)
Metolachlor	Herbicide Chloroacetanilide	Bacterium	*Vibrio fischeri*	-	Bioluminescence	Antagonism	(Lin et al., 1999)

The other two studies investigate the effects of UV-B radiation on the toxicity of the well-known carbamate pesticide carbaryl. Both studies evaluated the lethal and sublethal effects of the combination on amphibians. Bridges and Boone (2003) found no effect of UV radiation on carbaryl toxicity. The authors believe that dissolved organic carbon of the artificial ponds used in the test might have act as a protection. On the other hand, Zaga et al. (1998) results indicated a synergistic effect due to photoenhanced toxicity by UV-B radiation. In this case, the synergism seems to arise from the photomodification of the carbaryl molecule in the water.

One study investigated the interactive effects of environmental relevant UV doses (105 to 400 $J.m^{-2}$) on the toxicity of carbendazim (a benzimidazole fungicide) to *Daphnia magna*. Ribeiro et al., (2011) found two different response patterns for feeding rates and reproduction. For feeding inhibition, when UVR was the dominant stressor in the combination, antagonism was observed. Conversely, for reproduction, synergism was observed when UVR was the dominant stressor in the combination. In these case the response patterns differs according to the endpoint and UVR dose applied. Differently from the others studies, these authors do not associate the increased toxicity to photomodification/phototransformation of the pesticide.

The other three studies explored the UV-B influence on herbicides toxicity. Kasai and Arts (1997) and Scrano et al., (2002) observed a negative effect of UV-B radiation on triazine and acifluorfen to algae and daphnids respectively. On the other hand, Lin et al., (1999) studying the effect of simulated sunlight on atrazine and metolachlor toxicity to aquatic organisms observed a decrease in toxicity when increasing light intensity. The authors suggested a possible photodegradation of the pesticides when exposed to simulated sunlight as the responsible for the reduction in the toxicity.

Based on the knowledge obtained until now, the combination of UV radiation and pesticides seems to depend on the specific compound and its concentrations and also on the species and stage of development. However, further studies need to be conducted to comprehend how these stressors interact with one another and the possible mechanisms behind these interactions in order to predict the consequence of the increasing UV radiation. The synergistic effects should be thoroughly investigated to better define more realistic safe concentrations of pesticides. Furthermore, to realistic predict the consequences of increasing UV-B radiation and pesticides pollution under

the climate change conditions, it is of extremely and crucial importance to increase the knowledge on combined effects of these stressors.

3. Conclusion and future remarks

According to the literature reviewed, it is clear that climate change will have a wide range of effects not only on structure and function of freshwater ecosystems, but also on pesticide distribution and toxicity. From the 28 studies reviewed on interactive effects of pesticides and environmental factors showed that in the majority of cases (78%), environmental variables significantly modified the toxicity of pesticides on tested organisms. Although many studies refer the potential impact of environmental variables in pesticide toxicity such as pH, a lack of knowledge is still present when we address to the nature of this interactions. The terms synergism and antagonism still need to be clearly defined in what concern this relations. These terms do not imply that a direct effect is experienced due to interactions at the physiological level or the possible "side effects" that a given environmental parameter may induce. Also, it is clear that a temporal gap (from 2000 until now only 4 studies available) exists for certain parameters such as the verified in pH while there is a clear gap of knowledge concerning depletion of dissolved oxygen and the effects of UV radiation. Moreover, in the case of pH, the majority of studies evaluate its effects in the range between 6 and 9 which do not reflect their real toxicity. Climate change is a much more complex problem than what is addressed in individual research on sole environmental parameters and pesticides and therefore we need to keep in mind that in natural environmental conditions interactions of more than one parameter and more than one toxicant may occur. Although this review highlights the need of further research in what concerns pesticide toxicity in the climate change context, it also points out the need to conduct integrated studies in which data should include site specific conditions for better ecological risk assessment. Understanding the mechanisms underlying these effects is a pressing need and essential to build better predictive models and therefore implementing better policies for climate change mitigation. We hope that this review may inspire future studies in this matter, not only referring to effects of the interactions between environmental parameters and pesticide toxicity but also to the complexity of the questions underlined here and hopefully leading to creation of better tools for

ecological risk assessment and mitigation policies.

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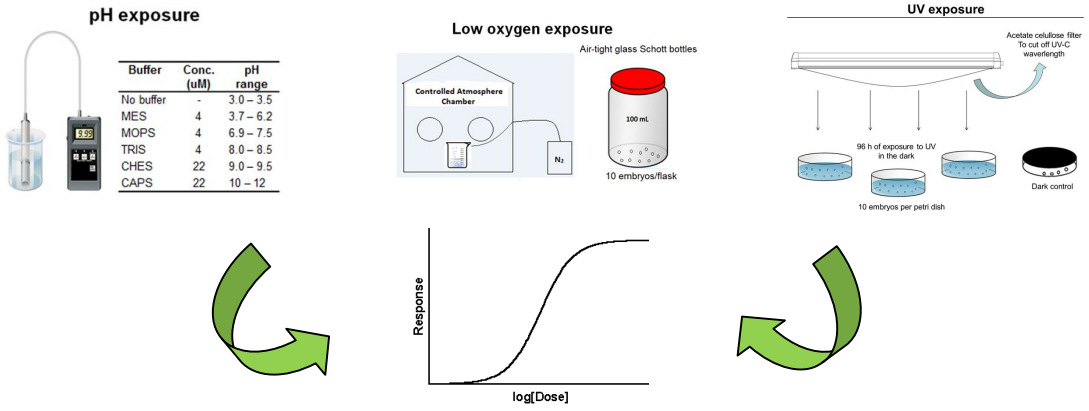
Chapter 3

Zebrafish embryo tolerance to environmental stress factors – concentration/dose response analysis of oxygen limitation, pH and UV-light irradiation



Zebrafish embryo tolerance to environmental stress factors – concentration/dose response analysis of oxygen limitation, pH and UV-light irradiation

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Abstract

During the last century the increase in the mean global temperatures has been shown to impact on freshwater physico-chemical parameters such as pH, dissolved oxygen or UV light abundance. Changes in these parameters could modify the toxicity of environmental pollutants. Therefore, in this study, we studied the tolerance of zebrafish (*Danio rerio*) embryos to variations in pH (3 - 12), dissolved oxygen (3.9 – 237 µmol/L) and UV intensity (55 – 467 mW/m^2) using selected endpoints. The zebrafish embryo is an established alternative test system that is widely used for assessment of the toxicity of chemicals. Embryos were exposed to different ranges of the sublethal endpoints, assessment included the quantification of hatching success, developmental delay, reduction of body length, and frequency of edema, and morphological abnormalities. 96 h-LC_{50}s of 3.68 and 10.21 were determined for acid and alkaline pH, respectively. Embryo survival appeared to be relatively resistant to oxygen depletion with a 96 h-LC_{50} of 13.17 µmol/L. However, concentrations of 187 mg/L and below caused edema and developmental retardations. Continuous exposure to UV radiation (UVR) affected zebrafish development by reducing survival and hatching rate and triggering a series of developmental abnormalities such as pericardial edema and deformities. A 72 h-LC_{50} of 227 mW/m^2 was derived from intensity-response modelling. Our data provide a useful basis for the subsequent assessment of combined effect of environmental stress parameters and chemicals in the context of climate change scenario using the zebrafish embryo model.

Key words: *Danio rerio,* global changes, embryo development, abiotic factors, co-stress

1. Introduction

Recent reports by the Intergovernmental Panel on Climate Change (IPCC, 2013) have indicated that the global mean surface temperature has increased since the late 19th century with a tendency to exceed a further 1.5º C increase until 2100 in almost all

scenarios. Temperature does not only have a direct effect on organism but is impacting also on water physicochemical parameters such as dissolved oxygen (DO) level and pH which could affect the toxicity of contaminants to aquatic organism. Indirectly, temperature may also change exposure of organism to UV light.

For instance, increasing temperatures and increased summer droughts are likely to favour acidic conditions in surface waters (Wright, 2008) due to a higher production of carbon dioxide. pH has been shown to affect survival rates, hatching success, reproduction, pigmentation, swimming performance behaviour and body chemistry of both fish species and invertebrate aquatic species (Fromm, 1980; Havas and Rosseland, 1995; Jordahl and Benson, 1987; Lechleitner et al., 1985; Okland and Okland, 1986; Ye and Randall, 1991), with early life stages of development being more sensitive to pH variations. Consequently, changes in the pH may also affect the community level and translate into via alterations in population structure and reduced species diversity. In acidic streams (pH of about 5), sublethal effects such as reduced larval activity and pigmentation, incomplete hatching as well as low survival of embryos could explain low population density and altered populations structure as observed e.g. in *Salvelinus fontinalis* brook trout populations (Jordahl and Benson, 1987). Eventually, low pH levels can constitute a risk for more sensitive fish and invertebrate species through effects on survival and development of embryos and larval stages. Moreover, an influence of pH on pesticides toxicity has been documented in various studies (Chen et al., 2004; Fisher, 1991; Folmar et al., 1979; Mount, 1973). Overall, these studies showed that fluctuations in pH exacerbate the toxicity of pesticides which may be attributed to increased bioavailability as has been shown for e.g. aluminium and other metals (Dietrich and Schlatter, 1989).

Increasing temperature is also expected to decrease saturation concentrations of oxygen due to a decrease in water capacity to carry oxygen. In aquatic ecosystems with fluctuating oxygen levels as common for many lakes or streams, the dissolved oxygen depletion could increase the frequency of hypoxia status. Hypoxia is commonly defined as an oxygen concentration below 2 mg/L in aquatic environments. Anthropogenic activities leading to organic and nutrient enrichment may contribute further to amplify this effect by eutrophication.

Reduced spawning success, sperm motility, fertilization success, hatching rate and larval survival have been described as hypoxia effect on wild fish populations and to

reduce reproductive performance (Wu et a. 2003). At the community level, hypoxia does not only change the structure by loss of species diversity in fish and benthic communities (Dauer et al., 1993; Diaz and Rosenberg, 1995) but also change functional groups and types of animal/plants in benthic, fish and planktonic communities. Given that many chemicals are known to interfere with reproductive success and development, combination effects with oxygen depletion could be anticipated. For instance, synergistic effects on survival and growth resulting from the interaction between low oxygen levels and pesticides were described by Ferreira et al. (2008) and Hanazato et al. (1995) in daphnids. Decrease in oxygen concentrations has also shown to affect the toxicity of phenolic compounds to fresh water fish *Nopterus nopterus* (Gupta et al., 1983).

Penetration depth of UV light can range from 0.5 to 4 m. Hence, UV-light is potentially damaging to species living in this zone (Speekmann, 2000). Projections show that despite the efforts to reduce atmospheric ozone depletion, the baseline levels of UVR before 1980 will not be restored in the next decades (Weatherhead and Andersen, 2006). Moreover, the UV exposure of inland aquatic ecosystems remains highly variable and could increase due to climate changes. For instance, alterations in dissolved organic carbon (DOC) could affect the UVR transparency since drier and warmer climates will reduce flooding and water saturation of soils within watersheds and hence, could reduce the entry of DOC to adjacent lakes and streams. This might increase exposure of aquatic species to UVR and affect distribution and abundance of planktonic and shallow benthic organisms as well as the benthic spawning zones of vertebrates such as amphibians and fish that deposit their eggs in shallow surface waters (Häder et al., 2007).

UVR is defined as the shortest wavelength that reaches the surface of the earth being divided into UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm) (Helbling and Zagarese, 2003; WHO, 2002; WMO, 2011). The UVR reaching the earth's surface is mainly composed of UV-A and a small quantity of UV-B while UV-C is almost completely absorbed by the stratospheric ozone. UVR is known to provoke several deleterious effects in fish such as DNA damage (Zeng et al., 2009) and reduction of hatching success and egg survival rates (Charron et al., 2000). Moreover, UV has shown to modify the toxicity of many chemical stressors such as metals (Preston et al., 1999), polycyclic aromatic hydrocarbons (PAH) (Huovinen et al., 2001; Nikkilä et al., 1999) and pesticides (Ribeiro et al., 2011; Zaga et al., 1998) with – in the majority of studies –

increasing toxicity of contaminants which may result in synergistic (i.e. higher than expected from the individual toxicity) effects.

In the context of global change, understanding how the above mentioned components can affect toxicity of environmental contaminants to aquatic organisms is pertinent. Therefore, this study intended to evaluate the effects of different ranges of pH, DO and UVR (280-400 nm) on zebrafish embryos, an important model for the hazard assessment of chemicals. The zebrafish embryo test is considered as an alternative model to toxicity tests with juvenile or adult fish. It offers the possibility to perform small-scale, high-throughput analyses compliant with the 3Rs approach (refinement, replacement and reduction of animal experiments; Embry et al., 2010; Halder, 2001; Strähle et al., 2012). The transparent embryos and its well characterized embryonic development represent further advantages of the model in toxicity assessment (Kimmel et al., 1995). Therefore, we set out to provide a detailed and comprehensive assessment of susceptibility ranges for pH, DO and UVR as a basis for the future assessment of the impact of stress factors related to climate change on chemical toxicity.

2. Material and Methods

2.1 Test organisms

Zebrafish were kept in a ZebTEC (Tecniplast, Buguggiate, Italy) recirculating system. Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with 0.34 mg/L salt ("Instant Ocean Synthetic Sea Salt", Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was 26.0 ± 1 ºC, conductivity 750 ± 50 µS, pH 7.5 ± 0.5 and dissolved oxygen equal or above 95 % (7.6 mg/L) saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd) and brine shrimp. Eggs were obtained by breeding of fish in aquaria with marbles in the bottom to protect eggs from predation by adults. The day prior to breeding, males and females were separated by placing a barrier in the holding container until the next morning. Early in the morning, barriers were removed. After removal of marbles eggs were collected, rinsed in water and checked under a

stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). Eggs with cleavage irregularities, injuries or other kind of malformations were discarded.

All tests were performed similar as described in the OECD testing guideline 236 (OECD, 2013), in charcoal filtered and deionised water supplemented with 0.34 mg/L sea salt (see above) at 26 ± 1 ºC and a 16:8 h (light:dark) photoperiod (except UV test, see below). Exposure was conducted from 3 to 96 hpf.

2.2 Analysis of pH effects

Zebrafish embryos were exposed to pH ranges from 3.0 to 12. A set of buffers were used in order to prevent pH drifts during the assay (Table 1). All buffers (MES, MOPS, TRIS, CHES and CAPS) were first tested at neutral pH. The neutral pH is outside of the optimal buffer range but was only used to estimate any potential toxic effect to zebrafish embryos (Fig S1 and S2 suppl. Data ('S' before the number indicates that the figure or table respectively is provided in the 'supplementary information')). This analysis indicated that buffers were used at concentrations ranging from 17- to 64-fold below concentrations that cause mortality. Except for MES, all buffers were used in their optimal pH range to test for pH toxicity. However, despite that MES was used outside the optimal range it was found to efficiently maintain the pH (Figure S3) and was not toxic to embryos at the used concentration. HCl and NaOH (1.0 N) solutions were used for pH adjustment; a portable multiparameter device (ProfiLine Multi 332) was used for pH measurements. Test solutions were daily renewed.

Embryos were exposed individually in 24-well plates for 96 h. For each test 10 embryos were used per replicate and a minimum of 3 replicates were used per treatment. The following endpoints were registered: survival, incidence of pericardial edema, heart rate, deformities, hatching success, body length and developmental delay. The heart rate was measured by counting heart beats under a stereomicroscope in 3 randomly selected 48 hpf-embryos of each replicate (n=9 per concentration) over a period of 15 s. Body length was measured by analysis of digital images of the embryos using the software NIS Elements D (Nikon Corporation, Tokyo, Japan) in 96 hpf-old embryos. Developmental delay was determined by comparison of controls and exposed embryos with developmental stages as described by Kimmel et al. (1995) and by calculating the difference between the

stages in comparison to normal development (in hours). A correction accounting for the different temperatures used in Kimmels' work and the present work was done using the formula H_T=h/ (0.055 T-0.57) described in Kimmel et al. (1995) with H_T= hours of development at temperature T, and h = hours of development to reach the stage at 28.5.

Table 1 – Buffers used for pH stabilization: concentrations used, respective pH range and toxicity data.

Buffer	Concentration used in test (mM)	pH range	NOEC	LC_{50}
No buffer #	-	3.0-3.5	-	-
MES- 2-(Morpholinoethanesulfonic) acid monohydrate	4	3.7-6.5	256 mM*	*n.d.*
MOPS- 3-(N-Morpholino)propanesulfonic acid	4	6.9-7.5	256 mM*	*n.d.*
TRIS- 2-Amino-2-(hydroxymethyl)-1,3-propanediol	4	8.0-8.5	256 mM*	*n.d.*
CHES- 2-(Cyclohexylamino)ethanesulfonic acid	22	9.0-9.5		396 mM
CAPS-3-(Cyclohexylamino)-1-propanesulfonic acid	22	10-12		380.7 mM

n.d. not determined due to low mortality rates at concentration up to the limit of solubility.
* Highest tested concentration
# pH levels at this range could be maintained stable without a buffer

2.3 Analysis of oxygen depletion

Embryos were exposed to oxygen concentrations ranging from 3.9 and 237 µmol/L (0.12 to 7.6 mg/L), reflecting an oxygen spectrum from hypoxic to normoxic conditions. The different oxygen concentrations were established by injecting compressed gaseous nitrogen to the exposure medium. This was done inside a controlled atmosphere chamber (model 855-AC, PlasLabs, USA), saturated with nitrogen gas in order to facilitate the establishment of the desired oxygen concentration in each bottle. Subsequently the test vessels with the embryos were transferred to an incubator with controlled temperature. Oxygen concentrations, pH and conductivity were measured using a portable multiparameter device immediately after adding the embryos and at the end of the test. Embryo exposure was performed with 10 embryos in 100 ml air-tight glass Schott bottles completely filled with test solution to avoid gas exchange during the assay. A minimum of

nine replicates per treatment were used.. At the beginning of the test, the pH values varied among the different DO treatments between 7.6 and 8.4. Given that the results from pH experiments demonstrated that pH effects were only observed at various levels above or below neutral conditions, the pH was not adjusted. (see section 3.1: *pH effects on zebrafish embryo survival and development*).

To avoid variation in the DO concentrations, flasks were not opened until measurement. Hence, separate replicates were used to analyse oxygen concentrations at 24, 48 or 96 hpf. Embryos were then visually inspected using a stereo microscope. Survival rate, hatching success, frequency of edema, heart rate, developmental delay and body length were recorded.

2.4 Analysis of UV effects

Zebrafish embryos were continuously exposed to the UVR intensities ranging from 55 ± 3.3 to 467 ± 25.7 mW/m^2 for a 96 h period with no other source of light. A continuous exposure was chosen to allow the modeling of data and the derivation of $L(E)C_{50}$ values on continuous exposure basis for future experiment on the combined effects of UV and chemical exposure. Additionally, a normal light control (16:8 h, light: dark; ≈500 lux) and three continuous light controls (3.2 ± 0.83 lux to resemble intensity under UVR exposure, 539 ± 7.63 lux, similar to the normal light control and 1804 ± 13.11 lux for comparison with previously published literature) were conducted in order to indicate whether the differential photoperiod alone may impact on the development and survival of the embryos. Organisms were placed in plastic petri dishes. Acetate cellulose filters (0.003 mm, Grafix plastics, USA) were used to reduce UVR irradiation and placed directly below the UV lamps and over the Petri dishes (were they also avoided medium evaporation). These filters were previously irradiated for 12 h to achieve stable reductions in UV transmission and to filter UV-C wavelengths (200-280 nm). In order to achieve different UVR intensities, the organisms were placed at different distances from two Spectroline XX15F/B lamps (Spectronics Corporation, NY, USA). Lamps had peak emission at 313 nm and 365 nm corresponding to UV-B and UV-A respective emission peaks, see Fig S4 supplementary data.

UVR intensities (280 – 400 nm) were measured every 24 h with a spectro-radiometer connected to a monochromator and analyzed with BenWin+ Software (Bentham Instruments, Reading, UK). Lethal and sublethal effects such as described in section 2.2 were monitored daily.

2.4. Data analysis

Lethal concentration values (LC_{10} and LC_{50}) and effect concentration (EC_{10} and EC_{50}) were calculated for each environmental parameter by fitting logistic dose-response curves using the package drc (Ritz and Streibig, 2005) in the software R (R Core Team, 2014). Model choice decision was made based on the the R^2, the log likelihood value, Akaike's information criterion (AIC) and the estimated residual standard error. The models used as well as the slopes for each concentration response curve are presented in Table S1 in the supplementary information.

Buffer LC_{50}s could only be calculated for CAPS and CHES. For all other buffers tested the low mortality rates up to concentrations at the solubility limit did not allow obtaining LC_{50} values. For these buffers an ANOVA (one-way analysis of variance) with appropriate post hoc test (- Dunnett's or Dunn's test) were conducted to potentially derive LOEC or NOEC values. The type of ANOVA (parametric or non-parametric) and post hoc test was chosen depending on whether normality and homocedasticity of data were demonstrated by analysis of the residuals with the Shapiro-Wilks test. Test statistics and analysis of normality were conducted using the software SigmaPlot V.11.0 (Systat Software, 2008) and a significance level of 0.05.

3. Results

3.1 pH effects on zebrafish embryo survival and development

The pH effects were tested for both acidic and alkaline conditions (pH 3-7 and pH 7-12) using various buffers well below lethal concentration ranges (Fig. S1 and S2, supplementary data) to stabilize the pH. Embryos exposed to pH below 3.5 or above 10.5 showed a 100 % of mortality. The observed lethality was established within 24 hours and

did not increase with prolonged exposure. A 96 h-LC_{50} value of 3.7 ± 0.03 pH units was determined (Table 2 and 3 and Fig. 1 a). Heart rate was affected (bradycardia) at 48 h and pericardial edema increased frequency was observed at 96 h (Table 2 and 3, Fig 2 b, Fig. S5 a and b). For the alkaline range, a 96 h-LC_{50} of 10.2 ± 0.03 pH units was calculated (Table 2 and 3, Fig. 1 b). Alkaline pH also affected hatching success at 72 hpf (Fig. S6 a) and the incidence of pericardial edemas (Fig. S6 b).

3.2 Oxygen depletion effects on zebrafish embryo survival and development

The effects of oxygen depletion were studied on the survival and development of zebrafish embryos exposed to DO in the range of 3.9 to 237 µmol/L. At 24 h no mortality was observed even at the lowest tested oxygen concentration. At 48 hpf survival was decreased at concentrations below 18.75 µmol/L. Hundred percent mortality was observed at 96 h at concentrations below 9.37 µmol/L. For the same stage a 96 h-LC_{50} of 13.17 ± 1.7 µmol/L (Table 2 and 3, Fig. 1 c) was calculated. At DO below 4 mg/L, sublethal effects could be observed such as decreased heart rate (48 hpf), increased incidence of pericardial edema (48 hpf and 96 hpf, Fig. S7 a, b) and developmental delay (48 and 96 hpf, Fig. 2 c, d and e, Fig S7 c). At 96 h embryos exposed to oxygen concentration of - 62.5 µmol/L showed a developmental delay of 20 h compared to the control and according to stage classifications based on Kimmel, et al. (1995) (with developmental time corrected for a temperature of 26º C). I.e. the pec fin stage that was reached 60 hpf of controls was only achieved at 96 hpf in exposed embryos. This developmental delay was coinciding with a decreased hatching rate (Fig S7 d) and a reduced body length (Fig S7 e). Body length was already reduced at DO concentrations where no other adverse effects were observed 125 µmol/L.

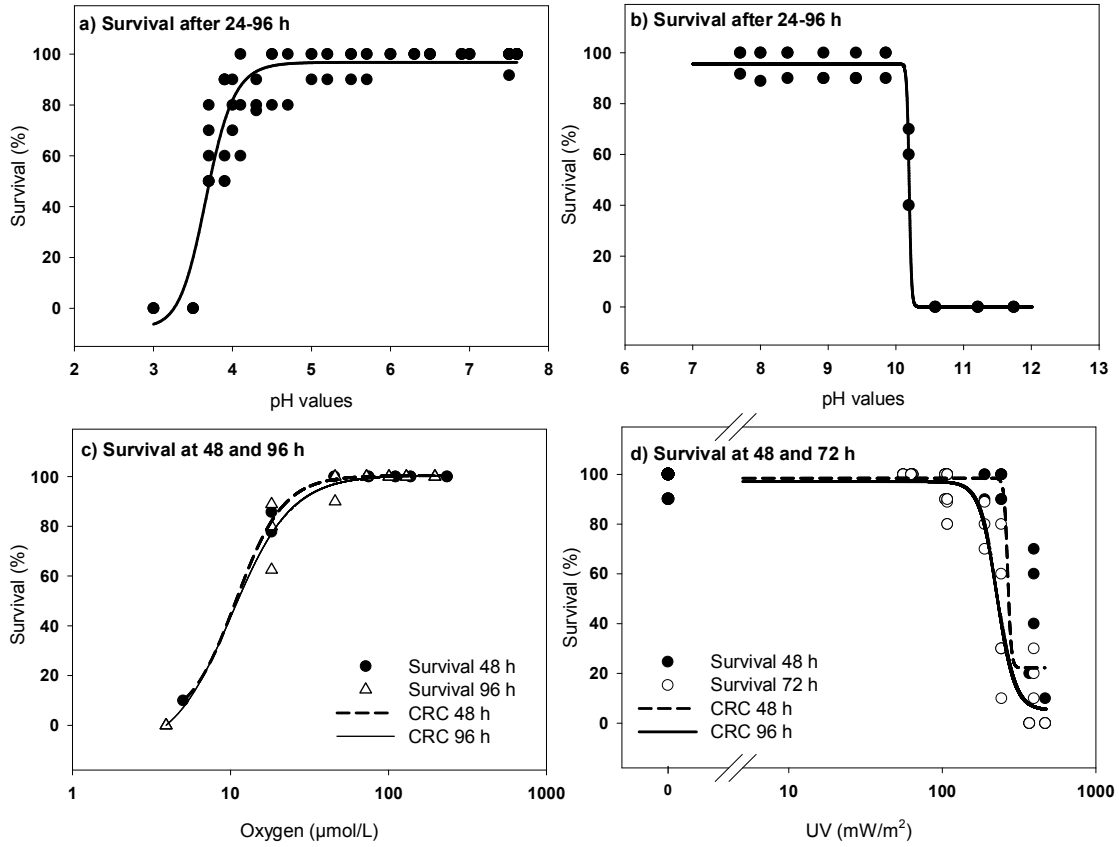

Fig. 1 - Zebrafish embryos survival after exposure to environmental stress: a) Survival after 24 - 96 h of exposure to acid pH (no increase in lethality after exposure for 24 h); b) Survival after 24 - 96 h of exposure to alkaline pH (no increase in lethality after exposure for 24 h); c) Survival after exposure to low oxygen concentrations at 48 and 96 h; and d) Survival rate at 72 h after exposure to UV radiation; CRC means Concentration Response Curve.

3.3 UVR effects on zebrafish embryo survival and development

In order to determine the effects of UVR on zebrafish survival and development, zebrafish embryos were continuously exposed to UVR (280 nm – 400 nm) over a 96 h period. The continous exposure was selected for compatibility with combinded stress/mixture experiments with chemicals, which requires a contineous exposure. Given that it is known that continuous illumination can already affect the development, a continuous light control was conducted. The control and the continuous light control groups developed normally, no mortality and/or developmental effects were observed in both normal photoperiod and the continuous light control. After 96 h, no survival was

detected already at the lowest UV level tested (3 mW/m^2). Due to technical constraints (availability of suitable UV filters) it was not possible to test lower UVR intensities in order to derive 96 h-LC_{50}. Therefore, effect concentration could only be determined for an exposure up to 72 h. The 72 h-LC_{50} was 227 ± 6.55 mW/m^2 (Table 2 and 3, Fig. 1 d). UVR exposure also induced a variety of sublethal effects on embryos including increased incidence of edema (Fig. S8 c), inhibition of hatching (Fig S8 a), reduced heartbeat (Fig. S8 b) and deformities. UVR intensities above 107 mW/m^2 significantly increased general malformations at 48 h (Fig. S8 d) and spine deformities at 72 h (Fig. S8 e). Fig. 1 g and h illustrates the type of anomalies observed in embryos exposed to UVR if compared to the control group.

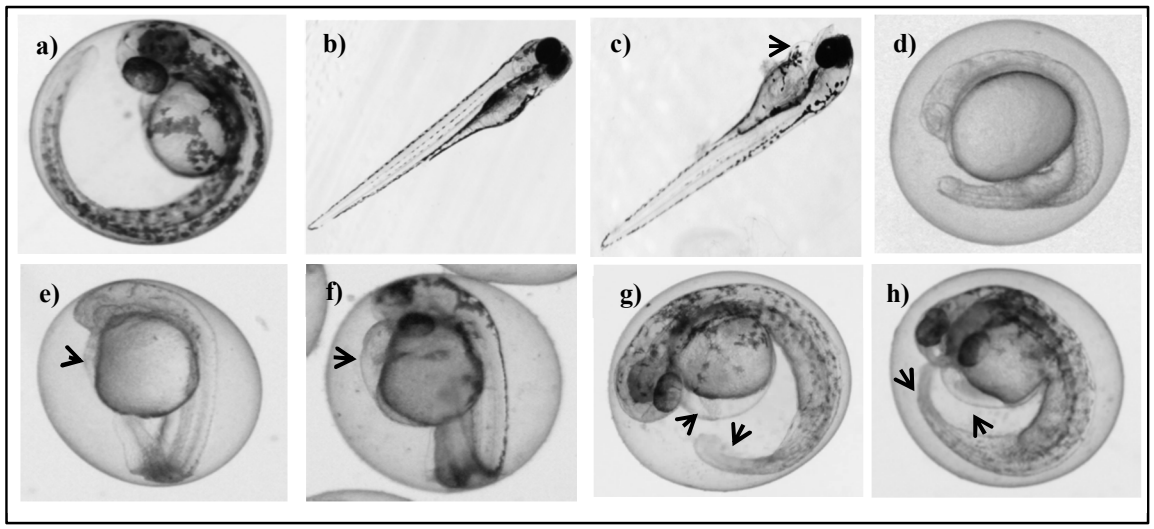

Fig.2 – Example of zebrafish embryos abnormalities during exposure to various environmental stress factors. a) Control embryos at 48 h of exposure (3x magnification); b) Control embryos at 96 h of exposure (3x); c) Embryos exposed to acidic pH of 3.7 units after 96 h of exposure presenting pericardial edema (2x); d) Embryos exposed to oxygen concentration of 0.5 mg/L at 48 hpf showing developmental delay (3x); e) Embryos exposed to oxygen concentration of 1 mg/L at 48 h with pericardial edema and developmental delay (2x); f) Embryos exposed to 1 mg/L of oxygen showing pericardial edema at 96 h (2x); (g) Embryos treated with 3.0 mW/m^2 presenting pericardial edema and bent tail at 72 h; (2x) (h) Embryos treated with 11.7 mW/m^2 demonstrating pericardial edema, tail deformities 72 h (2x);

4. Discussion

In this study the effects of changes in pH, low DO levels and increased UVR intensities on the survival and development of zebrafish embryos were evaluated. The impact of these environmental stress factors have been studied in various aquatic organism from algae to vertebrates (Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010). Data were also partially available for the zebrafish embryo but a comprehensive and detailed analysis including a time-course analysis as a basis to study combinatorial effects with chemicals was lacking. E.g. the pH effects on zebrafish development was previously only assessed for the acidic range by Dave (1984) who observed reduced survival at extreme acidic pH. However, in the study of Dave (1984) the pH fluctuations in some cases exceeded more than 1 pH unit and mortality at pH around 4 and 7 may have been partially provoked also by high buffer concentrations (1.1 mM citrate-HCl and 1.3 mM phosphate) used to stabilize the pH (Dave, 1985). The effects of oxygen limitation has been studied in zebrafish embryos from 48 to 168 hpf by Küster and Altenburger (2008), Shang and Wu (2004) and Strecker et al (2011) but none of them provided a concentration-response relationship for the endpoints assessed. Regarding UVR effects, the studies available (e. g. Charron et al., 2000; Dethlefsen et al., 2001; Dong et al., 2007) did not perform a continuous exposure to UV light which would facilitate to model data for subsequent experiments targeting to analyse combinatorial effects with chemicals.

The compromising effects of acidic or alkaline pH, oxygen depletion and UV radiations are also long known and our study confirmed the expected results for the zebrafish embryo. Our primary aim was to establish concentration-response curves which could be used for hazard and risk characterisation of combined effects with chemicals (Cassee et al., 1998). The data provided by our study will be a useful tool for the study of combined effect of environmental stressors and chemical toxicity.

Table 2 – Summary of LC_x and EC_x values (± Standard error) along 96 h of exposure to environmental parameters. L(E)C values are in pH units (or as specified) for acidic and alkaline pH, µmol/L for dissolved oxygen or mW/m² for UV radiation.

	Days of exposure	24 hpf		48 hpf		72 hpf		96 hpf	
	EC/LC	10	50	10	50	10	50	10	50
pH acid	Hatching	-	-	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	Heartbeat µM H_3O^+	-	-	n.d.	-	-	-	-	-
	Heartbeat (pH units)	-	-	6.94 ± 1.20	-	-	-	-	-
	Edema µM H_3O^+	n.e.	n.e.	n.e.	n.e.	n.d.	n.d.	16.05 ± 3.7	286 ± 44.7
	Edema (pH units)	n.e.	n.e.	n.e.	n.e.	n.d.	n.d.	4.79 ± 0.09	3.60 ± 0.05
	Deformities	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	Developmental delay	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	Body length	-	-	-	-	-	-	n.e.	n.e.
	Survival µM H_3O^+	136 ± 44.1	226 ± 11.4	120 ± 30.0	223 ± 13.9	152 ± 37.4	229 ± 10.7	92.7 ± 19.0	210 ± 16.6
	Survival (pH units)	4.01 ± 0.06	3.66 ± 0.02	4.03 ± 0.07	3.66 ± 0.02	4.00 ± 0.07	3.65 ± 0.02	4.09 ± 0.08	3.68 ± 0.03
pH alkaline	Hatching (µM OH^-)	-	-	n.e.	n.e.	118 ± 79.5	141 ± 32.7	n.e.	n.e.
	Hatching (pH units)	-	-	n.e.	n.e.	10.1 ± 0.16	10.1 ± 0.04	n.e.	n.e.
	Heartbeat	-	-	n.e.	n.e.	-	-	-	-
	Edema (µM OH^-)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	10.6 ± 6.3	35.4 ± 25.1
	Edema (pH units)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	9.02 ± 0.25	9.56 ± 0.32
	Deformities	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	Developmental delay	n.e.	n.e.	-	-	n.e.	n.e.	n.e.	n.e.
	Body length	-	-	-	-	-	-	n.e.	n.e.
	Survival (µM OH^-)	135 ± 8.92	246 ± 10.6	143 ± 34.25	172 ± 55.1	141 ± 22.8	176 ± 38.6	133 ± 95.2	160 ± 23.6
	Survival (pH units)	10.1 ± 0.03	10.4 ± 0.02	10.13 ± 0.02	10.26 ± 0.02	10.1 ± 0.02	10.2 ± 0.03	10.1 ± 0.12	10.2 ± 0.03
Oxygen	Hatching	-	-	-	-			64.2 ± 7.87	54.0 ± 3.42
	Heartbeat	-	-	202 ± 40.8	85.6 ± 31.7	-	-	-	-
	Edema	n.e.	n.e.	135 ± 23.52	61.8 ± 30.1	-	-	119 ± 16.2	83.7 ± 6.8
	Deformities	n.e.	n.e.	n.e.	n.e.	-	-	n.e.	n.e.
	Developmental delay	-	-	115 ± 26.8	45.7 ± 15.1	-	-	130 ± 21.6	55.5 ± 3.96
	Body length	-	-	-	-	-	-	181 ± 37.7	n.d.
	Survival	n.d.	n.d.	21.2 ± 3.55	13.9 ± 4.09	-	-	23.5 ± 2.86	13.2 ± 1.70
UVR	Hatching	-	-	n.d.	n.d.	> 55.5	> 55.5	#	#
	Heartbeat	-	-	n.d.	n.d.	-	-	#	#
	Edema	n.e.	n.e.	n.d.	n.d.	n.d.	n.d.	#	#
	Deformities	n.e.	n.e.	166 ± 10.9	214 ± 6.87	n.d.	n.d.	#	#
	Spine Malformation	n.e.	n.e.	-	-	133 ± 18.0	217 ± 17.8	#	#
	Developmental delay	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	#	#
	Body length	-	-	-	-	-	-	#	#
	Survival	n.d.	n.d.	251 ± 18.6	339 ± 55.8	170 ± 16.2	227 ± 6.55	#	#

-Endpoint not analysed; *n.e.* no effect on the endpoint analysed; *n.d.* not determined (no effect or only effects below a 50 % level); *hpf* hours post-fertilization; NA not available; # due to 100% of mortality the ECx and LCx could not be provided for this time point.

4.1 pH effects on zebrafish embryo survival and development

Lethal effects of pH were fully established after 24 h of exposure for both acidic or basic ranges (see Table 2). Generally our data agreed with previous literature on fish toxicity, where pH showed to significantly impact on fish survival at values below 5 (Dave, 1984; Jordahl and Benson, 1987) and above 10 (Kaur and Toor, 1980; Le Louarn and Webb, 1998). Cardiac effects, i.e. pericardial edemas and reduced heart rate were the most prominent observed sublethal effects for acidic and alkaline pH, however, they were observed at concentrations close to mortality and may be associated with overall toxicity rather than representing specific effects (see Table 2 and 3).

Furthermore, at the alkaline range a hatching delay was observed. This effect was also observed in *Silurus asotus* at alkaline pH (Gao et al., 2011). Hatching delay is often associated with a reduction in the activity of the enzyme chorionase (Yamagami, 1981) which seems to be pH dependent with an optimum at around pH 8.5 (for the fish *Coregonus albula* L and *C. lavaretus* L) (Luczynski et al., 1987). Alternatively, pH may increase the hardness of the chorion and reduce hatching capability (EL-Fiky, 2002).

Several processes may be involved in pH related toxicity: (i) the interference with structure and functioning of proteins (Kapetaniou et al., 2006), in case that intracellular pH cannot be maintained. Activity of proteins generally exhibit pH optima ranging from pH 5 to 9 (e.g. Talley 2010). (ii) more energy may be required to maintain intracellular pH-levels. If the intracellular pH is affected, the energy budget is further compromised, since the generation of ATP is enabled via proton gradients at the mitochondria (Mitchell, 1961). (iii) the interference in the osmotic regulation of essential ions. (iv) for alkaline condition, the disturbances in ammonia excretion and acid base regulations is considered as the major cause for toxicity (Baldisserotto, 2011; Bolner and Baldisserotto, 2007; Wilkie and Wood, 1996). Although fish can compensate changes in the external pH (Claiborne et al., 2002), the mortality observed in zebrafish embryo is likely to be associated with levels above the compensation capacity, with compromising effects on protein function and/or energy budget.

4.2 Oxygen depletion effects on zebrafish embryo survival and development

Contrary to pH, DO effects on zebrafish embryo were strongly dependent on the exposure duration (Table 2 and 3). The results were in concordance with previous studies in zebrafish which reported developmental effects such as delay, heart rate decrease, increased incidence of edemas and reduced body length at oxygen concentrations below 4.2 mg/L; (Küster and Altenburger, 2008; Strecker et al., 2011) and reduced survival rates at 0.5 mg/L (Shang and Wu, 2004). Padilla and Roth (2001) showed that zebrafish embryos exposed to anoxia conditions enter a stage of arrested development which can be survived up to 24 h without any deleterious effect on subsequent development if oxygen levels are restored. This capacity to survive low levels of oxygen is likely related to a decrease in metabolism as an organisms' strategy to reduce energy expenditure through arrest of developments and the corresponding demand for energy to synthesis cellular macromolecules such as proteins (Wu, 2002). Ton et al. (2003) has shown changes in gene expression including drastic suppression of ATP demand and shutting down of protein synthesis and cell division in zebrafish embryos under hypoxia. The observed reduction in body length and heart rate that were also observed by other zebrafish embryo studies (Bagatto, 2005; Shang and Wu, 2004; Strecker et al, 2011), may be related to the aforementioned biochemical adjustments but could also reflect a developmental delay and secondary toxic responses to hypoxia conditions.

4.3 UVR effects on zebrafish embryo survival and development

In order to establish concentration-response curves, embryos were continuously exposed to UVR throughout 72 hours (for technical reason longer exposure durations could not be used for deriving concentration-dependent effects). Although not corresponding to a natural exposure scenario, a continuous UVR exposure was selected to enable future studies on the assessment of combined UV-light and chemical exposure including the modelling of data. However, a continuous illumination cycle may interfere with the circadian clock and impact the development and survival of organisms as it is well documented for the zebrafish embryo in the literature (e.g. Jensen et al., 2012; Villamizar et al., 2013 among others). Villamizar et al., 2013 studied the effects of continuous light

exposure or lack of illumination and observed effects on body length and survival rate (reduction by below 10%). Nevertheless, significant effects were only detected at 7 and 12 dpf respectively. Similarly, in the study of Jensen et al., 2012, constant light exposure (1800 lux) did not affect viability or caused any abnormal phenotypes although effects on the development of vasculatures were observed in 24 and 72 hpf embryos. Despite the observed vascular defects under constant light, embryos development was not affected. In our study, no effects on survival or developmental endpoints were observed after exposure to constant light. Therefore, the effects observed under UVR exposure can be mainly attributed to UV light and not to the alteration of the dark:light cycle. In addition, as reviewed by Vatine et al., 2011, although clock gene expression are detected in zebrafish embryos at the first 24 hpf, no circadian rhythms of S phase are evident during the first 3 days of development demonstrating that these rhythms are dispensable for normal growth and development in the laboratory environment.

To our knowledge no other study has used a continuous UV exposure and hence, comparison to other results is difficult. E.g. in the study of Dong et al. (2007) a 24 h-LD_{50} of 2.32 J/cm^2 was obtained for zebrafish embryos exposed to short-term pulses of UVR during the first 24 h of development. The continuous exposure to UVR showed a strong time-dependent impact on embryo survival leading to 100% mortality at 96 hpf for all intensities tested.

In our study, UVR also affected hatching – in agreement with studies on amphibian (Blaustein et al., 1997) and others fish embryos (Dethlefsen et al., 2001; Dong et al., 2007). The hatching delay/inhibition may be related to a reduced spontaneous movement of embryos mainly caused by an increased incidence of malformations that may hinder movement and reduce embryos ability to move to breach the egg shell.

Developmental abnormalities have already been reported previously for zebrafish and other species exposed to UVR. In the study of Dong, Svoboda et al. (2007) embryos exposed to UVR doses $\geq$ 0.93 J/cm^2 exhibited spinal deformities while Fujimoto et al., (2007) observed edema, deformed head and double body axis in embryos of *Misgurnus anguillicaudatus*. These effects were discussed as a result of impairment of body axis formation through the UVR provoked disruption of the formation of the cellular microtubule array (Jesuthasan and Strähle, 1997; Strähle and Jesuthasan, 1993).

The observed EC_{50} or LC_{50} values of the environmental stressors tested are unlikely (with potentially the exception of O_2 levels in some areas) to be of direct relevance for climate change impacts. However, the detailed concentration-dependent analysis would allow studying the interaction with chemical effects at low effect levels of the stressors, using for instance established models of mixture analysis. Future research can now build on the availability of detailed description of the impact of stress factors on zebrafish development and survival.

5. Conclusion

The present study intended to comprehensively evaluate the effects of oxygen level, pH and UV-irradiation using a detailed concentration-response on zebrafish embryos. All stressors showed clear concentration- or intensity-dependent, respectively, effects on zebrafish embryo survival allowing the application of established concentration-response models and deriving half-maximal effect concentrations (LC_{50}, EC_{50}). These data provide an important source to study the interaction of environmental stress factors with contaminants in the zebrafish embryo model in the context of climate change scenario.

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Supplementary Data

Zebrafish embryo tolerance to environmental stress factors – concentration/dose response analysis of oxygen limitation, pH and UV-light irradiation

Thayres S Andrade, Jorge F Henriques, Rita Almeida, Stefan Scholz, Amadeu M.V.M. and Inês Domingues

The supplement provides additional data on the toxicity of acidic and alkaline pH, reduced oxygen levels and ultraviolet radiation to zebrafish embryos. The effects of the buffers MES, MOPS, TRIS, CAPS and CHES on embryos survival are shown to indicate that the buffer concentrations selected did not compromise the analysis of pH effects (Fig S1-S2). Fig S4 shows the UV light spectra used in this study, while Fig S5 to S8 shows the concentration response curves for almost all endpoints analyzed. In the cases where no difference between the curves at the different stages was observed, only one stage is shown. Heart rate and body length were only evaluated at 48 and 96 hours post fertilization (hpf), respectively.

Contents

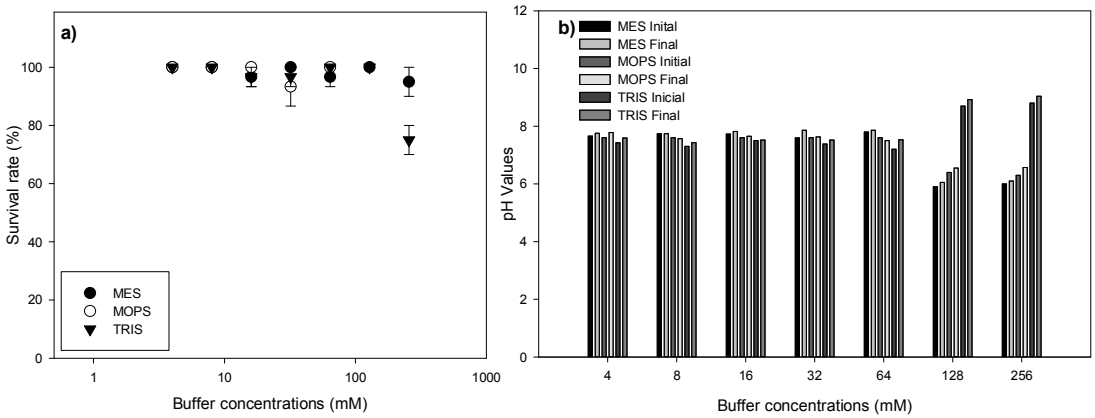


Fig. S1 a) Zebrafish embryos survival at 96h after exposure to a range (0 – 256 mM) of MES, MOPS and TRIS concentrations at neutral pH; no statistically significant differences to controls were observed; b) Initial and final pH values for MES, MOPS and TRIS toxicity tests. The buffers were tested outside of their optimal buffer range at or close to neutral pH in order to test the potential buffer toxicity without interference by pH. The pH was measured at the beginning and end of the experiment to guarantee exposure to neutral pH throughout the test.

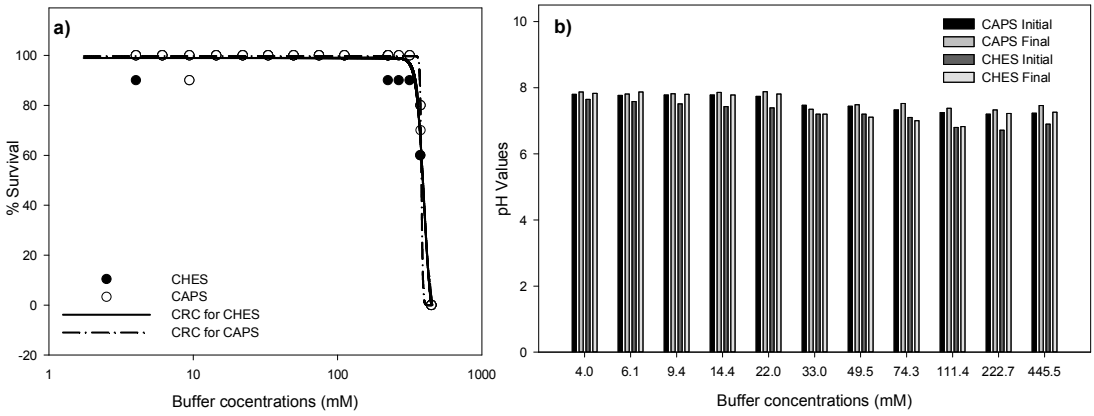


Fig. S2 a) Zebrafish embryos survival at 96 h after exposure to a range (0 – 445 mM) of CAPS and CHES concentrations at neutral pH (CRC means concentration response curve); **b)** Initial and final pH values for CAPS and CHES toxicity tests. The buffers were tested outside of their optimal buffer range at or close to neutral pH in order to test the potential buffer toxicity without interference by pH. The pH was measured at the beginning and end of the experiment to guarantee exposure to neutral pH throughout the test.

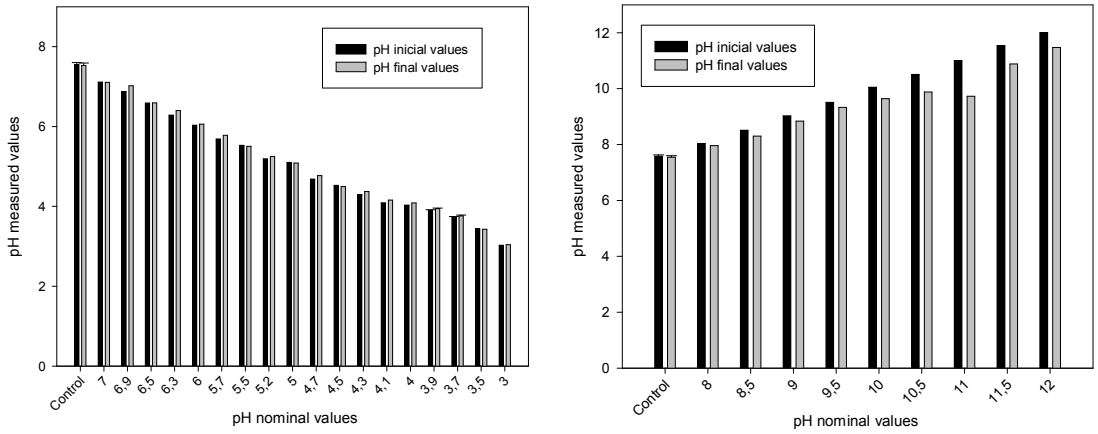


Fig. S3 a) Average initial and final pH values measured during acid pH tests; **b)** Average initial and final pH values measured during basic pH tests. The different pH levels were maintained by using the following buffers: MES for pH 3.7-6.5 (0.31-316 μM), MOPS for pH 6.9-7.5 (0.0316-0.13 μM), TRIS for 8.0-8.5 (1-3.16 μM) CHES 9.0-9.5(10-31.62 μM) and CAPS for pH 10-12 (100-10000 μM).

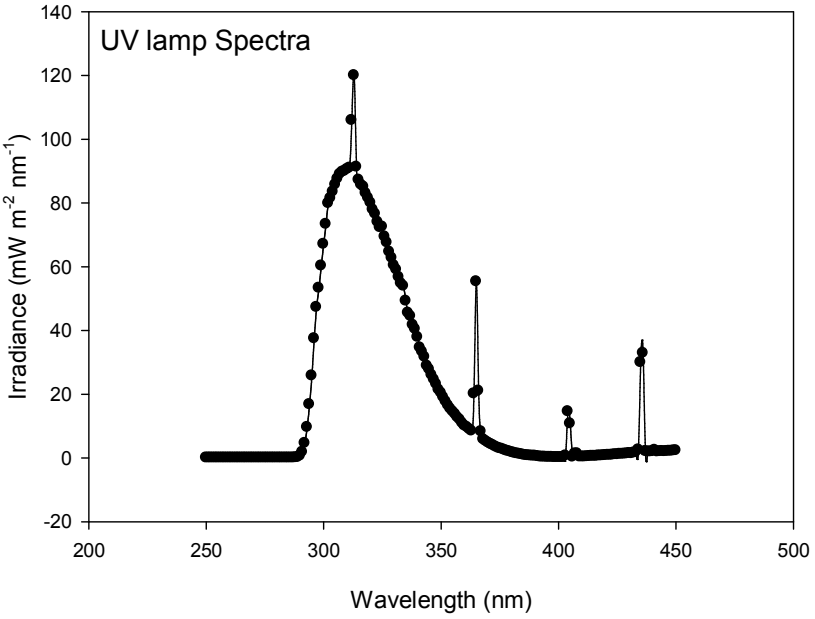


Fig. S4 – UV-light spectra of the lamp used for UV irradiation (UV Spectroline XX15F/B, Spectronics Corporation, NY, USA) showing two peak emission at 313 nm and 365 nm corresponding to UV-B and UV-A emissions peaks respectively.

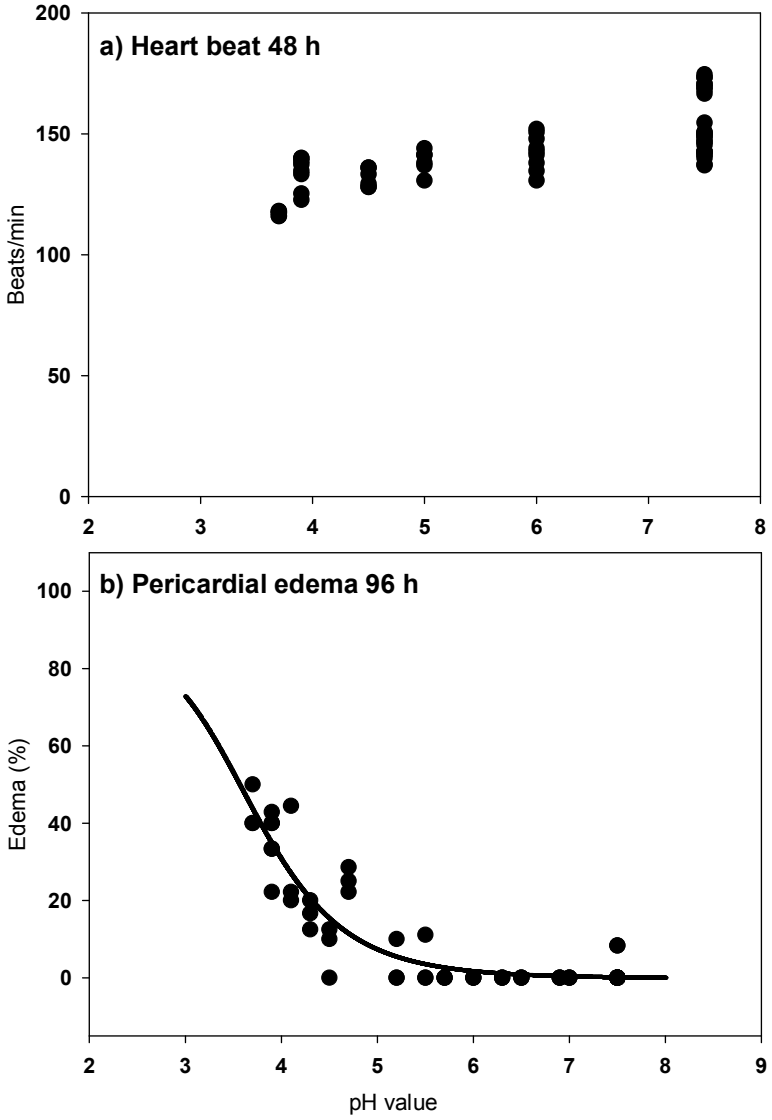


Fig. S5: Development of zebrafish embryos exposed to acid conditions (pH 3-7.5): (a) Heart rate of embryos at 48 h; (b) Incidence of pericardial edema at 96 h.

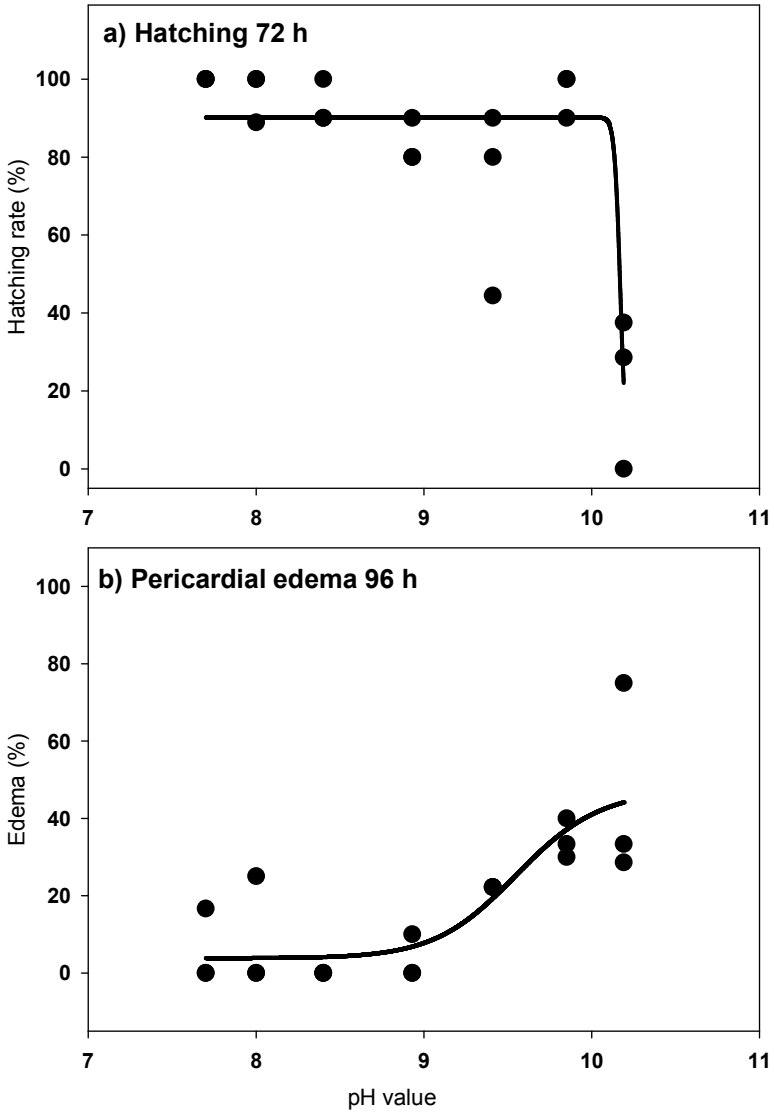


Fig. S6: Development of zebrafish embryos exposed to alkaline conditions (pH 8-12): (a) Hatching rate at 72 h of exposure; (b) Incidence of pericardial edema at 96 h.

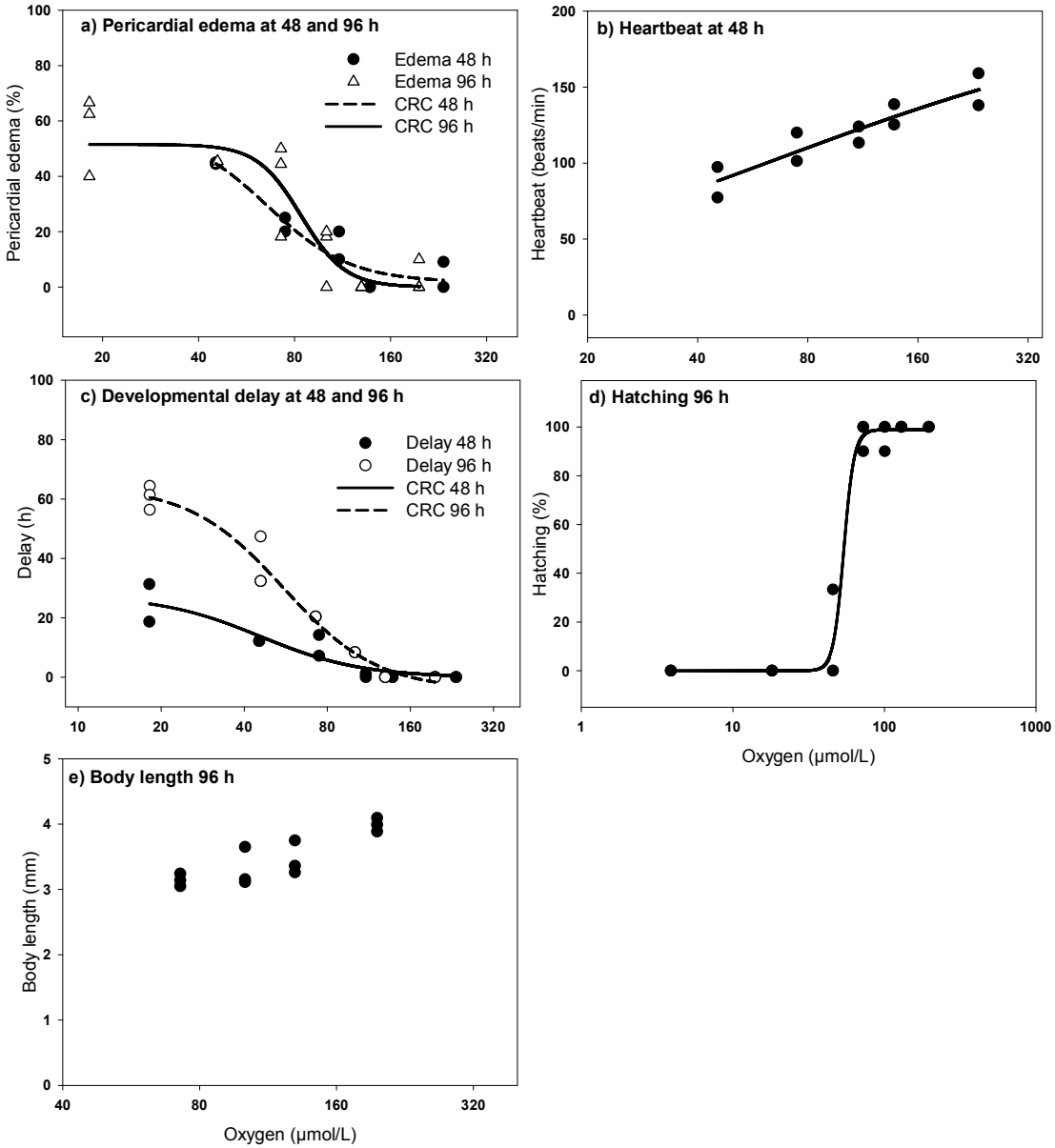


Fig. S7: Zebrafish embryos development under low oxygen concentrations after 96h of exposure: a) Incidence of pericardial edema at 48 and 96 h; b) Heartbeat at 48 h; c) Developmental delay at 48 and 96 h; d) Hatching rate at 96 h; e) Body length at 96 h. CRC - concentration response curve.

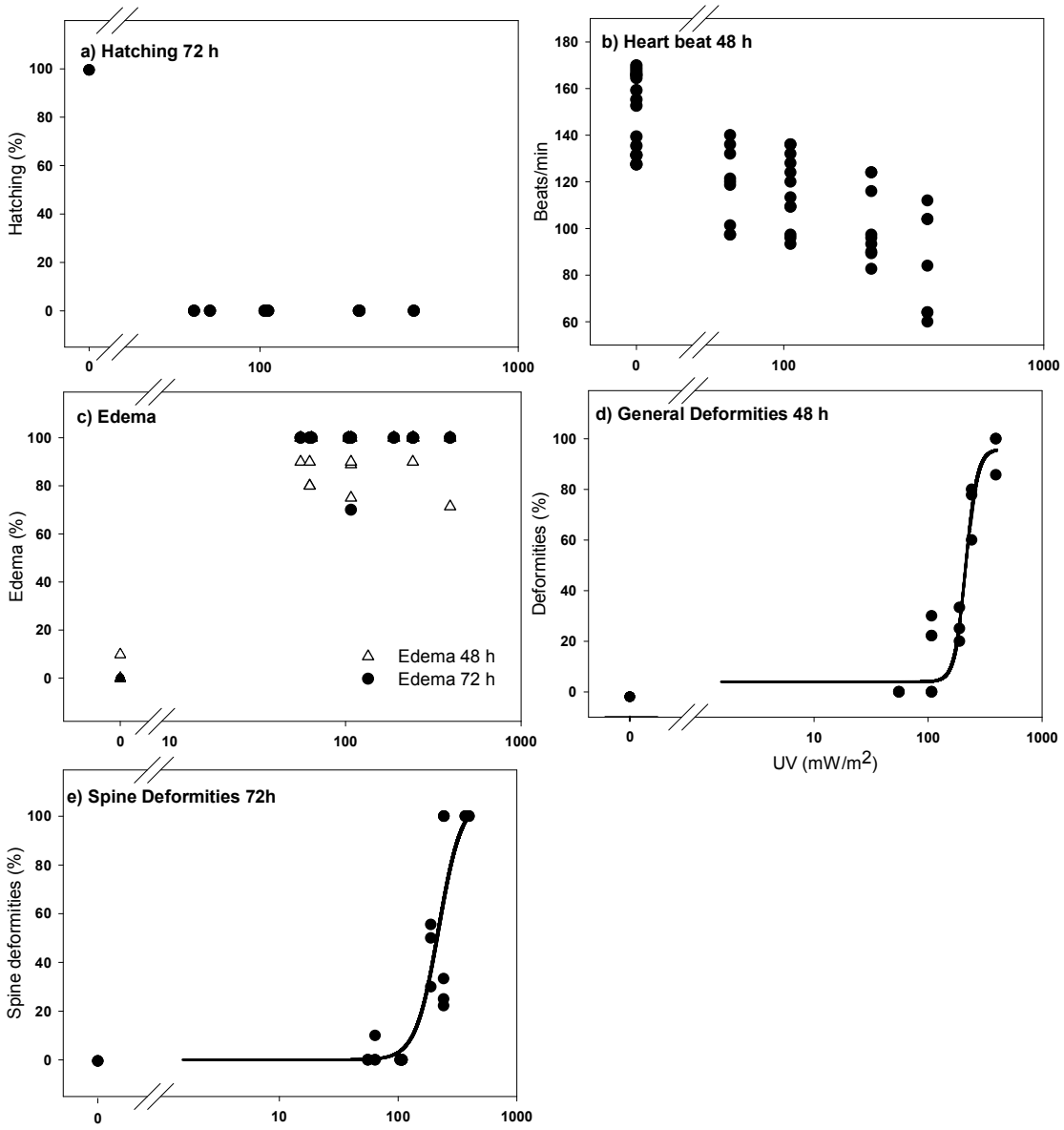


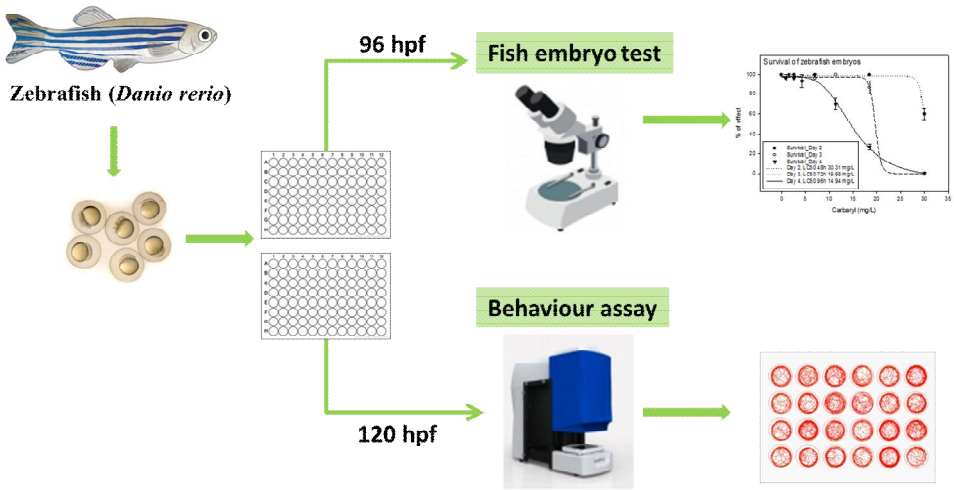
Fig. S8 - Development of zebrafish embryos exposed to UV radiation: **a)** Hatching success at 72 h; b**)** Heart beat rate at 48 h; **c)** Incidence of edemas at 48 and 72 h **d)** Deformities at 48 h and **e)** Spine deformities at 72 h of exposure. CRC means Concentration Response Curve.

Table S1 - Summary of models used to calculate concentration-response curves and the respective slope for each endpoint and environmental stressor.

	Days of exposure	**24 hpf**	**48 hpf**	**72 hpf**	**96 hpf**
	Model/Slope	**slope**	**slope**	**slope**	**slope**
pH Acidic	Heartbeat (µM H_3O^+)		$-1.59*10^{-1}$		
	Heartbeat (pH units)		8.16		
	Edema (µM H_3O^+)				-0.76
	Edema (pH units)				7.60
	Survival (µM H_3O^+)	4.35	3.54	5.35	2.68
	Survival (pH units)	87.57	168	168	182.7
pH alkaline	Hatching (µM OH^-)			12.15	
	Hatching (pH units)			179.48	
	Edema (µM OH^-)				-1.77
	Edema (pH units)				-38.30
	Survival (µM OH^-)	3.66	11.85	9.67	12.13
	Survival (pH units)	87.57	168	168	183
Oxygen	Hatching (µmol/L)				-12.63
	Heartbeat (µmol/L)		2.55		
	Edema (µmol/L)		2.89		6.30
	Developmental delay (µmol/L)		2.38		2.57
	Body length (µmol/L)				8.16
	Survival (µmol/L)		-5.20		-3.79
UV Radiation	Hatching				
	Heartbeat				
	Edema				
	Deformities		-8.72		
	Spine Malformation			-4.51	
	Survival		7.35	7.56	

Chapter 4

Effects of carbaryl in zebrafish embryos development, biochemical makers and locomotion



Effects of carbaryl in zebrafish embryos development, biochemical makers and locomotion

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This chapter is in preparation to be submitted as an original article in:

Environmental Science and Pollution Research

Abstract

Despite the efforts to reduce the use of pesticides in agriculture, they are still widely used to prevent and treat pests. The main concern with the use of pesticides is the impact in non-target wildlife with serious consequences to aquatic ecosystems. Typically, environmental concentrations of pesticides rarely cause lethality; therefore the risk assessment of these chemicals is better achieved if a battery of sublethal endpoints is used. Within this context, the main goal of this work was to evaluate the effects of Carbaryl – a carbamate insecticide – in zebrafish (*Danio rerio*) embryo survival, development, biomarkers and behaviour. Developmental endpoints included delay, hatching success, edema incidence, malformations, reduction of size and alterations in heartbeat rate. Biomarkers included Acetylcholinesterase (AChE), Glutathione-S-transferase (GST), Lactate dehydrogenase (LDH) and Catalase (CAT). Behaviour was assessed through a locomotion assay at 96 hpf (hours post fertilization) using the Zebrabox (Viewpoint, France) video tracking system. Carbaryl effects on zebrafish embryos survival was dose dependent with a 96 h-LC_{50} of 14.94 mg/L. At the sublethal level, carbaryl significantly affected heartbeat rate, decreased body length and increased incidence of edema and malformations. Moreover, carbaryl exposure resulted in significant decrease in enzymatic activity (AChE, GST and LDH) and alterations in the locomotor behaviour of zebrafish embryos reflected by a decrease in the total distance moved. When compared to the developmental endpoints (mortality/survival, edemas, heart beat and etc.), biomarkers and locomotor behaviour were the most sensitive parameters showing effects at very low concentrations. This study highlights the importance of considering the sublethal effects of environmental contaminants in risk assessment in order to better estimate their effects in aquatic ecosystems.

Keywords: *Danio rerio*, behaviour, carbamate insecticide, sublethal effects

1. Introduction

Pesticides have become a major environmental problem. The use (and abuse) of pesticides in agriculture is widespread all over the world and has increased in the last years (except in some regions) as a consequence of the increase in human population and crop production. According to EPA (Environmental Protection Agency), in 2007 pesticides use worldwide topped approximately 2.5 billion kilograms (EPA, 2007). Moreover, the increased in the incidence of existing pest, diseases and weeds predicted under climate change scenarios may imply a more extensive and frequent application of pesticides increasing its use and bioavailability (Chen and Mccarl, 2001; Koleva and Schneider, 2009; Mango et al., 2011; Reilly et al., 2003). The main problem with the use of pesticides is its migration from agricultural fields to aquatic environment by runoff or leaching (Larson, Capel et al. 1995, Battaglin, Thurman et al. 2003) which may increase their concentrations in the water, threatening aquatic biota.

Insecticides represent a large (33 %) proportion of total pesticides used worldwide (Stokstad and Grullón, 2013). Carbaryl (1-naphthyl-N-methylcarbamate) is among the most widely used carbamate insecticide and is applied to control a broad spectrum of insect pests on crop and non-crop sites including domestic gardens (CCME, 2009). Low concentrations of carbaryl ranging from 0.1 to 1737 µg/L (Vryzas et al., 2009; Walters et al., 2003; Wilsont and Foos, 2006) have been detected in surface waters both adjacent to agricultural fields and urban areas (Munn et al., 2006; Phillips and Bode, 2004). Generally environmental concentrations are in the sublethal range which strengthens the importance of using appropriate tools to assess the risks posed by pesticides in more realistic scenarios. Zebrafish early life stages tests focusing on developmental endpoints, biomarkers and behaviour have been showing promising results to evaluate sublethal concentrations of contaminants (Coelho et al., 2011; Oliveira et al., 2009).

Like most carbamates, carbaryl is a known acetylcholinesterase (AChE) inhibitor with a resulting disruption of nerve impulse transmissions. The presence of carbaryl prevents AChE from breaking down acetylcholine causing it to accumulate in the nervous system (Fukuto, 1990). As a consequence, the continuous stimulation of the muscle leads to uncontrolled, rapid movement of some muscles, paralysis, convulsions and even death (Gruber and Munn, 1998; Gunasekara et al., 2008; Mora et al., 2000; Scaps et al., 1997). In

addition, carbaryl is also teratogenic, causing developmental and hatching delay, defects in heart formation including defect in cardiac looping, pericardial edema and decrease in heart rate and affected body length in zebrafish embryos (Gallo et al., 1995; C C Lin et al., 2007; Schock et al., 2012; Todd and Van Leeuwen, 2002).

The fish embryo toxicity test (FET) has been validated and adopted by OECD (Organisation for Economic Co-operation and Development) (OECD, 2013) and is now widely used with zebrafish. Among many other advantages, the transparency of zebrafish eggs allow the monitoring of the entire organogenesis permitting the study of a wide range of sublethal endpoints including anomalies (edemas, tail deformities etc.) and developmental delay (hatching delay) (Lammer et al., 2009; Scholz et al., 2008). Moreover, the use of zebrafish early life stages for the assessment of behaviour is also becoming popular (Fetcho and Liu, 1998; Tierney, 2011). Behaviour is linked to a range of stress responses such as physiological and biochemical disturbances (Beauvais et al., 2000; Tierney et al., 2007) and represents an interface between internal (physiological) and external (environmental, social) forces that may have serious implications in health and organisms survival (Little et al., 1990). In this context locomotor behavioural analysis represents a sensitive tool for detection and evaluation of sublethal effects of chemicals as compared to conventional endpoints such as survival (Levin et al., 2004).

In the present study we aimed to assess the toxicity of carbaryl to zebrafish embryos focusing on sublethal endpoints such as developmental endpoints (hearbeat, edema, body length etc.), biochemical makers (Cholinesterase, Glutathione-S-transferase, Lactate dehydrogenase and Catalase) and behaviour (total distance moved). Although previous studies have already reported carbaryls' toxicity to zebrafish embryos, the present study provides detailed concentration-dependent analysis (LCx or ECx) throughout the whole embryonic and early larval development (0-96h). Moreover, we are unaware of any studies addressing the effects of carbaryl on locomotor behaviour of zebrafish eleutheroembryos.

2. Materials and Methods

2.1 Zebrafish maintenance and embryo collection

All the embryos used in the present study were provided by the zebrafish (*Danio rerio*) facility established at the Department of Biology, University of Aveiro (Portugal). Adults were maintained in carbon-filtered water, complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was kept at 26.0 ± 1 ºC, conductivity at 750 ± 50 µS, pH at 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. This reconstituted water was used in the preparation of test solutions of all assays performed. The above mentioned temperature and photoperiod conditions were constant in all assays. The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd) and brine shrimp. Zebrafish eggs were obtained by crossbreeding of individuals in aquaria; after 30 min of natural mating, eggs were rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope -SMZ 1500, Nikon Corporation); those unfertilized, with cleavage irregularities, injuries or other type of malformations were discarded.

2.2 Test Chemical and Preparation of Test Solutions

Carbaryl (1-Naphthyl-*N*-methylcarbamate) was purchased from Sigma-Aldrich. Stock solutions of carbaryl were prepared fresh by dissolving it in water and test solutions were prepared by diluting the stock. Ten ml of each tested concentration were sampled at the beginning and at the end of the assay and preserved at -20 ºC to further chemical analysis. The chemical analysis was performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in České Budějovice, Czech Republic. The results are presented in supplementary materials Table S1.

2.3 Embryo assay

The assay was based on the OECD guideline (236) on Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). The test started with eggs at 3 hours post fertilizations (hpf), previously selected and exposed to concentrations ranging from 1 to 30 mg/L of carbaryl. Ten eggs per treatment were distributed in 24-wells microplates in triplicate and run for 96h. Embryos were observed daily under a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation, Japan). The following endpoints were evaluated: survival, incidence of pericardial edema, heartbeat rate, presence of haemorrhage (clutch of red blood cell), malformations, hatching and body length (total length: snout to tail tip). Heartbeat rate (beats/15s) was measured by counting heart beats under the stereomicroscope in 3 randomly selected embryos of each replicate (n=9 per concentration) at 48h. Body length was measured using digital images of the embryos with the software NIS Elements D (Nikon Corporation, Japan).

For analysis of biomarkers and locomotory activity, sublethal concentrations of carbaryl were used ranging from 0.00075 to 0.75 mg/L and from 0.0001 to 5 mg/L respectively. These tests were carried out in the same conditions as the above described test. For biomarkers, at 96 hours of exposure, 10 clusters of eight larvae per treatment were snap-frozen in microtubes containing 0.8 ml of K-phosphate buffer (0.1M, pH 7.4) and stored at - 80 ºC for further enzymatic analysis (see section 2.4). For locomotory assay larvae were transferred to 96 well plates and analysed using the track system Zebrabox (Viewpoint, Lyon, France) at 96 h (see section 2.5).

2.4 Biomarker determinations

Enzymatic assays were performed to analyse cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) activities in zebrafish embryos. On the day of enzymatic analyses, samples were defrosted on ice, homogenised (KIKA Labortechnik U2005 Control) and centrifuged at 4 ºC, 10000 g, during 20 min in order to isolate the post-mitochondrial supernatant (PMS) posteriorly used as enzyme

extract for enzymatic activity determination. All determinations were made spectrophotometrically (Thermo Scientific Multiskan Spectrum, USA) using 96 wells microplates.

Protein quantification in samples was performed in quadruplicate according to the Bradford method (Bradford, 1976), at 595 nm, using ɣ- globulin to determine standard curve. The method for the determination of ChE, GST and LDH activity is described in Domingues et al. (2010). Briefly, ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm (every 20 s, for 5 min) the conjugation product between thiocoline (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase), according to the method of Ellman et al. (1961). Activity determinations were made using 40 µL of PMS, 250 µL of reaction mixture (acetylthiocholine (75 mM), and DTNB (10 mM)) in Kphosphate buffer (0.1 M, pH 7.2).

GST activity was determined at 340 nm by monitoring the increase in absorbance every 20 s, during 5 min, following the general protocol described by Habig and Jakoby (1981) adapted to microplate reader (Frasco and Guilhermino, 2002). Activity determinations were performed using 100 µL of PMS from the sample and 200 µL of reaction solution (10 mM reduced glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene in K-phosphate buffer (0.05 M, pH 6.5)).

CAT activity was measured at 240 nm in spectrophotometer quartz cell by monitoring (every 10 s, for 2 min) the decrease of absorbance due to degradation of H_2O_2, as described by Clairborne (1985). Fifteen microliters of PMS was mixed with 135 µL of reaction solution (H_2O_2, 30 mM) and 150 µL of K-phosphate buffer (0.05 M, pH 7.0).

LDH activity was measured at 340 nm and was based on the decrease of absorbance (5 min) due to the oxidation of NADH, following the methodology described by Vassault (1983) with the modifications introduced by Diamantino et al. (2001). Activity determinations were made using 40 µl of PMS of the sample, 250 µl of NADH (0.24 mM) and 40 µl of piruvate (10 mM) in Tris–NaCl buffer (100 mM, pH 7.2).

Enzymatic activity were determined in quadruplicate and expressed in nanomoles of substrate hydrolyzed per minute per mg of protein. A Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA) reader was used to determine all protein and enzymatic activity.

2.5 Locomotory activity assay

To assess carbaryl effects on locomotor activity, zebrafish larvae at 96 hpf were used. Locomotion was evaluated for 24 embryos per treatment including the control in 96 well plates (one embryo per well). Embryonic movement was tracked using the Zebrabox-ZEB 478 (software version 3.22, Viewpoint Life Sciences, Lyon, France) a system that monitors the movement by automated video recording with an infrared camera (25 images per second). Typically zebrafish larvae show less locomotion during light periods and more during dark. Therefore, movement was stimulated by applying light:dark intervals according to what was described in Irons et al (2010). Briefly, embryonic movements were recorded during light-dark intervals over a period of 20 minutes (5 min light, 10 min dark, 5 min light). For each replicate the distances moved in 1 minute intervals were recorded separately and only locomotion in the dark period was used to calculate the differences between control and treated embryos. The parameter total distance moved was calculated and refers to the total swimming distance of the larvae during each measurement period.

2.5 Statistical analysis

Sigmaplot for Windows V.12.5 (Systat Software, 2008) was used for statistical analyses. One-way ANOVA (analysis of variance) was used to test differences between the different treatments in normally distributed data sets. When datasets failed the Kolmogorov Smirnov normality test, an ANOVA on ranks (Kruskall-Wallis) was performed and the Dunnet's or Dunn's post-hoc test where used to compare each treatment against control. Lethal concentrations (LCx) and effective concentrations (ECx) values were calculated for each developmental endpoint by fitting dose-response curves. A significance level of 0.05 was used to infer statistically significant results.

3. Results

3.1 Effects of carbaryl on zebrafish early life stages

Carbaryl significantly affected zebrafish embryos survival and development. Table 1 presents all the calculated LC_{50} and EC_{50} for all developmental endpoints analysed and Table S2 presents the models used to calculate concentration response curves. Below 5 mg/L, survival was higher than 90 %; however, there was a severe decrease in survival from 15 mg/L reaching 0 % at 30 mg/L. Fig. 1 indicates a dose dependent response with a 96 h-LC_{50} of 14.91 mg/L (Table 1).

Table 1: Effects of Carbaryl ($E(L)C_{50}$ values) on lethality and developmental parameters of zebrafish early life stages

Endpoints	24hpf	48hpf	72hpf	96hpf
Somite formation	n.e.	-	-	-
Heart beat	-	28.05 ± 0.8	-	-
Haemorrhage (clutch of red blood cell)	n.e.	n.d	n.d	9.06 ± 4.1
Hatching rate	n.e.	n.e.	15.12 ± 0.4	n.d.
General deformities	19.36 ± 3.3	n.d.	n.d.	n.d.
Tail deformities	-	-	n.d.	16.1 ± 5.6
Pericardial edema	n.e.	12.30 ± 3.3	7.53 ± 0.6	$4.38 \pm 3.6*10^{3\#}$
Body length	-	-	-	3.47±0.5
Mortality (LC_{50})	n.d.	$30.38 \pm 1.8*10^{4\#}$	$19.68 \pm 2.4*10^{5\#}$	14.91 ± 0.7

Values represent concentrations in mg/L ± standard errors
n.e. no effect on the endpoint analysed; n.d. endpoint not determined (no effect or only effects below a 50 % level); - endpoint not analysed; hpf hours post fertilization
due to high standard error these values are merely indictive

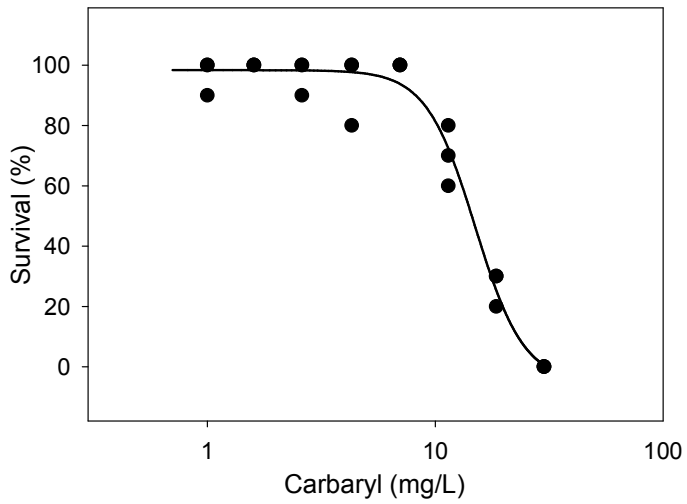

Fig. 1 Concentration response curve for zebrafish embryos survival after 96 h (0-96 hpf) of exposure to carbaryl.

The development of embryos was also affected by carbaryl. Teratogenic effects were found even at low concentrations. At 24 h of exposure concentrations above 15 mg/L increased the incidence of deformities and a 24 h-EC_{50} of 19.36 mg/L was calculated (Table 1). At 48 h, Carbaryl also significantly affected embryos heartbeat rate at concentrations above 3 mg/L inducing a decrease in heartbeat as can be seen in Fig. 2c. Embryos exposed to the highest concentration presented an average heart beat rate of only 67 beats, while controls had an average heart rate of 155 beats per min. Along with the decrease in heartbeat, carbaryl-treated embryos presented an increased incidence of edemas and haemorrhage (clutch of red blood cell) both at 48 and 72 h (Fig. 2a, Table 01). Moreover, at 72 hours a delay in hatching was observed in concentrations above 10 mg/L, with a 72 h-EC_{50} of 15.2 mg/L.

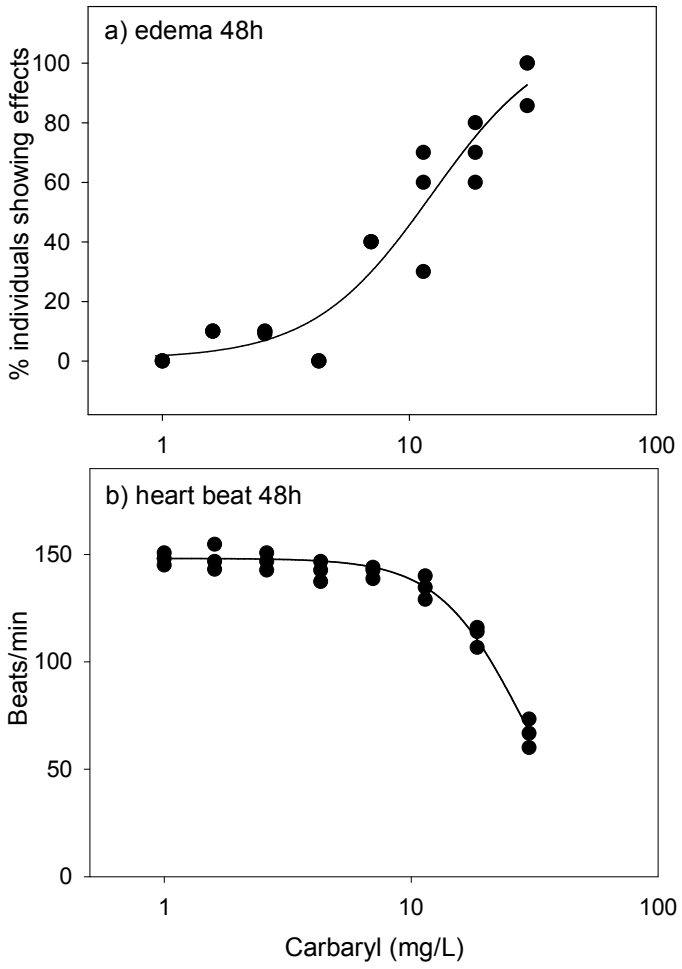

Fig. 2 Concentration-response curves for sublethal effects of carbaryl on zebrafish embryos at 48 hpf: a) percent of embryos showing edema and; b) heart beat rate of exposed embryos.

At 96 h, carbaryl strongly affected embryos body length at 96 hpf. Fig 3d shows a significant decrease of body length in treated embryos even at concentration as low as 1.6 mg/L were no other effect was observed. The carbaryl-treated embryos were considerably smaller; at the concentration 11.4 mg/L for example, embryos presented a mean body length of 3 mm, whereas control embryos exhibited a mean body length of 3.4 mm.

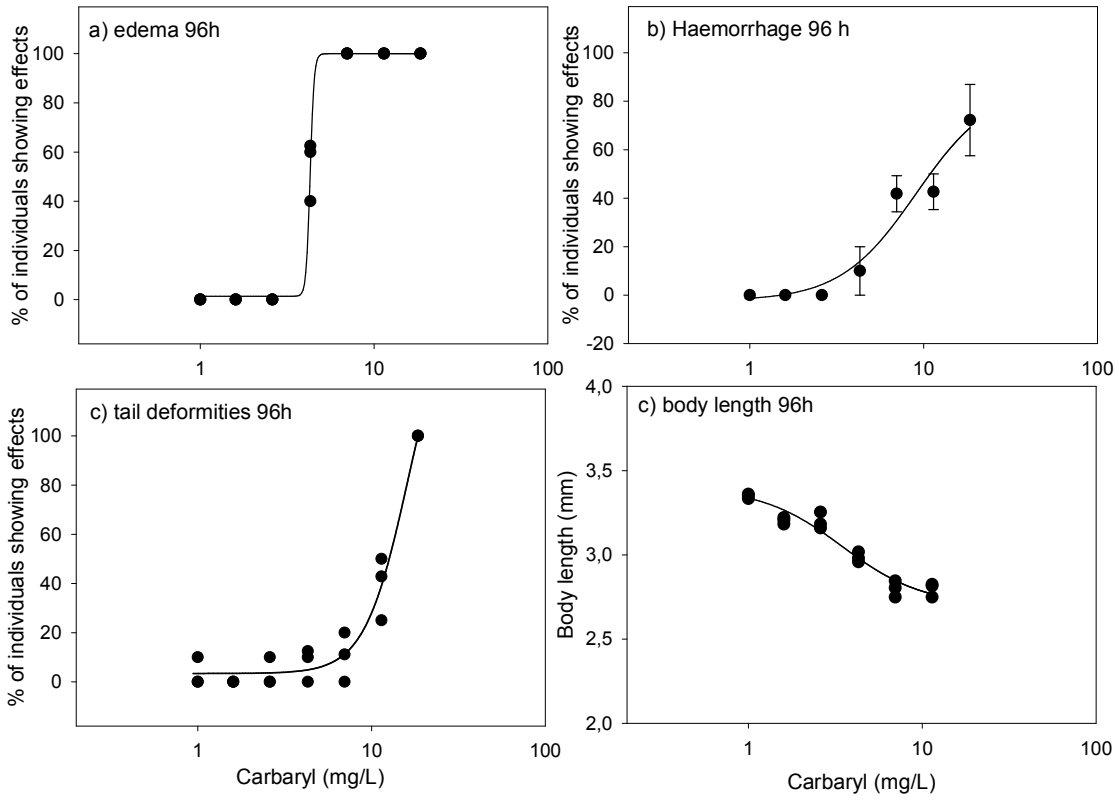

Fig. 3 Concentration response curves for sublethal effects of carbaryl on zebrafish embryos after 96 hpf: a) percentage of embryos showing edema; b) percentage of embryos that showed haemorrhage (clutch of red blood cell); c) percentage of embryos showing tail deformities and; d) body length of larvae at the end of the test.

Additionally, exposure to carbaryl also induced a series of morphological changes as can be observed in Fig. 4. These alterations can be characterized for the presence of haemorrhage (clutch of red blood cell) mainly in the heart (Fig. 4e) and in the tail (fig not showed), the incidence of pericardial edemas (Fig 4b, c, d and f), tail deformities (Fig 4d, f) and spine deformities (Fig 4b).

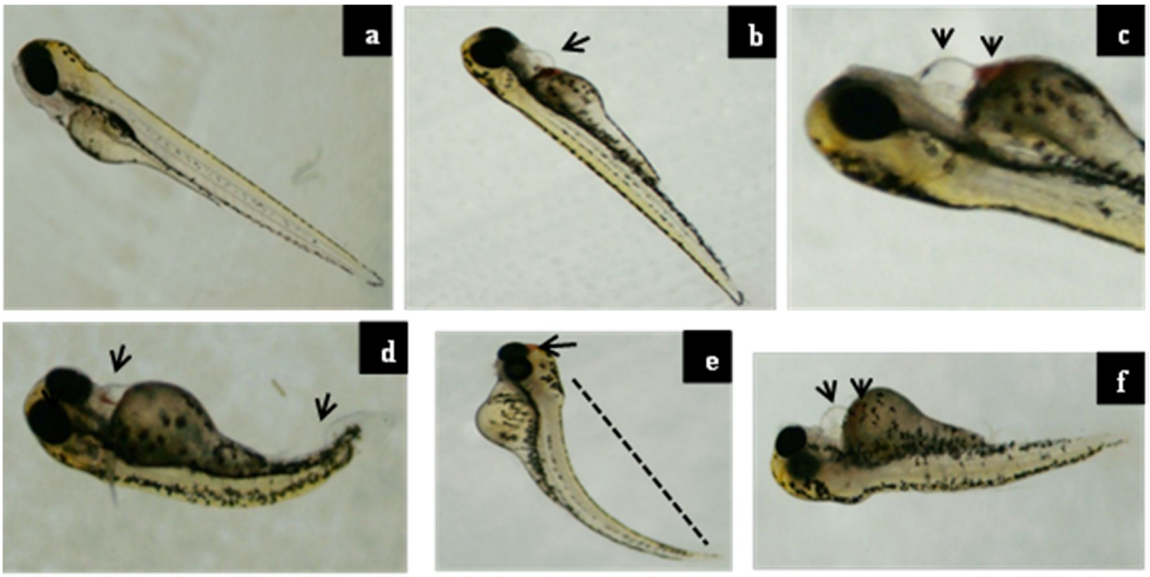

Fig. 4 Phenotypes of zebrafish embryos observed after 96 hours of exposure to carbaryl: a) control; b) embryo exposed to 4.3 mg/L showing a clutch of red blood cells a and pericardial edema (black arrows); c) embryo exposed to 7.0 mg/L with edema and a red blood cell clutch (black arrows); d) embryo exposed to 7.0 mg/L with pericardial edema and also tail deformity (black arrows); e) larvae exposed to 11.4 mg/L with red blood cell clutch (black arrows) and spine curvature (dotted line); f) larvae exposed to 11.4 mg/L displaying pericardial edema and blood cell clutch.

3.2 Carbaryl effects on enzymatic activity

The effects of carbaryl on the activities of biomarkers (ChE, GST, LDH and CAT) are presented in Figure 5. Exposure to carbendazim significantly inhibited ChE ($F = 7.11$; $P = 0.001$), GST ($F = 6.28$; $P = 0.001$) and LDH ($F = 4.49$; $P = 0.005$) activities at concentrations equal and above 0.0075 mg /L when compared to control group (Fig 5 a-c). The activity of CAT seems also to be altered; however, no statistically significant differences were observed ($F = 2.6$; $P = 0.046$).

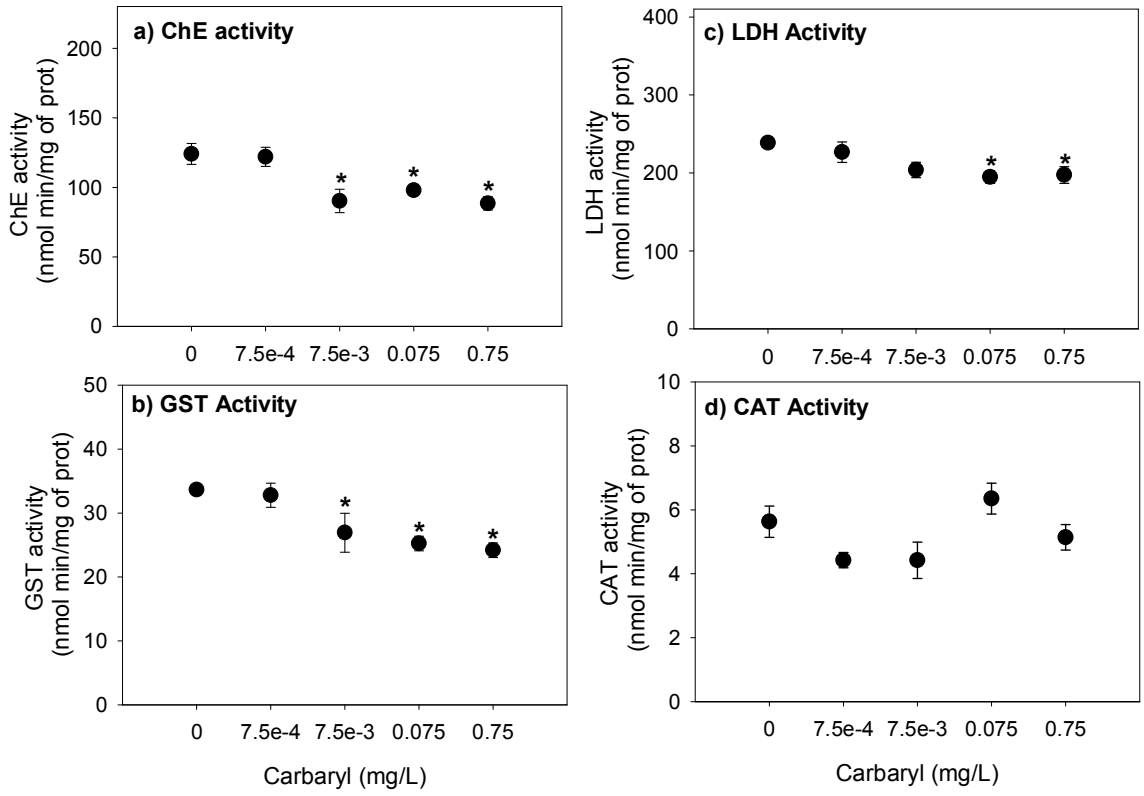

Fig. 5 Enzymatic activities (mean values ± standard error) on zebrafish embryos at 96 hpf. Asterisks mean significantly different from the respective control ($p \leq 0.05$).

3.3 Carbaryl impacts on larval behaviour

Carbaryl showed to significantly alter the locomotor behaviour of zebrafish embryos at 96 hpf. Fig. 6 shows the results for mean total distance moved (mm) across each measurement period. At low concentrations (≤ 0.01) exposure to carbaryl seem to do not affect the overall locomotor activity of the embryos as no differences were observed when compared to control (Fig 6). However, at the concentration 0.1 mg/L and above, the locomotor activity seems to be affected as significant reduction in total swimming distance was observed (Fig. 6).

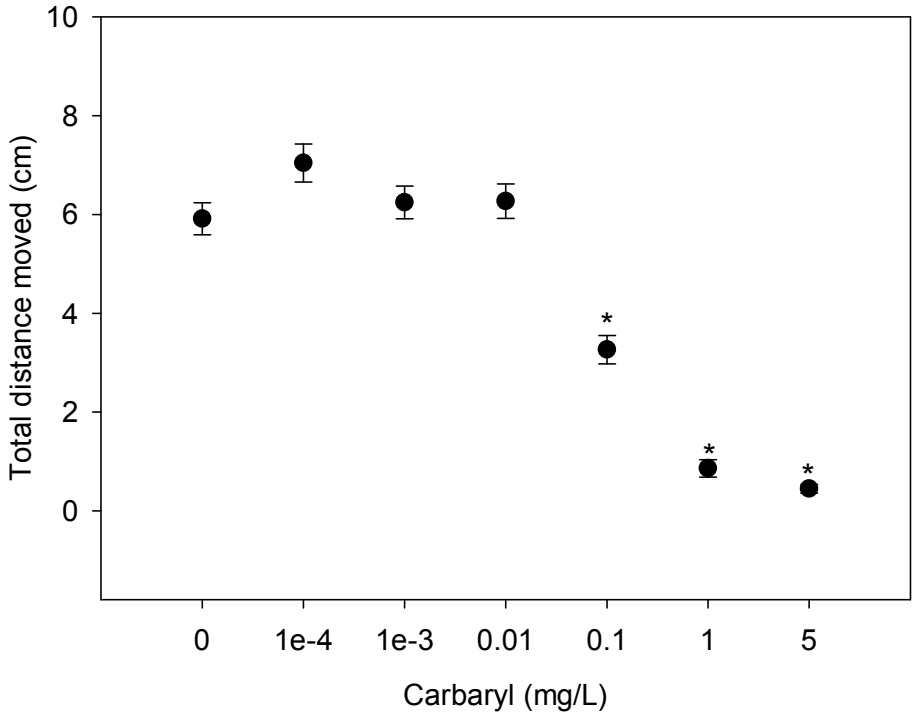

Fig. 6 Mean total swimming distance (mm) during dark period (10 min) at 96 hpf embryos exposed to carbaryl. * denotes statistical significance ($p<0.05$).

4. Discussion

4.1 Carbaryl effects on embryos survival and development

Pesticides are one of the major sources of ecological contamination and their impacts in the environment are an important target of research. Carbaryls' use started in the late 1950s (Chemical Watch) and in the past decades its toxicity has been studied in different model organisms. Carbaryl toxicity to fish early life stages varies accordingly to the fish species and stage of development. Table S3 (supplementary data) summarizes the literature review for carbaryl toxicity in fish early developmental stages. The toxicity of carbaryl to zebrafish embryos have been explored in three different studies, however, E(L)Cx values were reported only by Lin et al (2007) for the 24 hpf (LC_{50} = 44.66 and EC_{50} = 7.52 mg/l). For adult fish a 96 h-LC_{50} of 9.26 mg/L was reported for zebrafish (Gallo et al., 1995) while for other fish species varies between 2.51 and 7.89 mg/L. In our

study the 96 h-LC_{50} was 14.94 mg/L and the effects of carbaryl on zebrafish survival showed to be dose-dependent which are consistent with previous results (Schock et al., 2012) (Fig. 1).

At low concentrations, carbaryl exposure caused developmental and hatching delay and shortened body length of fish embryos (Kashiwada et al., 2008; Todd and Van Leeuwen, 2002). In our experiments, carbaryl caused hatching delay and was also responsible for a significant reduction of approximately 0.6 mm on body size of embryos. Carbaryl's mechanism of reducing body length has not been reported in the literature. This decrease in body length may be due to the metabolic costs associated with the detoxification of the pesticide leading to a reduction of individual fitness as hypothesized for fish and amphibians exposed to metal and pesticides (Cook et al., 2005; Diana et al., 2000; Schock et al., 2012). Development, hatching delay and reduced fitness may have serious implications in fish survival as it makes them more susceptible to predation.

Moreover, carbaryl has also shown to impair cardiovascular development of fish embryos causing heart malformations, pericardial edemas and bradycardia. Kashiwada et al (Kashiwada et al., 2008) studying carbaryl effects on medaka (*Oryzias latipes*) embryos, reported an increased rate of tubular heart and pericardial edema formation and a reduction in heartbeat rate at sublethal concentrations (5-10 mg/L). Morphological defects in cardiac development were also found in zebrafish embryos exposed to carbaryl. Schock et al., (2012) observed a decrease in cardiac precursor field as well as defects and delays in cardiac tube migration and fusion. In addition, they also observed delayed or defective cardiac looping in exposed embryos. In our studies, carbaryl induced a significant decrease in heart beat even at low concentrations (4.3 mg/L) (Fig. 2c) and also increased the incidence of pericardial edema (Table 1, Fig. 2b, 3a). Pericardial edema seems to be a common feature in carbaryl-exposed embryos as reported for medaka and zebrafish (Kashiwada et al., 2008; C. C. Lin et al., 2007; Schock et al., 2012). Generally, AChE inhibitors (the case of carbaryl) have been reported to decrease heartbeat rate (Kashiwada et al., 2008; Schock et al., 2012; Watson et al., 2014). Briefly, the increase in acetylcholine concentration in the synaptic cleft caused by the presence of carbaryl (AChE inhibitor) lead to continuous signals from the acetylcholine receptor causing the decrease in heart rate (McKim et al., 1987). However, Lin et al., (2007) hypothesized that the decrease in heart beat at early developmental stages (2 dpf) may be due to an alternate mechanism of

inhibition of calcium ion channels. Nevertheless, the exact nature of this mechanism is still unclear.

Carbaryl is also teratogenic, causing bent tail, notochord bending and axial skeletal defects in embryos of different species of amphibians (Bacchetta et al., 2008; Bridges, 2000; Kang et al., 2010) and inducing changes in tail morphology in zebrafish (Schock et al., 2012). In our study, carbaryl exposure triggered a series of different phenotypes in zebrafish embryos such as, haemorrhage, pericardial edema, tails deformities and spine curvature (Fig. 4b-f). This increase in developmental abnormalities may also be related to AChE inhibition action of carbaryl. Bacchetta et al., (2008) hypothesized cholinesterase inhibition with consequent repetitive muscular spasms to be responsible to the abnormal tail flexure of the amphibian embryos of the African clawed frog (*Xenopus laevis)*. Kang et al., (2010) sustain the same hypothesis as a consequence of several developmental abnormalities found in Boulenger (*Bombina orientalis*) embryos exposed to carbaryl. Behra et al., (2002) studying the AChE role in the neuronal and muscular development of zebrafish embryos concluded that AChE activity is essential for the correct development of the muscle apparatus preventing damage in subsequent developmental stages. In our study, tail deformities were common in zebrafish larvae at 96 hpf in a dose dependent manner and may be related to carbaryl ability to inhibit AChE activity.

4.2 Effects of carbaryl on biochemical makers and locomotor activity of zebrafish embryos

In the present study carbaryl was found to alter the swimming behaviour of zebrafish embryos exposed to concentration equal or higher than 0.1 mg /L. These alterations in swimming behaviour can disrupt feeding (capability to capture prey), impair the ability to attract mates, and increase vulnerability to predation (through an inability to remain inconspicuous) which may have serious implications for long term survival (Little and Finger, 1990) and makes locomotor activity an important and relevant parameter for the risk assessment of pesticides. Our results are in good agreement with previous studies where adverse effects of acute exposure to insecticides on fish swimming behaviour were also found. For example, a decrease in locomotor activity was also observed in juvenile goldfish (*Carassius auratus*) exposed to another carbamate insecticide carbofuran (50 and

100 µg/L) (Bretaud et al., 2001); a markedly decrease in swimming activity was observed in rainbow trout larvae (*Onchorhynchus mykiss*) after exposure to two organophosphate insecticide namely diazinon and malathion (Beauvais et al., 2000); similarly, Beauvais et al., (2001) reported a decrease in swimming speed of rainbow trout larvae (*Onchorhynchus mykiss*) exposed to carbaryl.

As expected for a carbamate insecticide, carbaryl significantly inhibited the activity of AChE (Fig. 5a). AChE is closely related with behavioural changes as pointed out by Scott and Slomman 2004. Therefore, AChE inhibition may be correlated to the decrease in swimming performance of zebrafish embryos observed in this study. Similarly, previous research also related AChE inhibition to changes in behaviour for other fish species exposed to carbaryl and other carbamate insecticides for example, in the rainbow trout larvae (*Oncorhynchus mykiss*) exposed to carbaryl (Beauvais et al., 2001) and the gold fish (*Carassius auratus*) exposed to carbofuran (Bretaud et al., 2001).

GST and LDH activities were also inhibited after exposure to carbaryl (Fig. 5 b and c). Both enzymes activities were also reported to be affected (decreased) after exposure to carbamate insecticides. Exposure to 0.25 mg/L of carbaryl decreased GST activity in the liver of nile tilapia (*Oreochromis niloticus*) (Matos et al., 2007). Similarly, the carbamate insecticide isoprocarb also significantly inhibited gill GST activity of gold fish (*Carassius auratus*) (Wang et al., 2012). Regarding LDH, Singh & Sharma (1998) demonstrated that exposure to carbofuran significantly decreased enzymatic activity in different body tissues of the teleost fish *Clarias batrachus*.

These two enzymes are directly involved in metabolic activity of organisms. GST play a crucial role in the biotransformation (metabolism) of xenobiotic compounds and its activity may be induced or inhibited after exposure to xenobiotics (Hyne and Maher, 2003). LDH is an important enzyme in the anaerobic pathway of energy production and is also involved in the metabolism of carbohydrates (Diamantino et al., 2001). Considering that metabolism is closely related with fish behaviour as reviewed by Sloman et al 2004, the observed inhibition of GST and LDH activities may have also be involved in the reduction of the total swimming distance of embryos exposed to carbaryl.

In this study, carbaryl exposure showed to significantly impact zebrafish embryos survival and development. Moreover, sublethal concentrations were responsible to decrease the total swimming distance and also inhibited ChE, GST and LDH activities

which effects are likely correlated. However, further studies correlating behavioural measures and biochemical marks are necessary to elucidate the specific mechanisms by which alterations in neurotransmission and/or metabolic enzymes result in changes in behaviour. Sublethal levels of carbaryl showed to affect embryos at concentrations more than ten times below the 96 h-LC_{50} (at 0.0075 mg/L for biomarkers and 0.1 mg/L for behaviour). This raises concern regarding carbaryl toxicity to aquatic biota since this insecticide is widely used and has been already detected in freshwater bodies in higher concentrations (0.1-1737 µg/L) (Vryzas et al., 2009; Walters et al., 2003; Wilsont and Foos, 2006). Therefore, our results highlight the importance of biomarkers and behavior endpoints in the risk assessment of pesticides. Although the total swimming distance was less sensitive compared to the biomarkers used, this endpoint is very important due to its ecological relevance and should be used as a complement in developmental studies.

5. Conclusion

The effects of carbaryl on zebrafish embryos development were analysed. Carbaryl showed to have a great impact in fish embryonic development reducing survival, delaying hatching, causing edema and deformities and affecting heartbeat and embryos size. The data presented here besides confirming previous findings, also give a more detailed analysis of carbaryl effects on zebrafish embryos overall development throughout the embryonic and early larval stage (96 h). Moreover, in this study we provide novel data demonstrating that sublethal concentrations of carbaryl affect locomotor behaviour of zebrafish which may indicate an overestimation of the effects of carbaryl specially in developing embryos. Compared to survival or developmental parameters, biomarkers followed by behaviour were the most sensitive endpoints been capable of detecting effects in very low concentration of carbaryl. Therefore, combining developmental parameters as well as the sensitiveness of biomarkers and behavioural endpoints seems to be an excellent approach to expand the sensitivity of standard toxicity tests and consequently improve the risk assessment of pesticides.

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Supplementary data

Effects of carbaryl in zebrafish embryos development, biochemical makers and locomotion

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Tabela S1: Analytical measurement of exposure media of the fish embryo toxicity test.

Nominal Exposure Concentrations (mg/L)	Measured Concentrations	
	(mg/)	% of Nominal concentrations
1	0.891	89
1.6	1.68	105
2.6	3.18	122
4.3	4.20	98
7.0	6.61	94
11.4	11.87	104
18.5	19.81	107
30	31.82	106

Table S2: Summary of models used to calculate concentration-response curves

Endpoints	24hpf	48hpf	72hpf	96hpf
Somite formation	n.e.	-	-	-
Heart beat	-	L3	-	-
Haemorrhage (clutch of red blood cell)	n.e.	n.d	n.d	L4
Hatching rate	n.e.	n.e.	L3	n.d.
General deformities	L4	n.d.	n.d.	n.d.
Tail deformities	-	-	n.d.	L4
Pericardial edema	n.e.	L4	L4	L4
Body length	-	-	-	L4
Mortality (LC_{50})	n.d.	L4	L4	L4

n.e. no effect on the endpoint analysed; n.d. endpoint not determined (no effect or only effects below a 50 % level); - endpoint not analysed; hpf hours post fertilization
L3 – Logistic 3 parameters
L4 – Logistic 4 parameters

Table S3: Toxicity of carbaryl to fish early life stages

Specie	Stage of development	Endpoint	Test	Conc. (mg/L)	Reference
Zebrafish (*Danio rerio)*	Embryos	Survival	24h-LC_{50}	44.66	(Lin et al., 2007)
		Pericardial Edema, malformations, red blood cell accumulation, altered heart beat rate	24h-EC_{50}	7.52	
Zebrafish (*Danio rerio)*	Embryos	Survival, Developmental delay, morphological defects, altered heart beat rate	72h	10-40	(Schock et al., 2012)
Zebrafish (*Danio rerio)*	Embryos	Developmental delay, hatching delay, embryo size	144h	5.3 -21.3	(Todd and Van Leeuwen, 2002)
Carp (*Cyprinus carpio*)	Fry	Survival	96h-LC_{50}	7.85	(De Mel and Pathiratne, 2005)
Rohu *(Labeo rohita)*	Fingerlings	Survival	96h-LC_{50}	8.24	(Mustafa and Mahboob, 2014)
Japanese Medaka (*Oryzias latipes*)	Embryos	Survival	14 days	0-10	(Kashiwada et al., 2008)
	Larvae	Survival	96h	0-10	
Indian Carp *(Catla catla)*	Fingerling	Survival	24h-LC_{50}	9.49 (8.91-10.08)[a]	(Mahboob et al., 2014)
			48h-LC_{50}	9.10 (8.50-9.76)[a]	
			72h-LC_{50}	8.42 (7.85-9.09)[a]	
			96h-LC_{50}	7.89 (7.31-8.67)[a]	

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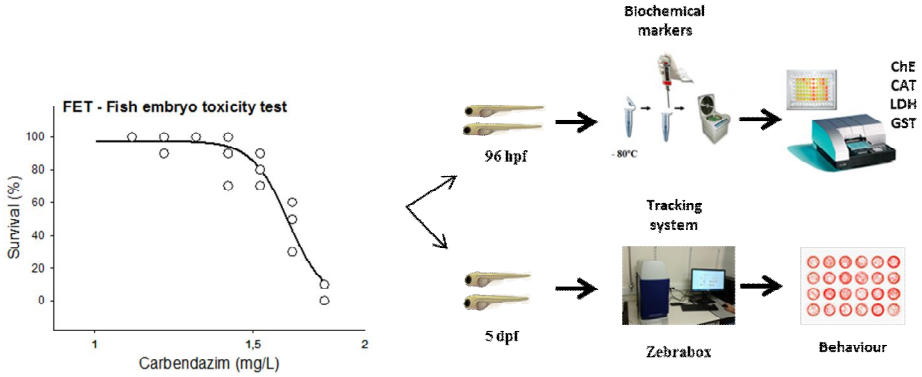
Chapter 5

Carbendazim exposure induced physiological, biochemical and behavior disturbance in zebrafish embryos



Carbendazim exposure induces developmental, biochemical and behaviour disturbance in zebrafish embryos

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Abstract

Carbendazim is a widely used broad spectrum benzimidazole fungicide; however, its effects to non-target aquatic organisms are poorly studied. The aim of this study was to investigate the toxic effects of carbendazim to zebrafish early life stages at several levels of biological organization, including developmental, biochemical and behavioural levels. A first embryo assay was done following the OECD guideline 236 and using a concentration range between 1.1 and 1.8 mg/L. Lethal and developmental endpoints such as hatching, edemas, malformations, heart rate, body growth and delays were assessed in a 96 hours exposure. A sub-teratogenic range (from 0.00016 to 0.5 mg/L) was then used to assess effects at biochemical and behavioural levels. Biochemical markers included cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) and were assessed at 96 hours. The locomotor behaviour was assessed using an automated video tracking system at 120 hours. Carbendazim showed high toxicity with a 96h-LC_{50} of 1.75 mg/L while sublethal effects such as hatching success, edemas, malformations, reduction of heart rate, body length and yolk sac consumption were observed with EC_{50s} ranging from 0.85 to 1.6 mg/L. Carbendazim exposure significantly altered biochemical parameters by inducing Che, GST and LDH activities at concentrations equal or above 0.004 mg/L. The locomotor response of zebrafish embryos was also affected as observed by alterations in swimming activity of larvae even at the lowest tested concentration (0.00016 mg/L). In this work, locomotion showed to be several orders of magnitude more sensitive than developmental parameters or lethality, highlighting the potential of behavioural endpoints as early warning signs for environmental stress. Further studies should focus on understanding how the behavioural disturbances measured in these types of studies translate into fitness impairment at the adult stage.

Keywords: *Danio rerio*, locomotor response, biomarkers, sublethal effects

1. Introduction

Carbendazim (methyl-1-H-benzimidazol-2-yl-carbamate) is one of the most widely used benzimidazole fungicides. It has a high toxicity to target organisms, inhibiting the development of a wide variety of fungi even at low doses. It is used in agriculture, horticulture, forest and home gardening and as a preservative in paint, papermaking, textile, leather industry, as well as a preservative of fruits (Selmanoğlu, G.Barlas, N.Songür, S.Koçkaya, 2001). Carbendazim is a metabolite of benomyl and it is known to target the tubulin in cells, causing disruption of microtubule assembly and cell division (Davidse, 1986). Many studies have reported the adverse effects of carbendazim on mammals mainly on reproductive organs (Farag et al., 2011; Ireland et al., 1979; Lim and Miller, 1997; Nakai et al., 2002; Urani et al., 1995). Low concentrations of carbendazim ranging from 0.2 to 200 µg/L have already been detected in surface waters near agriculture and forestry areas (Palma et al., 2004; Readman et al., 1997). Moreover, carbendazim has shown to be very persistent in the water with a half-life of 6 to 25 weeks (Cuppen et al., 2000a).

Although some of the toxic effects of carbendazim have been studied in mammals, its effects on aquatic organisms are poorly studied. The majority of studies available focus on zooplankton and macroinvertebrate communities where chronic exposure to carbendazim negatively affected these populations by decreasing survival, reproduction and feeding rates (Cuppen et al., 2000b; Daam et al., 2010; Ferreira et al., 2008; Ribeiro et al., 2011; Van den Brink et al., 2000). To our knowledge, there are only two studies available concerning carbendazim effects on fish early life stages. The study by Ludwikowska et al. (2013) showed that carbendazim could affect the survival and hatching success of *Prussian carp* embryos at concentrations above 0.036 mg/L and the study of Jiang et al. (2014) demonstrated that embryonic exposure to carbendazim led to significant changes in the expression of genes related to apoptosis, immunotoxicity and endocrine disruption in zebrafish (*Danio rerio*). In this later study concentrations between 0.004 and 0.5 mg/L of carbendazim were tested, although gene expression effects for most of the genes analyzed, did not follow a dose response pattern.

Risk characterization is better achieved by studying chemical effects at several levels of biological organization. Recently, behavioural parameters such as locomotion

(whose evaluation have been considered time consuming and lacking objectivity) have been increasingly used due to the development of technology for automated analysis. In the case of zebrafish, locomotion has been used as an endpoint to assess the neurotoxic effects of chemicals in early life stages (Irons et al., 2010; Padilla et al., 2011; Selderslaghs et al., 2010) and the sublethal toxicity of pollutants (Ulhaq et al., 2013). In fact, many contaminants disrupt fish behaviour after exposures much less severe than those causing mortality as demonstrated by Klüver et al., (2015) where behaviour of fish embryos was altered at concentrations 375-fold lower than the LC_{10}. Thus, behaviour has proven to provide very sensitive measures of stress exposure; furthermore it has high ecological relevance as effects can be translated in their long term health and survival (Scott and Sloman, 2004; Tierney, 2011).

Thus, in this work we aim at assessing the effects of carbendazim at several levels of biological organization using zebrafish embryos as model organism. Recently, the approval of the OECD Test Guideline nº 236 (fish embryo toxicity test) has consolidated the zebrafish embryo test as a true alternative for the acute fish toxicity test with adults (Braunbeck et al., 2014a) in the European Union. This test has been increasingly used to assess the toxicity of chemicals and waste waters as reviewed by Scholz et al. (2013, 2008). The low volume of test solutions needed (tests are deployed in 24 or 96-wells microplates), and the rapid development and transparency of embryos that allow the monitoring of the entire organogenesis are among the advantages of this test. Moreover, the possibility of measurement of behaviour (as referred above) and biochemical parameters such as AChE-acetylcholinesterase, GST – gluthatione-S-tranferase, LDH – lactate dehydrogenase, CAT - catalase (Oliveira et al., 2009), makes the zebrafish embryos a very good model to analyze effects of chemicals at several levels, providing complementary information on the mode of action of the chemicals (Braunbeck et al., 2014b; Küster and Altenburger, 2008) ultimately contributing to understand and establish the Adverse Outcome Pathway – AOP - (a recent framework proposed by Ankle et al (2010) that directly link molecular-level initiating events that ultimately lead to adverse outcome at higher levels) for this fungicide. Once described, an AOP can be a key factor for hazard identification in the risk assessment of chemicals (Ankley et al., 2010; Volz et al., 2011). This is particularly important considering the urgent need to reduce animal use for toxicity testing in the European Union.

Thus, in the present study, zebrafish embryos were used to assess the toxic effects of carbendazim at several levels:

i) survival,

ii) developmental level (including embryo development delays and malformations),

iv) biochemical level (including the measurement of the enzymes AChE, GST, LDH and CAT) and

v) behavioural level (by measuring locomotion of zebrafish eleutheroembryos expressed either by distance moved or time spent moving)

2. Materials and Methods

2.1 Zebrafish maintenance and embryo collection

All the embryos used in the present study were provided by the zebrafish facility established at the Department of Biology, University of Aveiro (Portugal). Adults were maintained in carbon-filtered water, complemented with 0.34 mg/L salt ("Instant Ocean Synthetic Sea Salt", Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was kept at 26.0 ± 1 ºC, conductivity at 750 ± 50 µS, pH at 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. This reconstituted water was used in the preparation of test solutions of all assays performed. The above mentioned temperature and photoperiod conditions were constant in all assays. Zebrafish eggs were obtained by crossbreeding of individuals in aquaria; after 30 min of natural mating, eggs were rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope -SMZ 1500, Nikon Corporation); those unfertilized, with cleavage irregularities, injuries or other kind of malformations were discarded.

2.2 Test Chemicals and Preparation of Test Solutions

Carbendazim (**Methyl 2-benzimidazolecarbamate, 97% purity**) was purchased from Sigma-Aldrich. Carbendazim solutions were carefully prepared by dissolving carbendazim on the zebrafish water system. Ten millilitres of each tested concentration was sampled at the beginning and at the end of the assay and preserved at -20 ºC for further chemical analysis. The chemical analysis aimed to assess the degradation of carbendazim in the test solutions and was performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in České Budějovice, Czech Republic.

2.3 Embryo assay

The assay was based on the OECD testing guideline 236 on Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). The embryos (approximately 3 hpf) previously selected were exposed to carbendazim concentrations ranging from 1.1 to 1.8 mg/L. Ten eggs in triplicate per treatment were distributed individually in 24-wells microplates. Test run for 96 h. Embryos were daily observed under a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation, Japan) and the following parameters were evaluated: survival, somite formation, incidence of pericardial edema, heart beat, malformations (general, spinal, tail and head), hatching, body length (total length: snout to tail tip), yolk sac length and developmental delay. The heart beat (beats/15s) was measured by counting heart beats under a stereomicroscope in 3 randomly selected embryos of each replicate (n=9 per concentration) at 48 h. The body and yolk sac length was measured using digital images of the embryos with the software NIS Elements D (Nikon Corporation, Tokyo, Japan). Development delay was obtained by matching the developmental stage of a given embryo with the developmental stages defined by Kimmel et al., (1995).

A sublethal range of carbendazim concentrations (0.00016, 0.0008, 0.004, 0.020, 0.1 and 0.5 mg/L) was used to set up the test for biochemical determinations and locomotory analysis. This test was deployed in the same conditions as the above described test. At 96 hours of exposure, 10 clusters of eight larvae per treatment were snap-frozen in

microtubes containing 0.8 ml of K-phosphate buffer (0.1M, pH 7.4) and stored at - 80 º C for further enzymatic analysis (see section 2.4). For locomotory assay larvae were transferred to 96 well plates and analysed using the track system Zebrabox (Viewpoint, Lyon, France) at 120 h (see section 2.5).

2.4 Biomarkers determinations

Enzymatic assays were performed to analyse cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) in larvae of zebrafish. Enzymatic determinations were made spectrophotometrically (Thermo Scientific Multiskan Spectrum, USA) using 96 wells microplates. On the day of enzymatic analyses, samples were defrosted on ice, homogenised (KIKA Labortechnik U2005 Control) and centrifuged (4 ºC, 10000 g, 20 min) in order to isolate the post-mitochondrial supernatant (PMS) posteriorly used as enzyme extract for enzymatic activity determination.

The methods for the determination of ChE, GST and LDH activity is described in Domingues et al. (2010). Briefly, ChE activity was determined at 414 nm according to the method of Ellman et al. (1961) adapted for microplate (Guilhermino et al., 1996). GST activity was performed at 340 nm as described by Habig and Jakoby (1981) adapted to microplate reader (Frasco and Guilhermino, 2002). The LDH activity was continuously monitored for 5 min at 340 nm, following the methodology described by Vassault (1983) with the modifications introduced by Diamantino et al. (2001). The CAT activity was measured at 240 nm in spectrophotometer quartz cell by monitoring the decrease of absorbance due to decomposition of H_2O_2, as described by Clairborne (1985).

Protein quantification in samples was performed in quadruplicate according to the Bradford method (Bradford, 1976), at 595 nm, using ɣ- globulin to determine standard curve. Enzymatic activity units were expressed in nanomoles of substrate hydrolyzed per minute per mg of protein. A Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA) reader was used to determine all protein and enzymatic activity.

2.5 Analysis of locomotor response

Zebrafish larvae were used to assess the effects of carbendazim on locomotory activity. At 120 hpf the embryos were removed from the exposure dishes and placed in 96 well plates (one per well). Locomotion was evaluated for 12 embryos per treatment in triplicate including the control. Prior to the assessment of behaviour, dead larvae or larvae that exhibited physical abnormalities were discarded and not included in analyses. Embryonic movement was tracked using the Zebrabox (Viewpoint, Lyon, France) tracking system using a 25 frame per second infrared camera over a period of 50 min. The temperature was maintained stable at 26 ± 1 ºC. Movement was stimulated by applying light:dark intervals according to what was previously described in Irons et al (2010). Briefly, the test consisted of acclimating the embryos in the light for 10 min, followed by a cycle of four alternating periods of light:dark intervals (10 min dark; 10 min ligth; 10 min dark; 10 min ligth). Typically zebrafish larvae show less locomotion during light periods and more during dark. For each replicate the distances moved in 2-minute time intervals were recorded separately for each dark and light period.

In this study the following parameters were calculated for each time interval except acclimation period that was not included in the analysis: total distance moved, relative small and large distance moved and relative swimming time. The total distance consists of the total swimming distance of the larvae during each measurement period. The relative small distance (%) is the ratio between small distance moved and total distance moved in each 10 min period. The relative large distance (%) is the ratio between large distance and the total distance moved in each measurement period. Finally, the relative swimming time refers to the time embryos spend moving (swimming) relative to the total measurement period. A threshold of 30 was used for background correction and a movement was considered “small” when individuals moved less than 0.5 mm/sec.

2.6. Determination of Carbendazim in water using liquid chromatography-tandem mass spectrometry

2.6.1. Chemicals

Liquid chromatography–mass spectrometry (LC-MS) grade methanol and acetonitrile (Li Chrosolv Hypergrade) were obtained from Merck (Darmstadt, Germany). Formic acid used to acidify the mobile phases was purchased from Labicom (Olomouc, Czech Republic). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All compounds used were analytical standards or of high purity (> 98%). $^{13}C_6$ labeled trimethoprim was purchased from Cambridge Isotope Inc. (Andover, MA, USA) and it was used as internal standard because it has quite similar retention time as carbendazim (elution time is 4.98 min for carbendazim and 5.35 min for labeled trimethoprim).

Stock solutions of carbendazim and labeled trimethoprim were prepared in methanol at a concentration of 1 $mg \cdot mL^{-1}$ and stored at -20°C. A spiking mixture was prepared for each compound by diluting stocks in methanol to concentration of 1 µg/mL and stored at -20°C.

2.6.2. LC-MS/MS analysis

A triple stage quadrupole MS/MS TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis of carbendazim in water samples. Thawed water samples were filtered through a syringe filter (0.45 µm, regenerated cellulose, Labicom, Olomouc, Czech Republic), after that 10 ng of internal standard was added to 1 mL of sample.

An analytical Cogent Bidentate C18 column (50 mm x 2.1 mm ID x 4 µm particles, Thermo Fisher Scientific) was used for chromatographic separation of the target analytes. Ionization of targeted compounds was carried out with heated electrospray ionization (HESI). The spray voltage was 3.5 kV. Nitrogen (purity > 99.999%) was used as the sheath gas (40 arbitrary units), auxiliary gas (10 arbitrary units) and collision gas. The vaporizer

was heated to 250°C and the capillary to 350°C. Chromatographic separation of targeted analytes was provided with acidified acetonitrile/ultrapure water gradient presented in the Table S1 (Supplementary data).

Two product ions from carbendazim 192→159, used for quantification, and 192→131, used for qualification, were monitored in positive ion mode during analysis. Developed method was validated in the range of tested concentrations. The method showed good linearity in the concentration range from 0.001 to 1 mg/L for carbendazim with R^2=0.998. Recovery of carbendazim from aquaria water was evaluated by spiking water samples with the target compound. Recovery value for carbendazim was 100%. The average carbendazim limit of quantification (LOQ) was 0.00016 mg/L and was calculated as one quarter of the lowest calibration point in the calibration curve where relative standard deviation of average response factor was < 30%.

Matrix-matched standard response was used as factors for correcting the response derived from the calibration curve. Matrix-matched standard was prepared from tested water blank by spiking with both internal standard and native compound at 0.01 mg/L and 1 mg/L, respectively.

2.7 Statistical analysis

Lethal concentration (LCx) and effect concentration (ECx) values were calculated for each endpoint by fitting dose-response curves using the package drc in the software R (R Core Team, 2014). For locomotor behaviour a one-way analysis of variance (ANOVA) was used to test differences between the different treatments among each of the light dark interval, except the acclimation period that was not included in the analysis. In the case datasets failed the normality and homoscedasticity test, an ANOVA on ranks (Kruskall-Wallis) was performed. When significant, differences were further explored with appropriate post hoc test (Dunnet's or Dunn's) to compare each treatment against control. Test statistics and analysis of normality were conducted using the software SigmaPlot V.12.5 (SysStat, San Jose, California, USA). A significance level of 0.05 was used to infer statistically significant results. The relationship between the different treatments/concentrations of carbendazin and the overall behavioural and biochemical endpoints was investigated by a Principal Component Analysis (PCA). Behavioural and

biomarkers data were standardized (scaled into 0-1 range), in order to be used in the same ordination plot. PCA was performed using CANOCO 4.5 software (Lepš and Šmilauer, 2003).

3. Results

3.1 Stability of carbendazim in the exposure medium

Chemical analysis of the exposure media showed stable exposure concentrations and within 80 – 120% of the nominal concentrations in what refers the FET test (1.1 – 1.8 mg/L). However, analysis of the exposure media of the sublethal range of concentrations (0.00016 – 0.5 mg/L) revealed some inconsistencies (Table S1, Supplementary data) probably because the tested concentrations were very close to the limit of quantification.

3.2 Effects on embryos development

The calculated LC_{50} and EC_{50} values for zebrafish embryos exposed to carbendazim are presented in Table 1. Carbendazim showed to be moderate to highly toxic to zebrafish embryos as can be observed in Fig S1, with a 96 h-LC_{50} of 1.76 mg/L (Table 1).

Table 1 – Effects of Carbendazim on the developmental parameters of zebrafish embryos. L(E)C values are presented in mg/L and followed by standard error.

Developmental parameters	24 hpf		48 hpf		72 hpf		96 hpf	
	$L(E)C_{10}$	$L(E)C_{50}$	$L(E)C_{10}$	$L(E)C_{50}$	$L(E)C_{10}$	$L(E)C_{50}$	$L(E)C_{10}$	$L(E)C_{50}$
Somite formation	n.e.	n.e.	-	-	-	-	-	-
General deformities	1.27 ± 0.03	1.48 ± 0.03						
Heart rate	-	-	1.18 ± 0.12	1.86 ± 0.05	-	-	-	-
Developmental delay	n.e.	n.e.	1.56 ± 0.11	1.59 ± 0.08	n.e.	n.e.	n.e.	n.e.
Head and eye deformity	n.e.	n.e	1.55 ± 0.07	1.60 ± 0.043	1.39 ± 0.02	1.52 ± 0.01	1.41 ± 0.03	1.53 ± 0.02
Tail deformities	n.e.	n.e.	n.e.	n.e.	1.44 ± 0.02	1.52 ± 0.01	1.38 ± 0.03	1.54 ± 0.02
Spine deformity	n.e	n.e.	1.42 ± 0.05	1.53 ± 0.02	1.33 ± 0.02	1.46 ± 0.02	1.31 ± 0.02	1.46 ± 0.01
Edema	n.d.	n.d.	0.88 ± 0.08	1.26 ± 0.08	0.85 ± 0.07	1.24 ± 0.06	0.89 ± 0.04	1.08 ± 0.02
Hatching rate	-	-	-	-	1.50 ± 0.04	1.57 ± 0.04	1.54 ± 0.03	1.62 ± 0.01
Body length	-	-	-	-	-	-	1.42 ± 0.02	1.72 ± 0.04
Yolk sac length	-	-	-	-	-	-	1.33 ± 0.09	1.35 ± 0.09
Survival	n.d.	n.d.	1.70 ± 0.11	1.75 ± 0.18	1.63±0.06	1.74 ± 0.61	1.48 ± 0.05	1.76 ± 0.13

n.e. no effect observed for the endpoint analyzed n.d. not determined (effects below a 50% level)
-endpoint not analyzed; hpf hours post-fertilization

Carbendazim also affected the development of embryos by triggering a series of developmental anomalies including the incidence of edema, spine, head and tail deformities and also reducing heart rate, body length and the rate of consumption of the yolk sac. Although carbendazim did not cause significant mortality at 24 hpf, embryos exposed to concentrations above 1.3 mg/L exhibited developmental anomalies such as tail and spine deformities presenting an EC_{50} of 1.48 mg/L (Table 1). At 48 hpf an increment in the frequency of edemas and spine curvature was observed with a EC_{50} of 1.26 mg/L and 1.53 mg/L respectively (Table 1, Fig S2 a and b). Moreover, carbendazim induced a significant reduction in heart beat rate affecting even embryos exposed to the lowest concentration of 1 mg/L. Embryos exposed to control exhibited a heart beat around 180 beats/min while embryos exposed to the highest concentrations presented a heart beat rate around 100 beats/min (Table 1, Fig S2 c).

At 72 hpf besides the increase in malformations, carbendazim reduced hatching rate in exposed embryos. The calculated 72 h-EC_{50} was 1.57 mg/L (Table 1, Fig S3 a). Concerning body anomalies the calculated 72 h-EC_{50} were 1.52, 1.52 and 1.46 respectively for head, tail and spine deformities (Table 1, Fig 3). The most important anomalies found were head and eye malformation, and spine curvature as can be observed in Fig 1 A-F. Moreover, carbendazim caused an increase in edema formation (mainly pericardial edema, see Fig S3 b and Fig 1 B, C and D) and the calculated 72 h-EC_{50} was 1.24 mg/L.

The same effects described above were still observed at 96 hpf (Fig S4), including hatching (96 h-EC_{50} =1.62 mg/L) where only around 50% of embryos exposed to carbendazim concentrations above 1.5 mg/L have hatched (Fig S4 a) and head, tail and spine deformities (96 h-EC_{50} = 1.53, 1.54 and 1.46 respectively) as can be observed in Table 1, Fig S4 b, d and f and Fig 1 G-I. In relation to pericardial edema embryos exposed to concentrations above 1 mg/L were severely affected (96 h-EC_{50} = 1.08 mg/L, Table 1, Fig S4 c). In addition, carbendazim significantly affected body and yolk sac length of embryos as can be observed in Table 1 and Fig. S4 e. The body length of embryos decreased as carbendazim concentrations increased and embryos were affected even in the lowest concentration tested. The body length of control embryos was around 3.5 mm while exposed embryos showed body length around 3.0 mm. In contrast a significant increase was observed in yolk sac length in concentrations above 1.30 mg/L which may be related to a delay in the consumption of the yolk.

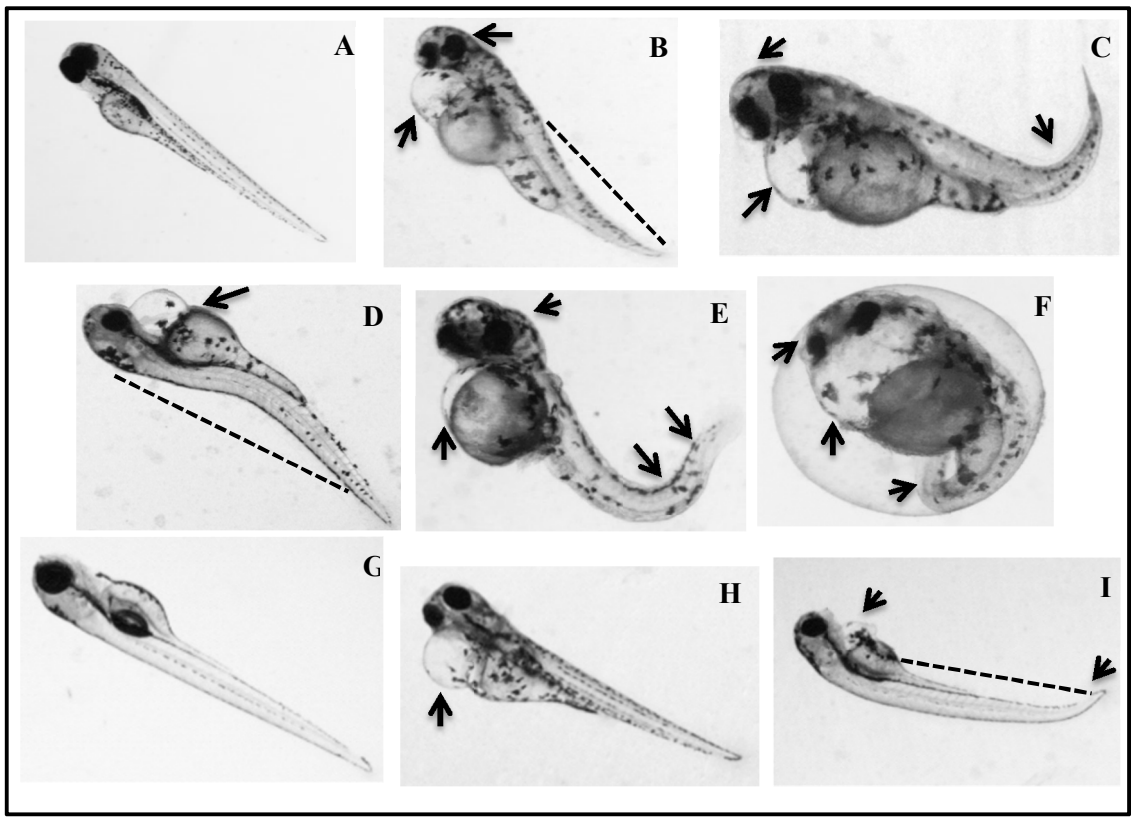

Fig 1 – Zebrafish embryos abnormalities during exposure to carbendazim. A) Control embryos at 72 h of exposure (2x magnification); B) Embryo exposed to 1.41 mg/L showing pericardial edema, head and spine deformities (3x); C, D and E) Embryos exposed to 1.53 mg/L of carbendazim after 72 h of exposure presenting pericardial edema, eye, head and spine with severe deformities (3x); F) unhatched embryo exposed to 1.66 mg/L presenting a severe pericardial edema, head, eye and tail deformities (3x); G) Control embryo at 96 h (1x); H) Larvae exposed to 1.19 mg/L presenting pericardial edema (1x) and I) Larvae exposed to 1.3 mg/L of carbendazim presenting pericardial edema and spine deformity(1x).

3.3 Biomarkers

The effects of carbendazim on the activities of biomarkers ChE, GST, LDH and CAT, are presented in Figure 2. Exposure to carbendazim significantly induced ChE ($F_{6, 34}$= 5.18; P= 0.001), GST ($F_{6, 28}$= 10.59; P< 0.001) and LDH (H= 20.70; P=0.002) activities at concentrations ≥ 0.004 mg/L when compared to control group (Fig 2 a-c). Regarding CAT activity, although a slightly decrease in activity was observed at concentration below 0.004 mg/L, no statistically significant differences were observed ($F_{6, 33}$= 1.36; P=0.268).

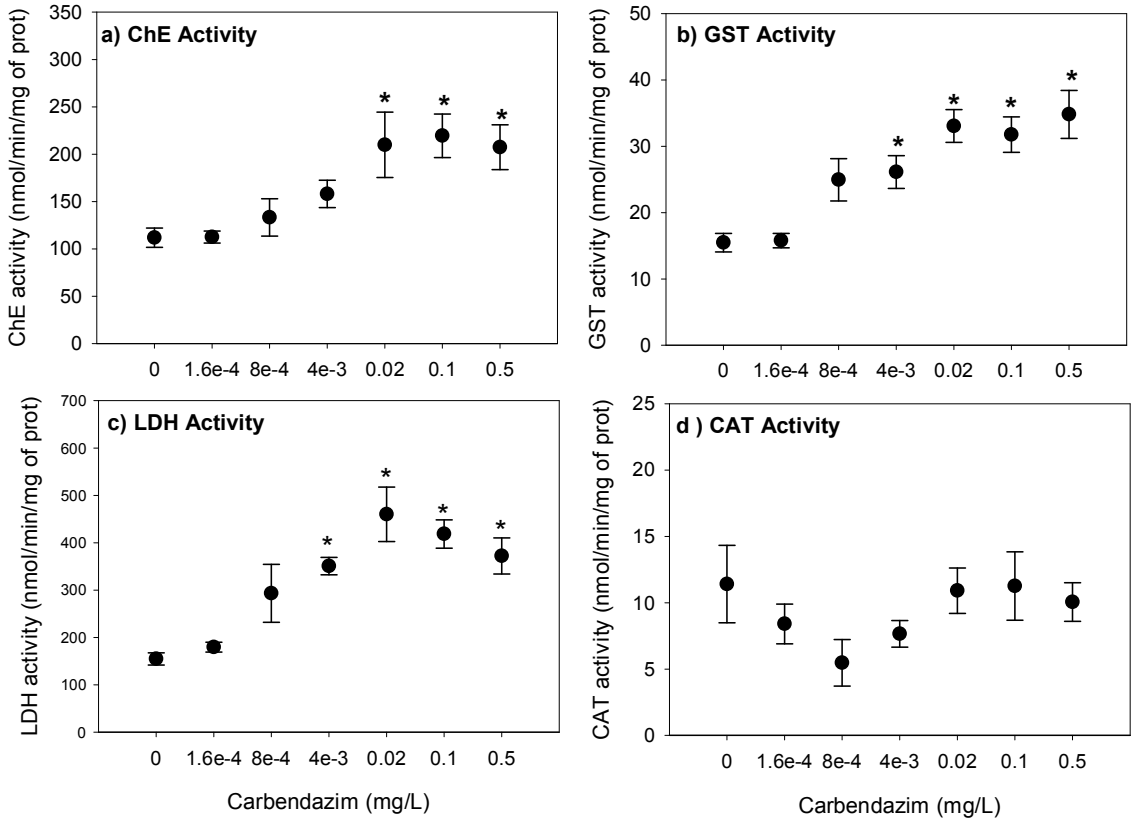

Fig. 2 – Enzymatic activities (mean values ± standar error) on zebrafish embryos after 96 hours of exposure to carbendazim: a) ChE activity; b) GST activity; c) LDH activity and d) CAT activity. Asterisks mean significantly different from the respective control ($p<0.05$).

3.4 Behavioural changes: locomotor response

Results within the two periods of light and within the two periods of dark tested were very similar and thus, here, only the results of the first dark and light periods will be presented (Fig 3). Carbendazim induced changes in the locomotor activity of zebrafish larvae at 120 hpf. Fig. 3 a-b shows the results for total distance moved (mm) in the dark and in the light period. During the dark period, no statistical differences were found on any treatment when compared to control although the One way Anova revealed an effect of carbendazim ($H= 16.49$; $P= 0.011$). On the other hand, during the light period a significant ($F= 2.13$; $P= 0.002$) decrease in the distance moved was observed at concentration above 0.0008 mg/L (Fig 3 b).

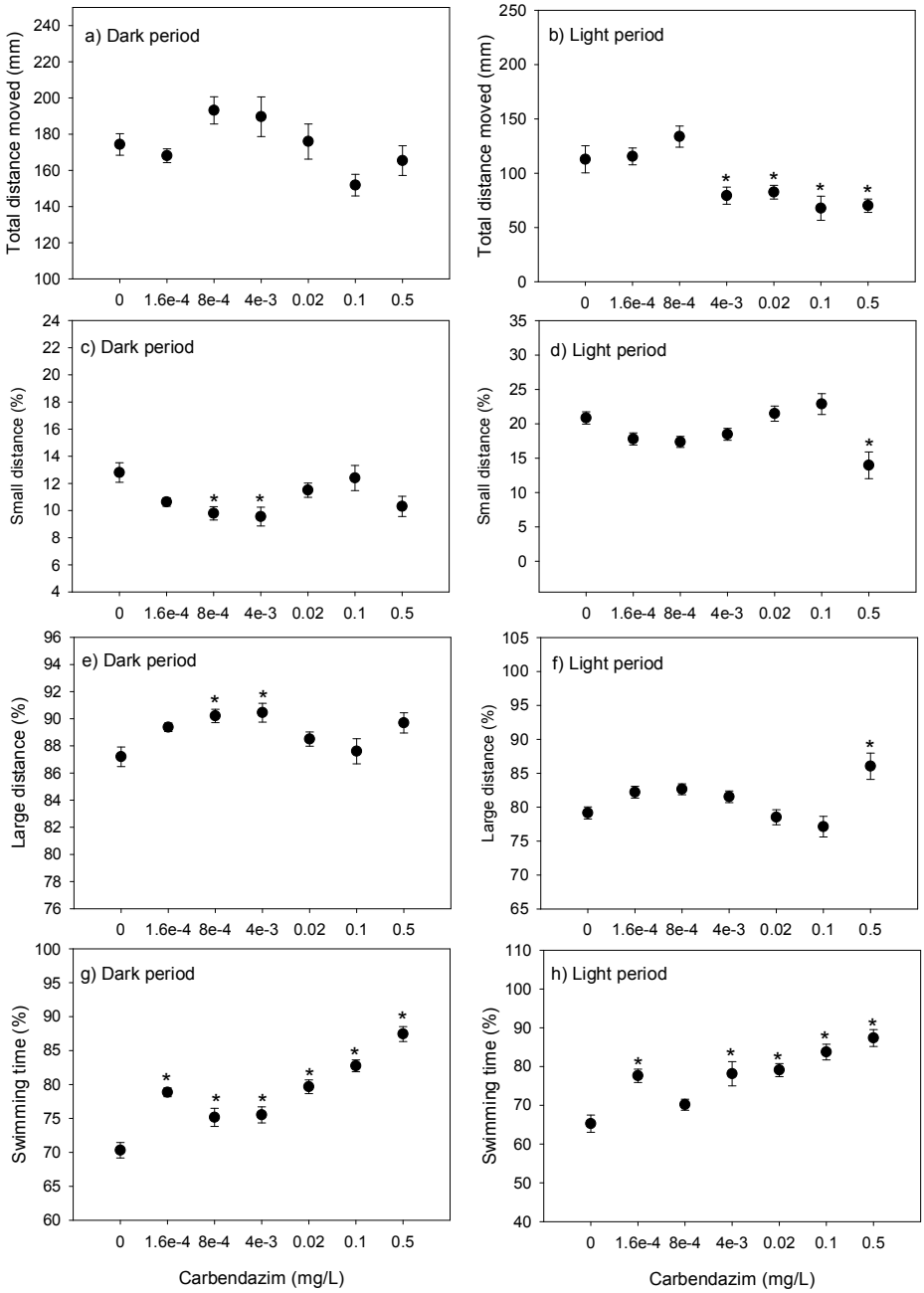

Fig. 3 – Effects of carbendazim on embryos locomotion in the first dark and light period: a na b) total distance moved by larvae; c) and d) refers to the small distance in relation to total distance moved; e) and f) large distance in relation to total distance moved for each time interval; g) and h) refers to the time larvae spend moving in relation to the total time. Asterisks indicate significantly different from control ($p<0.05$).

Small and large distances are complementary parameters as can be seen in Fig 3 c and e and d and f. Although an effect was observed for these parameters, they do not present a dose-response pattern. Generally, in the dark periods, organisms exposed to intermediate concentrations of carbendazim presented lower percentages of small distance movements (and higher % of large distance movements) when compared to control, Fig 3 c and e. In the light period only organisms exposed to the highest concentration presented a differentiated behaviour compared to control, translated by a decreased percentage of small distance movements (and increased % of large distance movement), Fig 3 d and f. In relation to the relative swimming time, larvae exposed to carbendazim presented a longer swimming time when compared to control group in both dark ($F= 27.44$; $P= <0.001$) and light ($H= 47.30$; $P= <0.001$) (Fig 3 g-h). This effect was concentrations dependent and differences could be perceived even at the lowest concentrations tested either for dark or for light periods.

3.5 PCA

In the PCA related to the light period (Figure 4-A), the first two ordination axes explained 72.2% of the total variation. The primary axis represented 55.4% of the variation and described an increase on the activity of all the measured biomarkers and also swimming time (ST) along with the decrease of total swimming distance (TD). Higher enzymatic activity and longer swimming time are related with higher concentration of carbendazim, while control samples and lower concentrations of the chemical are related with greater swimming distance. The second axis explained a variation of 16.8% and described a gradient of increasing percentage of long distance swimming but there was no clear pattern between these behaviour parameter and carbendazim concentrations.

The PCA for the Dark period (Figure 4-B) presented an overall similar pattern. The first two axes represented 75.0% of the total observed variation. Primary variation captured 53.1% of the global variation and described an increase of the enzymatic activity, followed by swimming time (ST). Again, the increase of these parameters is associated to higher concentrations of carbendazim. Secondary axis, that explains 21.9% of the total variation,

describes an increase in both total distance (TD) and large distance percentage (LD), but without a clear relationship with carbendazim concentrations.

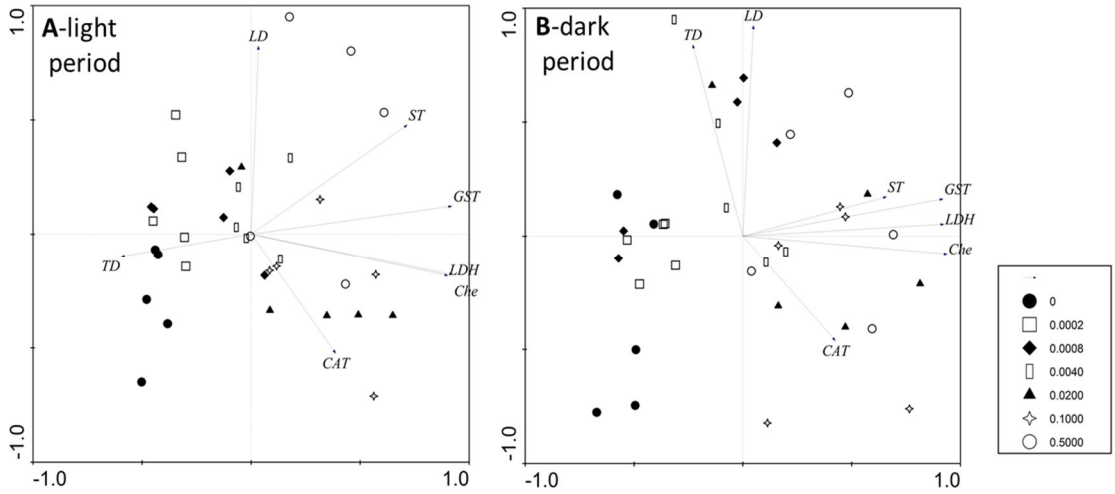

Fig. 4 – Biplot of the Principal Components Analysis (PCA) performed on different concentrations of carbendazim (symbols). The ordination was made in relation to behaviour endpoints TD (Total distance), LD (Large distance) and ST (Swimming time) and biochemical (GST, LDH, ChE, CAT) response variables (arrows).

4. Discussion

Our study revealed effects of carbendazim at developmental, biochemical and behavioural levels. Lethality of carbendazim to zebrafish embryos did not increase with time and was fully established after 48 h of exposure. This is probably due to the mode of action of carbendazim that inhibits the assembly of tubulin and the formation of microtubules in fungi, and also in mammals (Davidse, 1986; Ireland et al., 1979; Lim and Miller, 1997). For instance, the lethal effects of carbendazim in Clawed frog (*Xenopus laevis*) at 4-cell stage embryos proved to be higher than in embryos exposed later in blastula stage as concluded by Yoon et al., (2008). In our study, zebrafish embryos were exposed as early as 2 - 3 hpf and possibly, the establishment of toxicity at the first 48 h is the result of carbendazim inhibition of microtubule assembly and mitosis in the early embryonic stages. One of the few studies conducted in fish early life stages available also demonstrated that carbendazim strongly affected survival of Prussian carp (*Carassius gibelio*) embryos by causing 100% of mortality after 24 h of exposure to 0.216 mg/L

(Ludwikowska et al., 2013). Fingerlings of Milkfish (*Chanos chanos)* exposed to carbendazim showed a 96 h-LC_{50} of 0.013 mg/L (Palanikumar et al., 2014) which is very low compared to the one found in this study for zebrafish embryos (1.75 mg/L). Another study conducted in Tambaqui (*Colossoma macropomum*) alevins showed a 96 h-LC_{50} of 4.16 mg/L (Rico et al., 2011).

Furthermore, carbendazim exposure strongly affected the development of embryos by triggering a series of developmental anomalies (spine curvature, head deformity), decreasing heart rate and body length; increasing pericardial edemas and delaying yolk sac consumption and hatching. This is consistent with studies conducted in amphibian (Yoon et al., 2008) and rodent embryos (Farag et al., 2011) where carbendazim showed to be teratogen increasing the incidence of malformations such as pericardial edema, spinal lordosis, elongated heart, narrowed head among others. The body length of Clawed frog (*X. laevis*) exposed to carbendazim were also shown to be significantly shorter at concentrations $\geq$ 0.38 mg/L (Yoon et al., 2008). A previous study carried out with the parent compound of carbendazim (benomyl) in zebrafish embryos also showed to decrease hatching and heart rate and increase incidence of malformations causing the same type of anomalies observed in our study in concentrations as low as 30 µg/L (Kim et al., 2009).

Regarding the effects of carbendazim on biomarkers, a significant induction of ChE, GST and LDH activities was observed in exposed embryos. To the best of our knowledge, there is no study available which describes alterations in the enzymatic activity in zebrafish embryos caused by carbendazim. AChE plays an important role in neurotransmission being responsible for the hydrolysis of acetyltiocholine at the cholinergic synapses and neuromuscular junction (Olsen et al., 2001). In addition, several studies have pointed out evidences of AChE being involved in other physiological process including the participation in the regulation of cell proliferation and apoptosis as reviewed by Jiang & Zhang (2008). A recent study conducted in zebrafish has demonstrated that embryonic exposure to different carbendazim concentrations ranging from 0.004 to 0.5 mg/L lead to significant changes in the expressions of many genes that play critical roles during cell apoptosis (Jiang et al., 2014). However, the mechanisms that regulate AChE expression and participation in apoptosis are not fully understood yet (Soreq and Seidman, 2001; Zhang et al., 2002). Considering apoptosis is related to up regulation of acetylcholinesterase genes (Zhang et al., 2002), the overexpression of ChE activity in

zebrafish embryos observed in our study is probably linked to carbendazim potential to induce cell apoptosis.

GST activity was also induced after exposure to carbendazim. GST represent a family of enzymes with a central role in the biotransformation of xenobiotics and endogenic compounds and their activity can be enhanced in response to xenobiotics which make them a stress indicator that have been increasingly used as an environmental biomarker (Hyne and Maher, 2003). GST induction has also been observed following carbendazim and benomyl exposure (parent compound of carbendazim) in a midge species (*Kiefferulus calligaster*) and in adult Nile tilapia (*Oreochromis niloticus*) (Domingues et al., 2009; Min and Kang, 2008) respectively.

Similarly, LDH activity was induced in embryos exposed to carbendazim. LDH is a key enzyme in the anaerobic pathway of energy production and is involved in the carbohydrate metabolism (Diamantino et al., 2001). Increased LDH activity levels have been observed in conditions of chemical stress when high levels of energy are required as demonstrated in Nile tilapia exposed to benomyl, the parent compound of carbendazim (Min and Kang, 2008). It is possible that a metabolic hypoxia due to detoxification process increases the anaerobic pathways causing the LDH induction. Our results showed no effect of carbendazim in the activity of CAT, indicating that the antioxidant response was not activated. This agrees with the current knowledge since oxidative stress is not pointed out as a mechanism of action of carbendazim.

In our study, the locomotor response of zebrafish embryos was sensitive to the effects of embryonic exposure to carbendazim as observed by alterations in swimming activity of larvae even at the lowest tested concentration (0.00016 mg/L). Organisms exposed to carbendazim, spent more time swimming, either in the dark as in the light period. However, in both cases the increment of time spent swimming was not translated in an increment of the distance moved (meaning that organisms swam slower). In the light periods, unexpectedly, the total distance moved even decreased, suggesting that fish were not able to swim at their regular speed probably due to energy depletion or energy allocated to other physiological processes to cope with chemical stress or due to developmental impairment in key processes for the locomotion. To our knowledge the only study available concerning carbendazim effects on fish behaviour is the recent study conducted in juveniles of the African sharptooth catfish (*Claria gariepinus*). Fish exposed

to sublethal concentrations of carbendazim (0.22 – 0.43 mg/L) showed abnormal behavioural responses such as spiral swimming, hyperactivity, frequent surfacing to gulp water, jerky movement and loss of equilibrium. The authors attributed this altered behaviour to the toxic action of carbendazim and its effect on the nervous system of exposed fish (Nwani et al., 2015).

Behaviour effects measured as swimming time, are associated in the PCA, to the biochemical parameters ChE, GST and LDH. While ChE is probably involved in apoptose mechanism as suggested above, the GST and LDH, being general biomarkers of chemical and environmental stress, suggest that the metabolic cost involved on detoxification processes may be compromising other important functions such as behaviour including the locomotor response of zebrafish larvae. Results of previous study in fish exposed to copper related the partitioning of energy utilization (between metabolism maintenance and locomotion) to altered fish behaviour (Handy et al., 1999), corroborating this hypothesis.

Changes on fish behaviour may also be caused by disruption of the hormonal system. Many studies have shown the correlations between endocrine disruption and abnormal behaviour in fish (Bell, 2001; Clotfelter et al., 2004; Sárria et al., 2011). Actually, behaviour has been proposed as an endpoint in the environmental risk assessment of endocrine disrupter chemicals (Sárria et al., 2011). As demonstrated by Jiang et al., (2014) carbendazim had the potential to induce endocrine disruption in zebrafish embryo. Their results showed that carbendazim exposure caused down regulation of estrogen receptors and also decreased vitellogenin (used as a biomarker indicator of endocrine disruption) expression at concentrations as low as 0.004 mg/L. The altered locomotor behaviour of zebrafish larvae observed in our study which used a similar range of carbendazim concentrations as Jiang and colleagues (0.004 – 0.5 mg/L), may also be correlated to the effects of carbendazim on the endocrine system. However, our results showed that carbendazim affects behaviour even at lower concentrations (0.00016 mg/L).

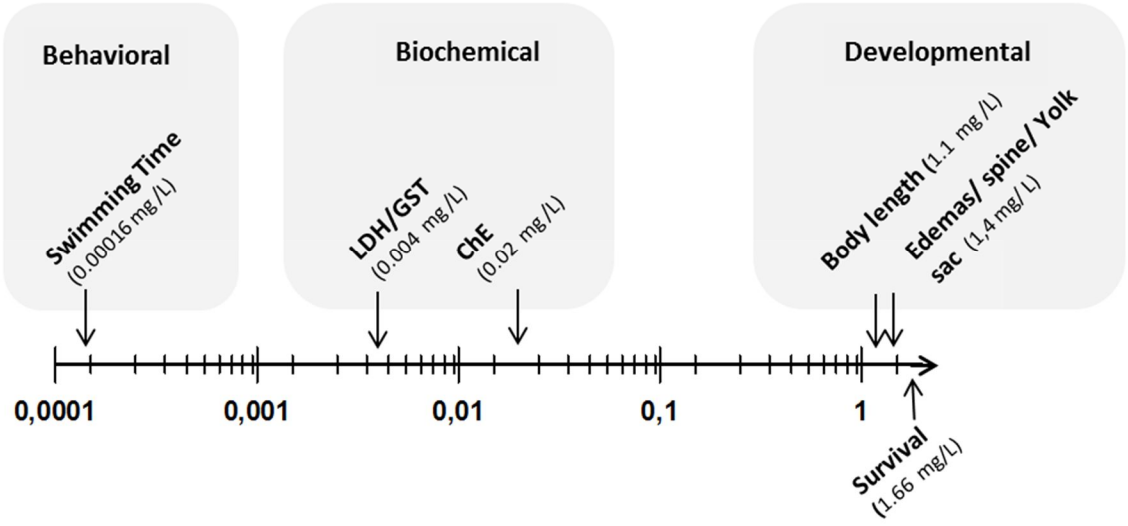

Fig. 5- Diagram comparing the relative sensitivity of endpoints used in the present work to assess effects of carbendazim in *Danio rerio* embryos. Values between brackets are Lowest Observed Effect Concentrations (LOEC).

From an ecological point of view, the alterations in the swimming behaviour (which were observed at environmental relevant concentrations) can have important consequences for the fitness of the organisms as they can further originate feeding disruption (capability to capture prey) and increase vulnerability to predation (through an inability to remain inconspicuous) among other processes which may poses serious risks to the success of fish populations (Little and Finger, 1990). From Fig 5, which summarizes the Lowest Observed Effect Concentrations of the several endpoints analyzed in this work, one can easily observe that behavioural endpoints are several orders of magnitude more sensitive than developmental parameters and thus have the potential to work as an early warning signal for environmental stress. Further studies should focus on understanding how the behavioural disturbances measured translate into fitness impairment at the adult stage.

5. Conclusion

To our knowledge, this was the first study to evaluate the effects of carbendazim at developmental, biochemical and behavioural levels in zebrafish early life stages. Data showed that carbendazim affects embryos survival and development causing a series of anomalies including pericardial edemas, body and tail deformities, decreased heart rate and body length among others. At the sublethal level, carbendazim induced alterations in ChE, GST and LDH activities. And at behaviour level caused increase in swimming times, but not increased swimming distances. Locomotory behaviour showed to be several orders of magnitude more sensitive that developmental parameters or lethality, highlighting the potential of behavioural endpoints as early warning signs for environmental stress. Since behavioural endpoints may translate in ecological relevant effects such as feeding behaviour of antipredatory behaviour disruption, these findings corroborate the importance of the emergent field of behavioural ecotoxicology as a relevant approach in ecological risk assessment strategies.

Acknowledgements

This study was supported by a PhD grant (SFRH/BD/74501/2010) attributed to Thayres Andrade and by the Post-Doc grant (SFRH/BPD/90521/2012) attributed to Inês Domingues by the Portuguese Science and Technology Foundation (FCT), funding by FEDER through COMPETE and Programa Operacional Factores de Competitividade and by National funding through FCT, within the research project Climatox—Impact of climatic changes on toxicity of pollutants (Ref. FCT PTDC/AAG-GLO/4059/2012).

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Supplementary data

Carbendazim exposure induces developmental, biochemical and behavior disturbance in zebrafish embryos

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Index

Table S1: LC gradient for the elution of target compounds.

Time (min)	Mobile phase composition		Flow rate (µL/min)
	water (0.1 % FA)	ACN (0.1 % FA)	
0.00	100	0	300
1.00	100	0	300
7.00	60	40	350
9.00	0	100	400
10.00	0	100	400
10.01	100	0	300
13.00	100	0	300

FA – formic acid

Table S2: Analytical measurement of exposure media of the fish embryos toxicity test and biochemical and behaviour quantification.

	Measured Exposure Concentration			
Nominal exposure concentrations (mg/L)	**0 h (mg/L)**	**0 h (% of nominal concentration)**	**96 h (mg/L)**	**96 h (% of nominal concentration)**
0.00016	0.00099	618	<LOQ	<LOQ
0.0008	0.0008	100	0.0014	175
0.004	0.0156	390	0.0065	163
0.020	<LOQ	<LOQ	<LOQ	<LOQ
0.1	<LOQ	<LOQ	<LOQ	<LOQ
0.5	0.64	128	0.76	152
1.1	1.24	113	1.09	99
1.19	1.22	103	1.47	124
1.3	1.26	97	1.55	119
1.41	1.41	100	1.55	110
1.53	1.70	111	1.50	98
1.66	1.80	108	1.75	105
1.8	1.94	108	1.84	102

< LOQ – Below limit of quantification

Table S3: Summary of models used to calculate concentration-response curves and the respective slope for each endpoint.

Days of exposure	24hpf	48hpf	72hpf	96hpf
LC/EC	**Slope (model)**	**Slope (model)**	**Slope (model)**	**Slope (model)**
Somite formation				
General deformities	-14.3±3.13 (LL.4)			
Heart rate		4.16 ± 0.97 (W1.3)		
Developmental delay		-59.87 ± 68.3 (W1.3)		
Head and eye deformity		-52.2 ± 38.04 (W1.3)	-23.20 ± 4.45 (LL.3)	-29.21 ± 8.78 (LL.3)
Tail deformities			-39.50 ± 9.47 (LL.4)	-19.62 ± 4.05 (LL.3)
Spine deformity		-28.2 ± 12.68 (LL.4)	-23.20 ± 4.63 (LL.3)	-20.45 ± 3.46 (LL. 3)
Edema		-6.25 ± 2.24 (LL.3)	-5.82 ± 1.67 (LL.3)	-10.94 ± 2.18 (LL.3)
Hatching rate			41.00 ± 35.55 (W1.4)	46.62 ± 14.25 (LL.3)
Body length				11.63 ± 2.24 (LL.3)
Yolk sac length				-0.017 ± 0.03 (LL.4)
Survival		42.68 ± 105 (W1.3)	-17.5 ± 80 (W1.3)	-12.79 ± 3.62 (LL.3)

LL.3 - Log-logistic three parameters
LL.4 - Log-logistic four parameters
W1.3 - Weibull three parameters (type 1) with lower limit at 0
W1.4 - Weibull four parameters (type 1)

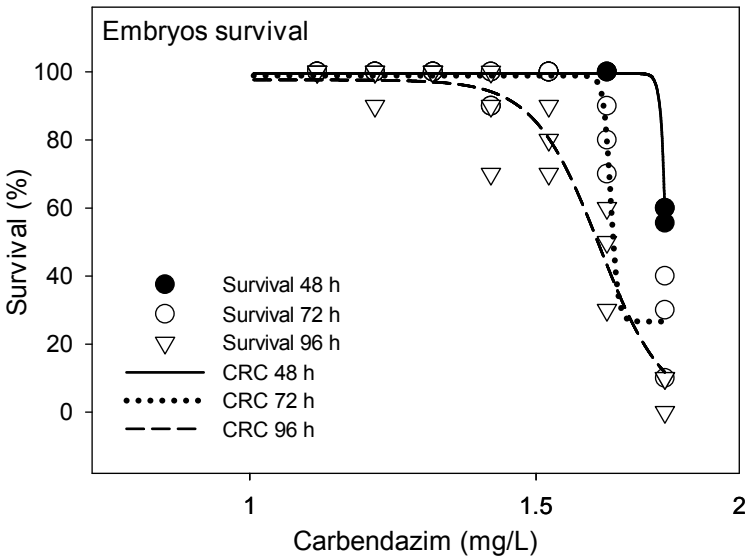


Fig.S1 - Concentration response curves (CRC) for zebrafish embryos survival after 48, 72 and 96 h of exposure to carbendazim.

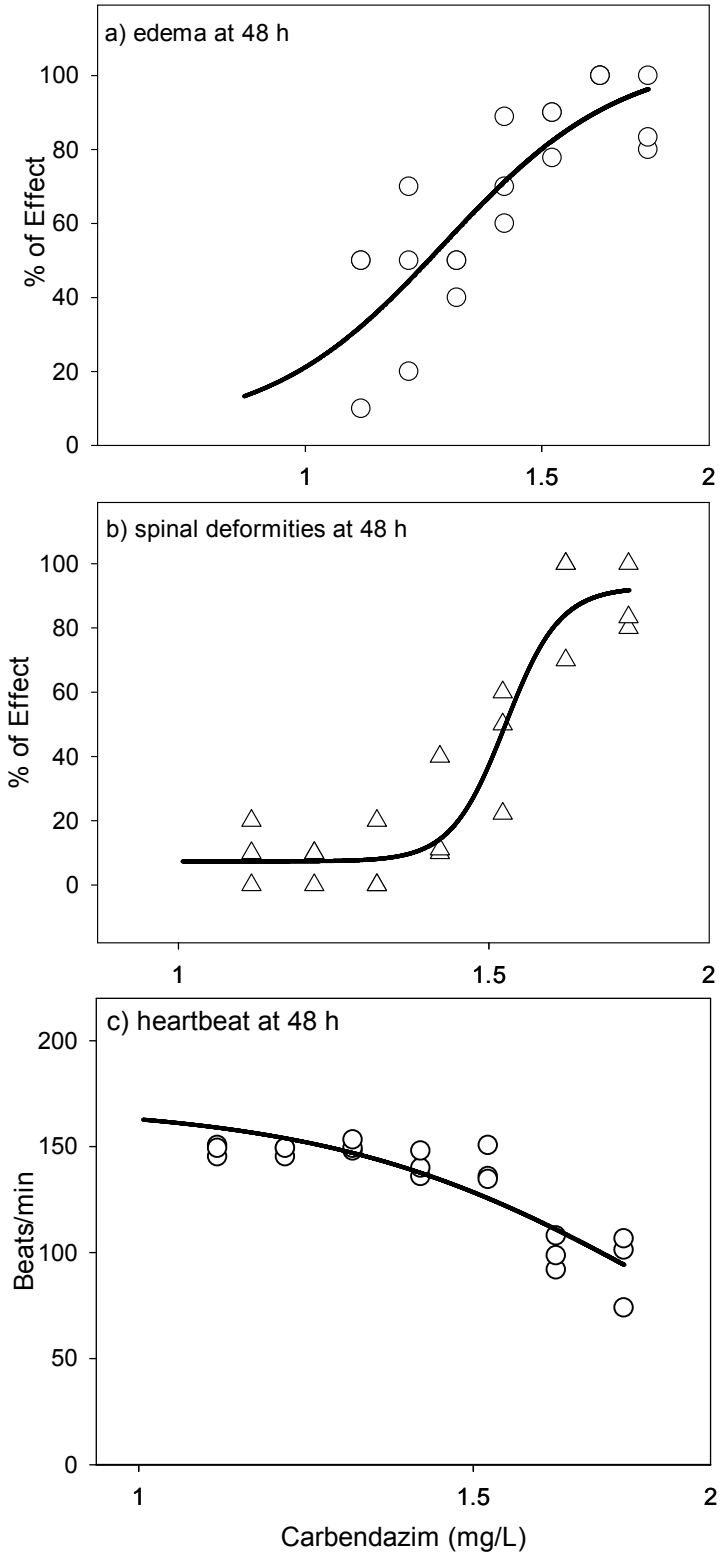


Fig. S2 - Overview of the effects of carbendazim on zebrafish embryos at 48 hours of exposure: a) incidence of edema; b) incidence of spinal deformities; and c) heart rate of exposed embryos.

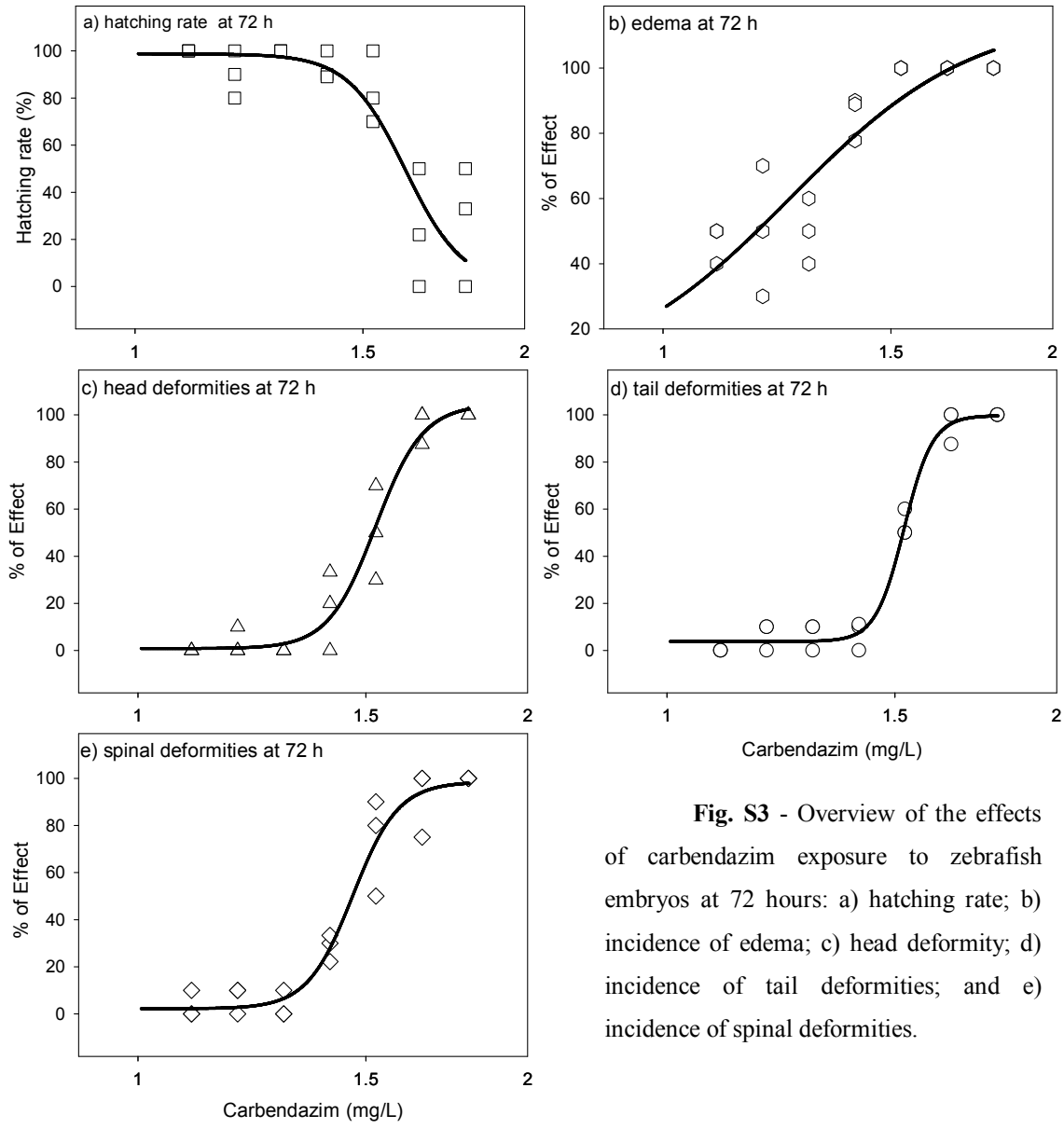


Fig. S3 - Overview of the effects of carbendazim exposure to zebrafish embryos at 72 hours: a) hatching rate; b) incidence of edema; c) head deformity; d) incidence of tail deformities; and e) incidence of spinal deformities.

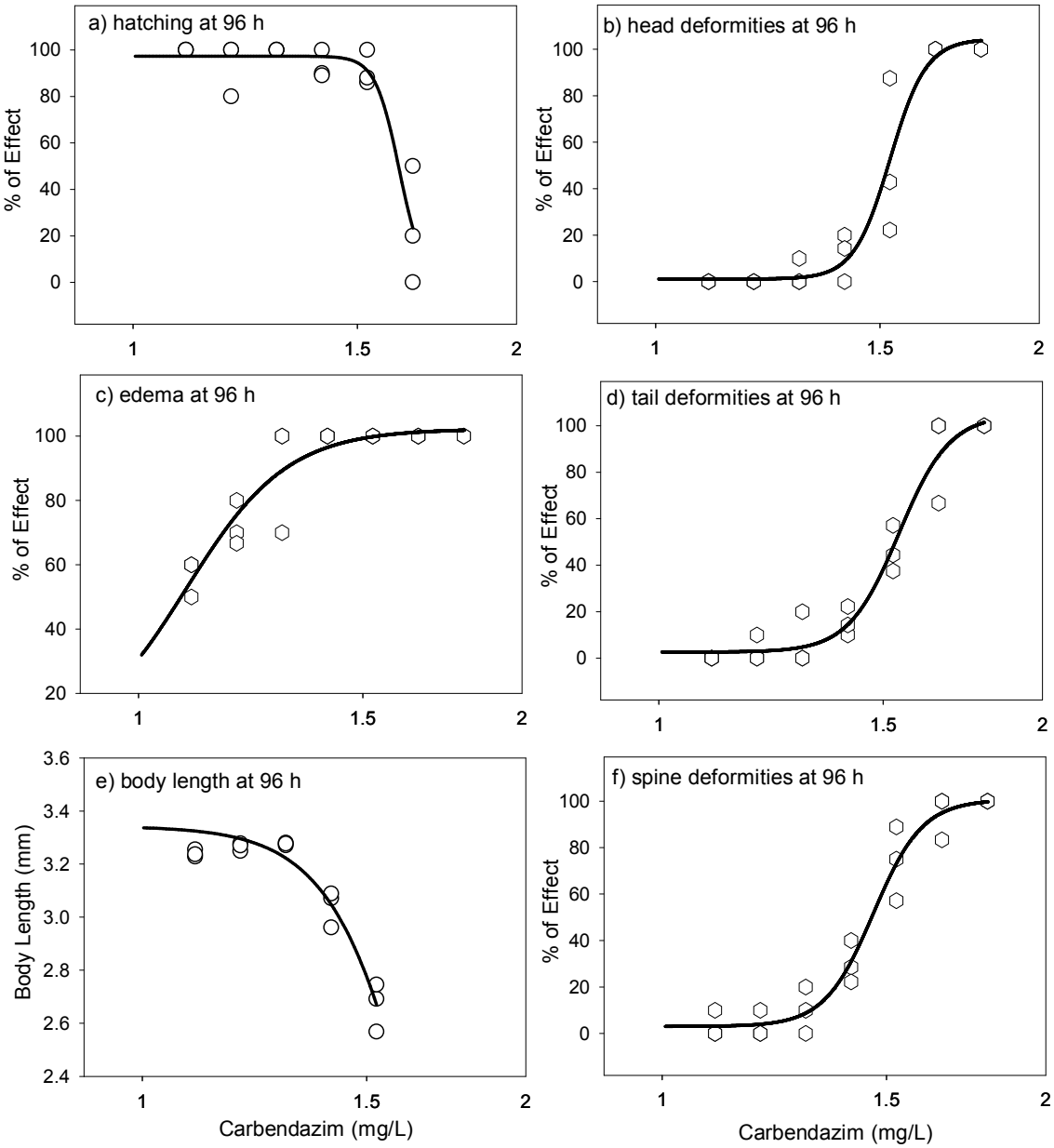
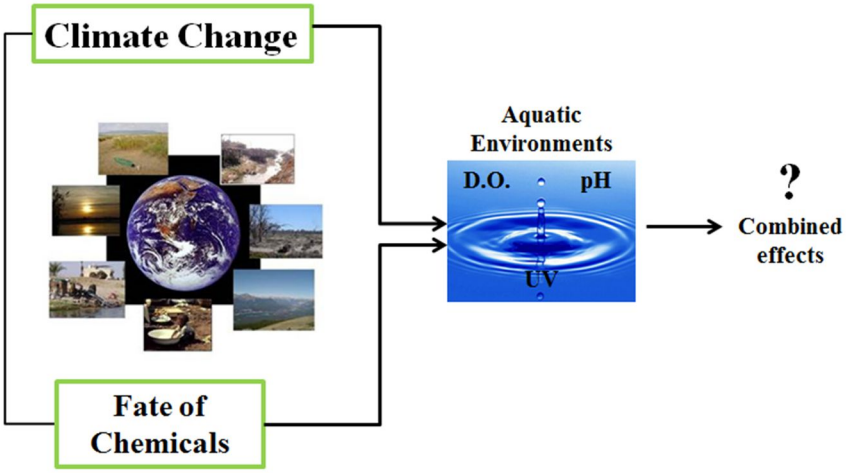


Fig. S4 - Overview of the effects of carbendazim exposure to zebrafish embryos at 96 hours: a) hatching rate; b) head deformity; c) incidence of edema; d) incidence of tail deformities; e) body length; and f) incidence of spine deformities.

Chapter 6

Influence of pH on the toxicity of carbaryl



Influence of pH on the toxicity of carbaryl to zebrafish early life stages

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Abstract

There are strong evidences that the climate is globally changing. These changes are expected to modify water quality mainly by alterations in environmental parameters such as temperature, pH, and dissolved oxygen. Slight shifts in these environmental factors may compromise the physiological performance of aquatic organisms and their capacity of response to additional stress. In natural systems, organisms are very often are forced to cope with environmental and chemical stress simultaneously. Understanding how the environmental changes may determine the physiological response of aquatic life and interact with contaminants, must be considered in risk assessment. Therefore, in the present study, we aimed at assessing the joint effects of pH (acid and alkaline) and carbaryl to zebrafish embryos.In ecotoxicology, the prediction of combined effects is normally based on the effects of the individual components of the mixture by using the well-known pharmacological concepts of concentration addition (CA) and independent action (IA). The toxicity of the binary combination was determined using the fish embryo toxicity test with zebrafish. Results showed that for the acid range the observed mixture toxicity was rather well predicted by CA. The median lethal concentration (LC_{50}) of the mixture was predicted with an error of only 8%. Considering the alkaline range, both concepts underestimate the LC_{50} by a factor of 3 for CA and by a factor of 4.6 for IA. The increased toxicity of the combination of alkaline pH and carbaryl was likely due to the hydrolysis of carbaryl at elevated pH values generating the more toxic product 1-naphthol. Our results highlight the necessity of incorporating the effects of environmental factors such as pH in risk assessment procedures, in order to avoid underestimation of effects and adequately protect aquatic organisms.

Keywords: carbamate pesticides, *Danio rerio*, concentration addition, independent action, mixtures.

1. Introduction

Currently, for the risk assessment of chemicals, aquatic toxicity tests are conducted according to established standard protocols where physicochemical – temperature, pH and etc. – conditions are maintained stable according to requirements for a given test species. Moreover, the risk management primarily focuses on the assessment of single/individual stressors while in the environment organisms are rather exposed to multiple mixtures of stressors (Altenburger and Greco, 2009). Furthermore, the problematic of climate change and global warming raises the idea that organism and biological systems may have to cope with multiple stressors including nonchemical adverse factors such as unfavorable pH, temperature and salinity in combination with chemical stress.

pH is one of the most determinant parameters for any chemical reaction and has been pointed out as the most important factor conditioning survival and fitness of many freshwater species. Variations in pH showed to have a significant impact on survival, hatching success, reproduction, pigmentation, swimming performance behaviour and body chemistry of both fish and invertebrate aquatic species (Fromm, 1980; Haines, 1981; Havas and Rosseland, 1995; Ikuta et al., 2000; Jordahl and Benson, 1987; Lechleitner et al., 1985; Okland and Okland, 1986; Ye and Randall, 1991). The effects of pH on the toxicity of chemical stressors have also been explored in the past decades, however, the majority of studies focuses mainly on metals and phenols toxicity (Bervoets and Blust, 2000; Dave, 1985; Dietrich and Schlatter, 1989; Grosell et al., 2006; Reader et al., 1989; Stouthart et al., 1996). Nevertheless, the toxicity of pesticides is also affected by pH (Mayer and Ellersieck, 1986) and only few studies evaluating the combined effects of these two stressors are available.

Mayer & Ellersieck (1986) reviewed the effects of pH and other physicochemical factors on chemical toxicity and reported that of any toxicity modifying factor tested, pH caused the greatest average change in chemical toxicity (in 96 h-LC50's). According to the studies reviewed, the toxicity of pesticides tended to increase due to the formation of more toxic hydrolysis products at high pH as in the specific case of mexacarbate zectarn, which was found to be 38 times more toxic at pH 9.5. Therefore, the effects of physicochemical factors such as pH, should be considered in hazard assessment in order to assess risk accurately and protect aquatic biota avoiding under or overestimation of effects. Within

this context, the present study aimed at investigating the influence of pH (acid and alkaline) on the toxicity of carbaryl to zebrafish embryos.

In aquatic toxicology, our current understanding of mixture toxicity is adopted from pharmacology and it is based on the concepts of concentration addition (CA) (Loewe and Muischnek, 1926) and independent action (IA) (Bliss, 1939). These two concepts are based on the assumption that the composition of the mixture is known and are capable of predicting the toxicity of the mixture from the toxicity of the individual components (Altenburger and Greco, 2009). The concept of CA has been introduced by Loewe & Muischnek (1926) and is based on the assumption that the components of a given mixture act in the same way, by the same mechanisms (Groten, 2000). CA is mathematically expressed as:

$$\sum_{i-1}^{n} \frac{c_i}{ECx_1} = 1 \qquad (1)$$

where n is the number of mixture components, ECx_i is the concentration of the ith mixture component that provokes x% of effect when applied singly, and c is the concentration of the respective component in the mixture. In contrast, the concept of IA is based on the idea that the compounds of a given mixture/combination have dissimilar modes of action and is mathematically expressed as follows:

$$E(c_{Mix}) = E(c_1 + \cdots + c_N) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] \qquad (2)$$

where $E(c_{Mix})$ denotes the predicted effect (scaled from 0 to 1) of an n-compound mixture, c_i is the concentration of the ith compound, and $E(c_i)$ is the effect of that concentration if the stressor is applied singly.

Both concepts have been extensively used to study binary and multiple mixtures of various chemicals, using different organisms and endpoints demonstrating their predictive power (Altenburger et al., 2000; Backhaus et al., 2000; Deneer, 2000; Kortenkamp, 2007). For example, in the study of Altenburger et al., (2000), CA has been successfully used to predict the toxicity of 16 similarly and specifically acting chemicals and showed to have an

excellent predictive power. Likewise, independent action showed to have an excellent predictive power when used to predict the toxicity of chemicals with dissimilar modes of action (Backhaus et al., 2000; Faust et al., 2003). In this study, these two concepts were used to predict the combined toxicity of pH (acid or alkaline) and carbaryl to zebrafish embryos and to detect deviations to the expected toxicity.

2. Materials and Methods

2.1 Test organisms

All the organisms (zebrafish embryos) used in this study were obtained from the zebrafish facility established at the Department of Biology, University of Aveiro (Portugal). Adult zebrafish (*Danio rerio*) were maintained in a ZebTEC (Tecniplast, Buguggiate, Italy) recirculating system. Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with 0.34 mg/L salt ("Instant Ocean Synthetic Sea Salt", Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was 26.0 ± 1 ºC, conductivity 750 ± 50 µS, pH 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd) and brine shrimp. Eggs were obtained by breeding of fish in aquaria. The eggs collected were rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). Eggs with cleavage irregularities, injuries or other kind of malformations were discarded.

All tests were performed similar as described in the OECD testing guideline 236 (OECD, 2013), in charcoal filtered and deionised water supplemented with 0.34 mg/L sea salt (see above) at 26 ± 1 ºC and a 16:8 h (light:dark) photoperiod. Exposure was conducted from 3 to 96 hpf.

2.2 pH effects on zebrafish embryos

In order to derive concentration response curves, zebrafish embryos were exposed to acid pH ranging from 3 to 7 (0.1 to 1000 µM of H_3O^+) and to alkaline pH ranging from 8 to 12 (1 to 1000 µM of OH^-). A set of buffers in concentrations ranging from 17- to 64-fold below concentrations that cause mortality (Fig S1, Supplementary Data ('S' before the number indicates that the figure or table respectively is provided in the 'supplementary information')) were used to prevent pH drifts during the assay (Table S1). HCl and NaOH (1.0 N) solutions were used for pH adjustment; a portable multiparameter device (ProfiLine Multi 332) was used for pH measurements. Test solutions were daily renewed. Embryos were exposed individually in 24-well plates for 96 h. For each test 10 embryos were used per replica and a minimum of 3 replicates were used per treatment. Survival was observed and registered daily until the end of the test.

2.3 Carbaryl effects on zebrafish embryos

To also derive concentration response curves for carbaryl, zebrafish embryos at 3 hpf were exposed to eight concentrations of 1, 1.6, 2.6, 4.3, 7.0, 11.4, 18.5 and 30 mg/L. Ten eggs per treatment were distributed in 24-wells microplates in triplicate and run for 96h. Embryos were observed daily under a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation, Japan). Survival was evaluated daily until the end of the assay at 96 h.

2.4 Combined exposure to carbaryl and pH

Once the LC_{50} values were calculated for the single exposures (pH and carbaryl separately), the mixture experiments were conducted following a fixed ratio design. For this, pH was converted into concentrations of ions H_3O^+ for acid and OH^- for alkaline range. Therefore, the two stressors were mixed in a ratio corresponding to 50:50% of effect. The mixture ratio was kept constant and the total concentration of the mixture was

varied in order to describe experimentally the complete concentration-response relationship of the mixture. For acid range only the MES buffer (4 mM) was used to avoid pH drifts likewise, for basic range, only CAPS buffer (22 mM) was used (please refer to Supplementary data). We aimed at using one buffer for the acid and one buffer for the basic pH range - albeit the buffers may have a limiting buffer capacity in part of the pH range (in order to avoid drifts, the test solutions were daily renewed). The advantage of using one buffer is that in mixture analysis and modelling only one buffer component would need to be considered. In order to discard any interference of the buffers on the combinations of acidic or alkaline pH and carbaryl, the tests were repeated using buffers concentrations 10 times higher (40 mM for MES and 220 mM for CAPS). Results are presented in the supplementary data Figs S2. The zebrafish embryos to the combinations followed the described in section 2.2 (*pH effects on zebrafish embryos*).

2.5-1-naphtol effects on embryos survival

1-naphthol is the major hydrolysis product of carbaryl. As it has been proved that carbaryl is highly susceptible to hydrolysis, we also tested the effects of 1-naphthol in zebrafish embryos in order to better understand the combined effects of pH and carbaryl. Zebrafish embryos at 3 hpf were exposed to 1-naphthol concentrations ranging from 0.38 to 30 mg/L following the same procedure described in the section 2.3 (*Carbaryl effects on zebrafish embryos*).

2.6 Chemical analysis: analysis of carbaryl concentrations at different pH's

In order to check the stability of carbaryl under acidic and alkaline pH, chemical analysis of the exposure medium were carried out. Ten millilitres of each combined treatment were sampled at 0 and 24 h and preserved at -20 ºC until chemical analysis. The analyses were performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in České Budějovice, Czech Republic. The results are presented in supplementary data (Table S2).

2.7 Data analysis

Buffer LC_{50}s could only be calculated for CAPS and CHES. For all other buffers tested the low mortality rates up to concentrations at the solubility limit did not allow obtaining LC_{50} values. For these buffers an ANOVA (one-way analysis of variance) with appropriate post hoc test (Dunnett's or Dunn's test) were conducted to potentially derive LOEC or NOEC values. The type of ANOVA (parametric or non-parametric) and post hoc test was chosen depending on whether normality and homocedasticity of data were demonstrated by analysis of the residuals with the Shapiro-Wilks test.

The concentration-response relationships of the single stressors as well as of the combinations were obtained by fitting dose-response curves using the software SigmaPlot V.12.5 (SysStat, San Jose, California,USA). Model choice decision was made based on the the R2 and the estimated residual standard error. The concentration response relationships as well as the statistics and analysis of normality were conducted using the software SigmaPlot V.12.5 (SysStat, San Jose, California,USA) and a significance level of 0.05.

2.7.1 Prediction of mixture toxicities

For the prediction of mixture toxicity the two well-known conceptual models CA and IA were used. The calculation of the mixture effects followed the procedures described in Backhaus et al 2000 and Altenburger et al 2000. To calculate the predictions by CA model, equation 1 was used as a starting point. As the ratio of the mixture components (H_3O^+/OH^- and carbaryl) is quantitatively known, the concentration of each component can be expressed as a fraction of the total concentration (p_i). Consequently, by rearranging Equation 1, the effect concentrations predicted by CA can be calculated as follows:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \quad (3)$$

Where ECx_{mix} is the total concentration of the mixture provoking x% of effect and p_i denotes de fraction of component i in the mixture. Using Equation 3, the total concentrations of each mixture giving 1.5 to 99% effect were calculated and the resulting concentration/effect pairs were connected by straight lines, providing a visualization of the predicted concentration-response curve.

To calculate the mixture effects according to independent action, Equation 2 was rearranged so that the concentration response relationships F_i of the two individual components of the mixture could be used to calculate their effects $E(c_i)$ as follows:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] = 1 - \prod_{i=1}^{n}[1 - F_i(c_i)] \quad (4)$$

Again, the individual components of the mixture can be expressed as fractions, p_i, of the total concentration, c_{mix}, therefore, the overall effect of the mixture concentration can be calculated by rearranging Equation 4 as follows:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n}[1 - F_i(c_i)] = 1 - \prod_{i=1}^{n}[1 - F(p_i . c_{mix})] \quad (5)$$

Using equation 5, the total concentration of the mixture provoking up to 100% of effect was calculated. The calculated concentration/effect pairs were connected by straight lines, providing a graphical representation of the predicted concentration response relationship of the mixture predicted by independent action. To calculate an arbitrary effect concentration $EC_{X_{mix}}$ according to independent action, Equation 5 can be rewritten and under the condition that total effect $E(c_{mix})$ equals $x\%$, c_{mix} is defined as

$EC_{X_{mix}}$,therefore, the overall effect of any given total mixture concentration can be calculated as:

$$x\% = 1 - \prod_{i=1}^{n}(1 - F_i\left(p_i\left(EC_{X_{mix}}\right)\right) \quad (6)$$

Equation 6, solved iteratively for a c_{mix} that provokes an effect of $x\%$, implicitly provides a prediction of effect concentrations of a mixture under the hypothesis of independent action.

3. Results

3.1 Stability of carbaryl at acidic and alkaline pH

In order to check the stability of carbaryl, chemical analyses were performed for the whole tested pH range. The analysis of the exposure media for acid condition showed stable exposure concentrations, remaining within 80 – 120% of the nominal concentrations with only two exception at pH 3.7 (129% of nominal concentration) and pH 4 (131% of nominal concentration). However, the measured concentrations were far below the nominal ones varying from 0.002 to 32% of the nominal concentrations. At pH above 9.5 the nominal concentrations could not be detected been below the limit of quantification (Table S1, Supplementary data).

3.2 Single stressors toxicity

Clear dose-response relationships were observed in the single exposure experiment as can be observed in Fig. 1. The toxicities of each stressor are summarized in Table 1. The pH effects were tested for both acidic and alkaline conditions (pH 3-7 and pH 7-12) Embryos exposed to pH below 3.5 or above 10.5 showed a 100% of mortality. The observed lethality was established within 24 hours and did not increase with prolonged exposure. For the acidic range, a 96 h-LC_{50} value of 225 ± 16.6 µmol/L was determined

and for the alkaline range, a 96 h-LC_{50} of 160 ± 23.6 was calculated (Table 1). For carbaryl a time and dose dependent relationship was observed with a 96 h-LC_{50} of 14.9 mg/L (Fig.1, Table 1).

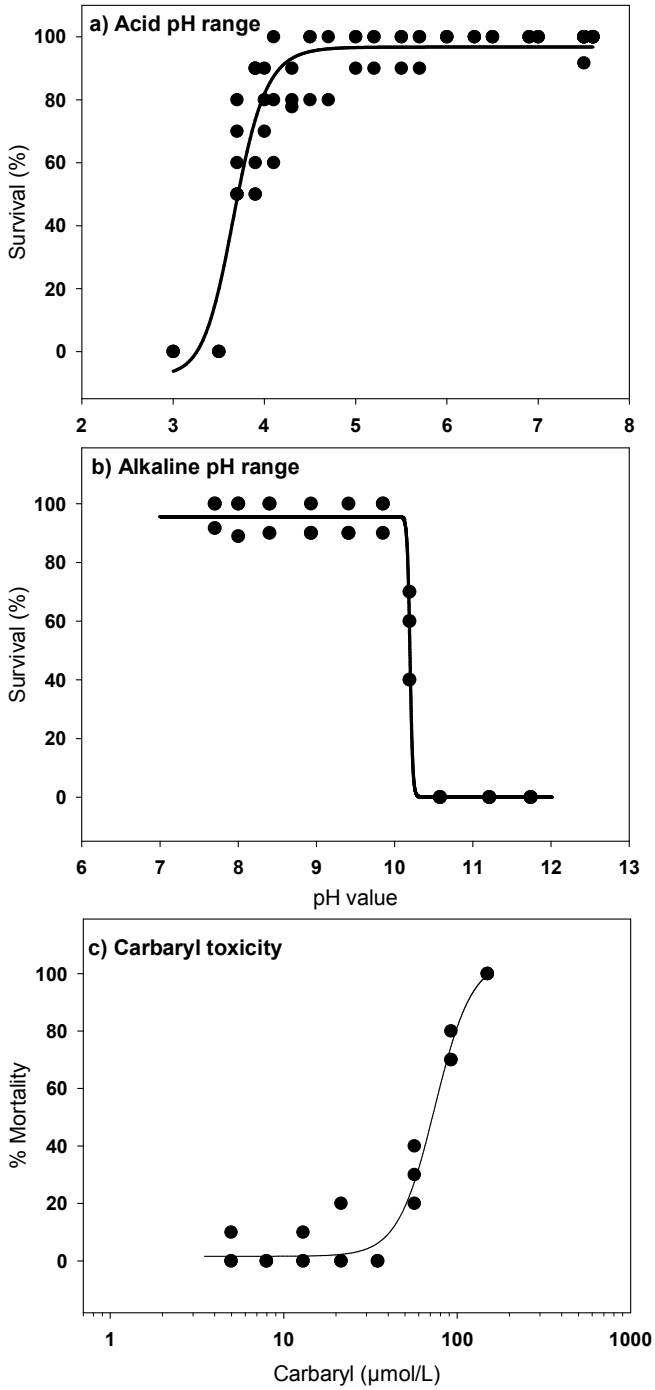

Fig 1 - Concentration-response curves of the single stressors experiments at 96 hpf: a) acid pH; (b) alkaline pH and (c) carbaryl.

3.3 Combined experiments

As stated in the methodology section, in order to discard any interference of the buffers used to stabilize the pH on the combined toxicity tests, two assays were conducted increasing 10 times the concentrations of the buffers used for acid and alkaline range. Fig. S2 shows the observed toxicity for acid range (MES concentration of 40 mM) and for alkaline range (CAPS concentration of 220 mM). When compared with the toxicities of assays using the lower buffer concentration (concentrations proved to have no toxicity to embryos) a slightly difference on the toxicity of 34% for MES and 35 % for CAPs was observed. Moreover, the concentrations used of 4 mM and 22 mM are far below the ones that pose risk to zebrafish embryos as demonstrated by the toxicity test conducted for each buffer used in the combined experiments (Table S1, supplementary data). For MES, the concentration of 4 mM is 64 fold less toxic than the established NOEC and for CAPS, the concentration of 22 mM is 17 fold less toxic than the LC_{50}.

Table 1 – Parameters values of concentration-response relationships of the single and combined experiments

	LC_{50} (µmol/L)
Toxicity of the single mixture components	
Acid pH	225 ± 16.6
Alkaline pH	160 ± 23.6
Carbaryl	74.3 ± 3.8
Toxicity of the Mixture	
Acid pH x Carbaryl – Observed effects	139 ± 31.0
Predicted CA	150 ± 8.9*10^{-2}
Predicted IA	222 ± 1.5
Alkaline pH x Carbaryl – Observed effects	42.1 ± 0.77
Predicted CA	124 ± 0.12
Predicted IA	192 ± 0.51

The results of the combined toxicity of pH (acid or alkaline) and carbaryl as well as the predictions made by CA and IA are shown in Fig. 2. Table 1 summarizes the concentration-response relationships for mixture predictions and experimental determinations. For both pH ranges CA predicts higher mixture toxicity than IA as can be observed in Fig. 2. However, there is only a factor of ~1.5 between the LC_{50}s predicted by CA (150 µmol/L for acid and 124 µmol/L for alkaline pH) and those predicted by IA (222 µmol/L for acid and 192 µmol/L for alkaline pH).

As can be seen from Fig 2a in the case of acid range, the mixture toxicity is rather precisely predicted by CA. The observed LC_{50} for the combination was 139 µmol/L, which is only a difference of 8% (a factor of 1.07) in relation to the predicted value of 150 µmol/L. In contrast the IA underestimates the combination of acid pH and carbaryl at the LC_{50} level. The LC_{50} predicted by IA was 222 µmol/L which is a difference of 60% (a factor of 1.6) compared to the observed toxicity.

Regarding the alkaline range, Fig 2b shows that both concepts underestimate the combined toxicity. The observed LC_{50} for the combination was 42 µmol/L which is a difference of 200% (a factor of 3.0) regarding the LC predicted by CA and a difference of 356% (a factor of 4.6) regarding the one predicted by IA.

Overall, both concepts (CA better than IA) adequately estimated the joint-effects of acid pH and carbaryl if the components were mixed in the ratio of their respective LC_{50}s. However, for the combination of alkaline pH and carbaryl, an increase in the toxicity was observed due to the higher toxicity of the degradation product of carbaryl 1-naphthol.

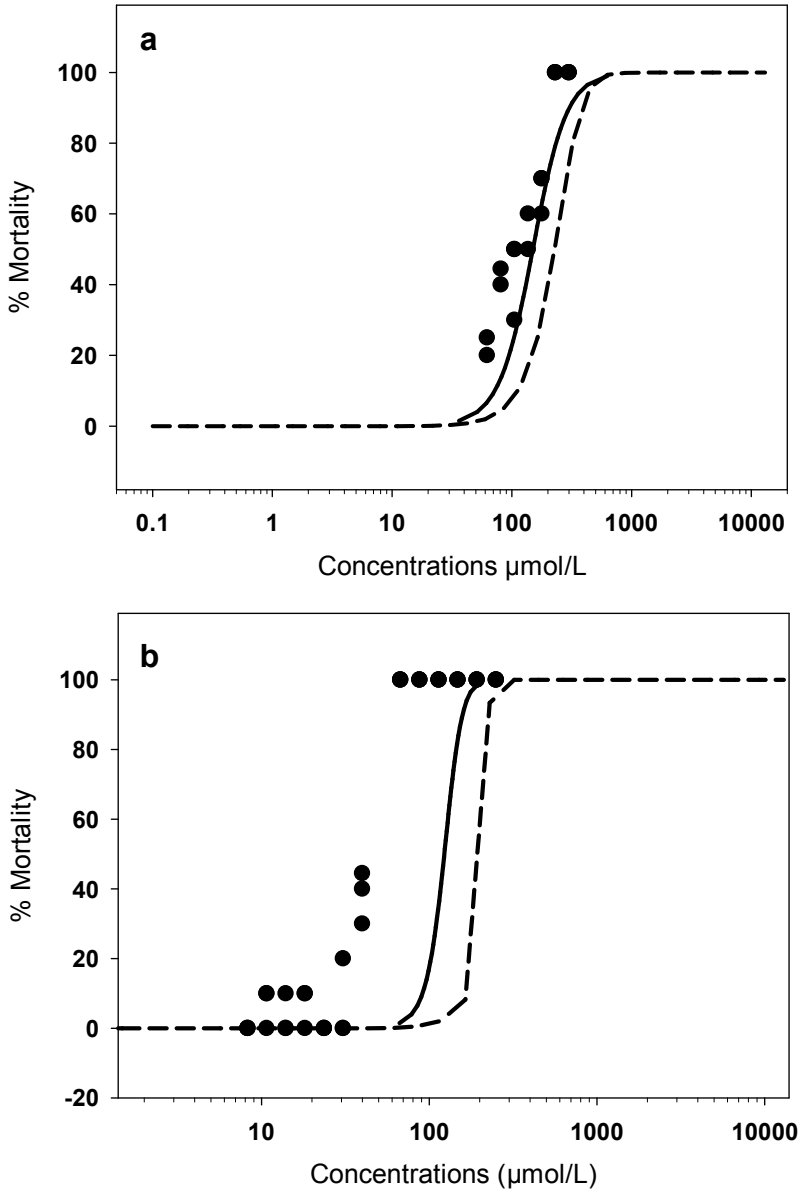

Fig 2 - Predicted and observed mixture toxicity. a) mixture ratio for acid pH; b) mixture ratio for alkaline pH. ——, prediction according to concentration addition; - - - - , prediction according to independent action.

4. Discussion

Extreme pH values showed to significantly impact zebrafish embryos survival. Generally our data are in good agreement with previous studies on fish toxicity, where pH

values below 5 and above 10 demonstrated to decrease fish survival (Dave, 1984; Jordahl and Benson, 1987; Kaur and Toor, 1980; Le Louarn and Webb, 1998). The detailed toxicity profile of the entire pH range is subject of a separate chapter (chapter 3). The insecticide carbaryl showed to be moderately toxic to zebrafish embryos. The toxicity of carbaryl has been studied in several fish species including zebrafish where LCs varied from 5.3 to 44.66 mg/L (De Mel and Pathiratne, 2005; Lin et al., 2007; Mahboob et al., 2014; Schock et al., 2012). As for pH, the detailed toxicity profile of carbaryl is also subject of a separated publication (chapter 4).

The joint effect of carbaryl and acid pH was correctly predicted by the CA model as demonstrated in Fig. 2a. pH in the acidic range seems not to be a determinant factor on the toxicity of carbaryl. This has been shown by other studies investigating the effects of pH on the toxicity of carbaryl in invertebrates and amphibians. In the study of Lohner & Warwick Fisher, (1990) acid pH had minimal effects on the toxicity of carbaryl to the midge *Chironomus riparius*. Similarly, the study conducted by Relyea (2006) demonstrated that a decrease in pH from 8 to 6 had no effect on survival of *Rana catesbeiana* and *Rana calmitans*. Although these two stressors do not have the same mode of action, it seems that CA may quite precisely describe the combined effects of carbamate insecticides and acidic pH, probably due to the stability of carbaryl under these conditions (see Table S2).

Regarding the alkaline pH range, both concepts clearly underestimated the toxicity of the combination at the ratio of LC_{50} as can be observed in Fig 2b. This strong deviation from the predicted toxicity is probably due to the higher toxicity of the main degradation product of carbaryl. The chemical analyses done (please refer to Table S2 in the supplementary data) have demonstrated that carbaryl undergo rapid degradation under alkaline pH confirming the findings of Aly and El-Dib (1971) which showed that carbaryl is susceptible to hydrolysis at alkaline pH with a half-life of only 2.5 h at pH 9. As 1-naphthol is the main degradation product a toxicity test was conducted with this compound to assess if the observed deviation in the toxicity could be due to the higher toxicity of 1-naphthol when compared to carbaryl. The Fig S3 (supplementary data) shows the concentration-response curve for zebrafish embryos exposed to 1-naphthol (96 h-LC_{50} 52 µmol/L) which proved to be 42% more toxic than carbaryl (96 h-LC_{50} 74.3 µmol/L). The effects of alkaline pH on carbaryl toxicity have also been explored in the past in different

experimental designs without the use of any approach for the calculation of the expected combinatory toxicity. Woodward & Mauck, (1980) investigated the effects of insecticides to the Cutthroat trout (*Oncorhynchus clarkii*) and found that an increase in pH from 6.5 to 8.5 was responsible for an increase in the toxicity of carbaryl by a factor of 5 and also for other carbamate insecticide aminocarb by a factor of 20. In another study also conducted with carbamate insecticide Mauck et al., (1977) demonstrated that at pH 9.5 mexacarbate (zectran) toxicity increased 38 times to the bluegills (*Lepomis macrochirus*). The authors attributed this high toxicity at alkaline pH to the breakdown product of zectran, 4-amino-3,5-xylenol that was 70 times more lethal to bluegills than mexacarbate. From our results and from the literature, it seems that the impact of alkaline pH on the toxicity of carbamate insecticides is primarily due to hydrolysis of the compounds into more toxic products.

As observed by our study, changes in environmental factors such as pH may enhance the acute toxicity of pesticides and underestimate their effects to aquatic biota. Some recent reviews have highlighted the importance of the possible interactive effects of environmental stressors and chemical pollution (Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010; Noyes et al., 2009). Heugens et al., 2001 reviewed the combined effects of stressful environmental conditions (temperature, nutritional state and salinity) and various classes of chemicals including pesticides. They concluded that in general the toxicity increased with increasing temperature and decreasing nutrient supply. In other review conducted by Laskowski et al., 2010, natural environmental factors showed to significantly modify the effects of toxicants on the tested organisms and the authors argued the necessity of incorporating natural stressors in ecological risk assessment.

Pesticides and environmental factors can "interact" in a variety of ways; natural factors can, for example, change the physicochemical properties of the pesticides modifying the adsorption, desorption, volatilization and degradation rates and directly influencing their bioavailability (Heugens et al 2001). Toxicokinetics may also be influenced by environmental factors through alterations in the uptake or detoxification rates (Løkke et al., 2013). Furthermore, the combined effects of natural stressors and toxicants can also affect the physiological state of organisms especially when the natural stressor reaches extreme levels such as extreme acidic and alkaline pH (see compilation of examples in Heugens et al 2001, Holmstrup et al 2010 and Laskowski et al 2010). This is particularly important when applying mixture concepts such as CA and IA for the

prediction of combined effects of environmental factors and chemicals. Neither CA nor IA relates to biochemical and physiological process of exposed organisms, or consider physicochemical characteristics of chemicals pollutants (Backhaus and Faust, 2012). However, these concepts have been successfully applied to investigate the combined effects of natural stressors and chemical pollutants (Jonker et al., 2005).

CA seemed to be the best model to predict the combined effects of pH and carbaryl. This model has proven to provide good to excellent predictions of mixture toxicities regardless the similarity or dissimilarity of the modes of action of the mixture components. Belden et al., (2007) reviewing the effects of mixture of pesticides concluded that for 88% of the cases CA model had observed effective independent of the mode of action of the mixture components. On the other hand, for mixtures with different modes of action, they observed that IA was more accurate than CA; however, in most cases the differences between the two models were relatively small. Irrespective of the mode of action, CA has empirically shown to provide a more conservative combined effect estimations (Faust et al., 2003) and has been proposed as the general solution for mixture toxicity analysis even though the mechanisms of action are unknown (Backhaus and Faust, 2012; Backhaus et al., 2000; Berenbaum, 1985).

Nonetheless, when using either CA or IA for prediction of the combined toxicity of environmental factors and chemical stress this may not always be the case. In this type of combination these concepts must be used with caution and knowledge of physicochemical properties of a given chemical seems to be crucial for an accurate prediction of effects (at least for abiotic factors known to modify physicochemical properties of chemicals). For the risk assessment of binary and multiple mixtures of chemicals (with specific and unspecific modes of action) many scientific concepts have been developed and validated for use (Altenburger and Greco, 2009; Backhaus and Faust, 2012). However, when dealing with combination of chemical and nonchemical stress only few approaches are available but are not ready yet for extrapolation for risk assessment practice as pointed out by Altenburger and Greco (2009). There is an urgent need to improve our understanding of the effects of environmental factors and chemical pollutants as the rapid climate change process is already altering structure and function of natural ecosystems and also impacting the fate and behaviour of chemical pollutants.

In this regard, the joint-effects of pH and carbaryl observed in this study raise the concern towards the risk assessment of pesticides. The potential toxicity of carbaryl is likely to be underestimated by established procedures where pH is held constant. Although in the climate change context such extreme scenario (alkaline pH) may be exception rather than the rule, our study - focusing in the lethal effects only – provide a first approach for effects screening. The use of sublethal endpoints would further give a more comprehensive understanding of the combined effects eventually detecting effects at pH levels more realistic in terms of ecological relevance.

5. Conclusion

We have shown that environmental factors such as pH can modify the toxicity of the pesticide carbaryl. The conceptual model CA allowed a precise prediction of the toxicity of the joint-effects of acid pH and carbaryl. Nevertheless, for alkaline condition both concepts failed in predicting the effects. Deviations to the model were however easy to explain as high pH values favour the hydrolysis of carbaryl with the consequent formation of the more toxic degradation product 1- naphthol. Although in the present study such explanatory process was easy to establish, for many other combinations the "interactive" nature is not so evident. In the context of the climate change few scenarios predict such increase in the pH of aquatic systems, however this was a first approach focused in the lethal effects only. In a second tier assessment effects at sublethal level would be sought and it is expectable that more subtle pH changes (more realistic in terms of climate changes scenarios) may also have an effect at physiological and biochemical levels with possible long term consequences for the population fitness.

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Supplementary data

Influence of pH on the toxicity of Carbaryl to zebrafish early life stages

Thayres S Andrade[1], Jorge F Henriques[1], Rita Almeida[1], Stefan Scholz[2], Amadeu M.V.M. Soares[1,3] and Inês Domingues[1]

The supplement provides additional data on the toxicity of acidic and alkaline pH, as well as for the combinatory effects of carbaryl and pH to zebrafish embryos. The effects of the buffers MES, MOPS, TRIS, CAPS and CHES on embryos survival are shown to indicate that the buffer concentrations selected neither compromise the analysis of pH effects (Table S1, Fig. S1) nor the combined effects. Table S2 shows the results of chemical analysis. Fig. S2 shows the predicted and observed effects of the combination of pH and carbaryl for both pH ranges increasing ten times buffer concentrations in order to exclude any possible effects of the buffer in the mixture. Fig S3 shows the concentration response curve for 1-naphthol, the main degradation product of carbaryl.

INDEX

Table S1 – Buffers used for pH stabilization: concentrations used, respective pH range and toxicity data.

Buffer	Concentration used in test (mM)	pH range	NOEC	LC_{50}
No buffer #	-	3.0-3.5	-	-
MES- 2-(Morpholinoethanesulfonic) acid monohydrate	4	3.7-6.2	256 mM*	*n.d.*
MOPS- 3-(N-Morpholino)propanesulfonic acid	4	6.9-7.5	256 mM*	*n.d.*
TRIS- 2-Amino-2-(hydroxymethyl)-1,3-propanediol	4	8.0-8.5	256 mM*	*n.d.*
CHES- 2-(Cyclohexylamino)ethanesulfonic acid	22	9.0-9.5		396 mM
CAPS-3-(Cyclohexylamino)-1-propanesulfonic acid	22	10-12		380.7 mM

n.d. not determined due to low mortality rates at concentration up to the limit of solubility.
* Highest tested concentration
# pH levels at this range could be maintained stable without a buffer

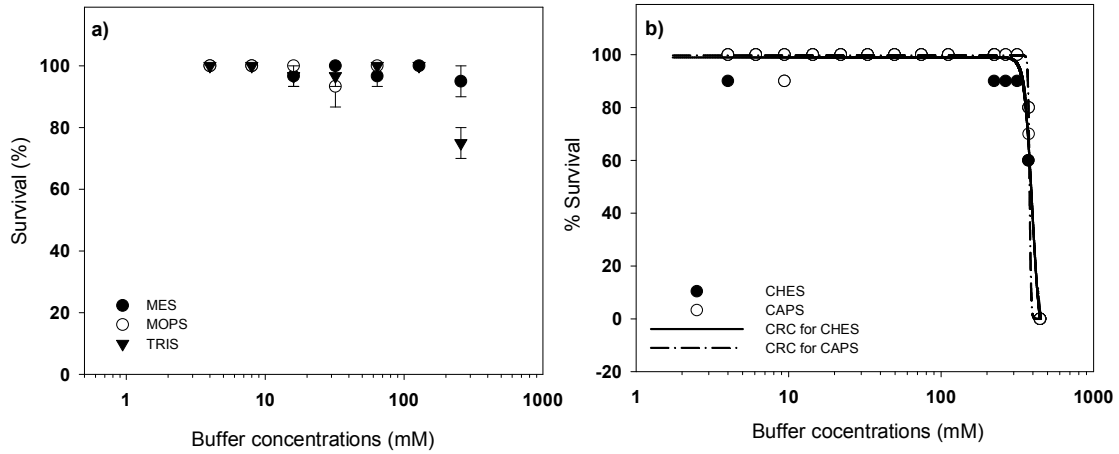

Fig S1 - Survival rate and concentration response curves for zebrafish embryos survival exposed to the buffers used to stabilize the pH. a) Survival rate after exposure to a range (0 – 256 mM) of MES, MOPS and TRIS concentrations. b) Concentration response curve for zebrafish embryos survival at 96h after exposure to a range (0 – 445 mM) of CHES (closed circles) and CAPS (open circles) concentrations. All the assays were conducted at pH around 8.0. This pH does not represent the optimal buffer range. However, in order to test buffer toxicity not related to the pH it was necessary to test the buffer toxicity at a pH range that is not toxic to zebrafish embryos

Table S2- Analytical measurements of exposure media

Nominal exposure concentration	pH of exposure	Measured exposure concentration			
		0 h ($mg.L^{-1}$ / % of nominal concentration)		24 h ($mg.L^{-1}$ / % of nominal concentration)	
3.78	4.44	4.66	123	4.21	111
4.92	4.33	5.24	107	4.78	97
6.39	4.21	7.26	114	6.79	106
8.31	4.10	9.67	116	10.90	131
10.81	3.99	12.03	111	10.33	96
14.05	3.87	15.87	113	15.11	108
18.26	3.76	21.97	120	23.52	129
0.41	8.79	0.13	32	0.030	7
0.7	9.02	0.008	1.15	0.002	0.3
1.18	9.25	0.012	1	<LOQ	<LOQ
1.99	9.50	0.040	2	<LOQ	<LOQ
3.36	9.70	<LOQ	<LOQ	<LOQ	<LOQ
4.37	9.82	<LOQ	<LOQ	<LOQ	<LOQ
5.68	9.93	<LOQ	<LOQ	<LOQ	<LOQ
9.61	10.16	0.050	<LOQ	<LOQ	<LOQ
12.49	10.27	<LOQ	<LOQ	<LOQ	<LOQ

<LOQ – Below limit of quantification

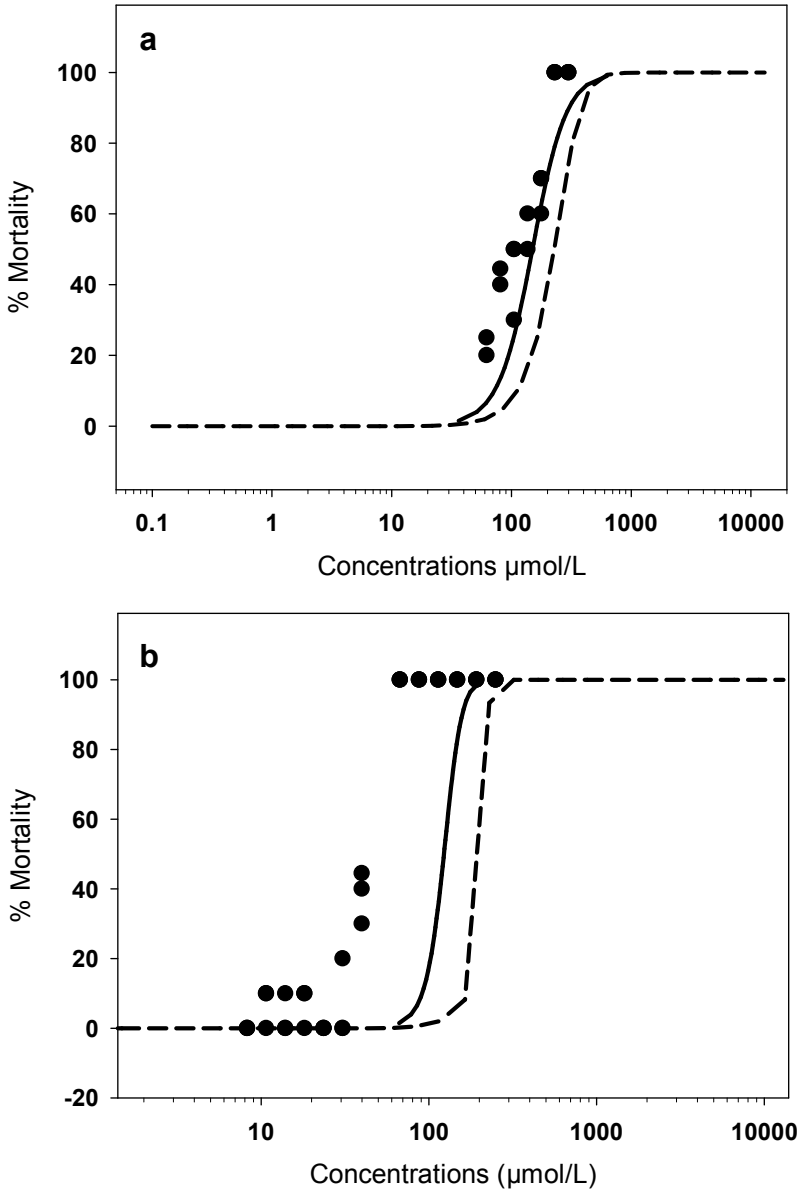

Fig S2 - Predicted and observed mixture toxicity increasing buffers concentrations. a) mixture ratio for acidic conditions using a buffer (MES) concentration of 40 mM; b) mixture toxicity for alkaline conditions using a buffer (CAPS) concentration of 220 mM. ——— , prediction according to concentration addition; – – – –; prediction according to independent action.

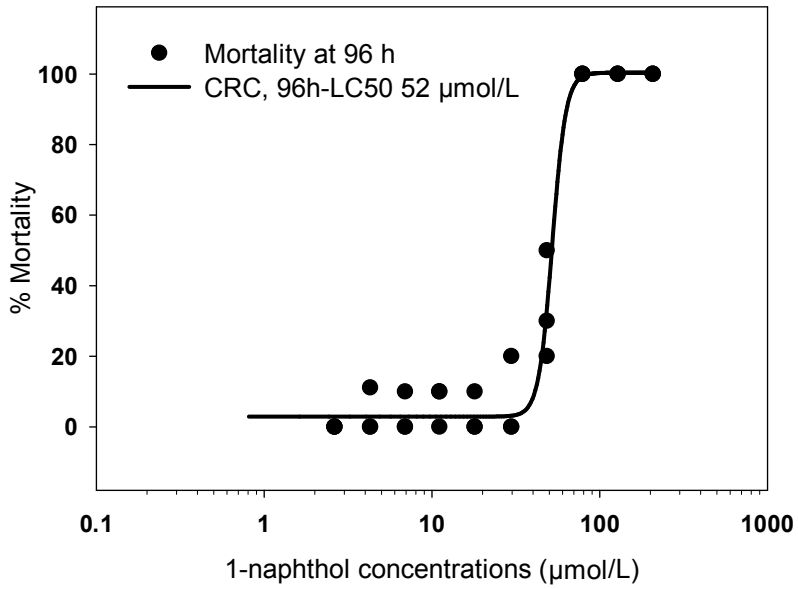

Fig S3 - Concentration response curve for zebrafish embryos exposed to 1-naphthol at 96 hpf.

Chapter 7

General Discussion

Getty images

7. General Discussion

This thesis addressed the problem of environmental stress and its role on the toxicity of pesticides, more particularly under a scenario of global changes in which aquatic systems have to cope with multiple sources of stress simultaneously.

In the last decades, the progressive changes on climate have had widespread impacts on natural systems changing not only the structure and function of many ecosystems (Benítez-Gilabert et al., 2010; Fenoglio et al., 2010; Mooij et al., 2005), but also impacting distribution and toxicity of chemical pollutants such as pesticides (Bloomfield et al., 2006). The assessment and prediction of effects elicited by many stressors (natural and chemical) acting simultaneously in a ecosystem implies a deep knowledge on the toxicity of the individual components. Thus, in this thesis, the zebrafish acute embryo toxicity test was firstly used to evaluate the effects of selected environmental parameters (pH, Dissolved Oxygen (DO) and UV radiation) and pesticides (carbaryl and carbendazim), providing a detailed and comprehensive analysis encompassing concentration-response curves and respective half-maximal effect concentrations (LC_{50}, EC_{50}) that can be further used in the assessment of combined stress. Zebrafish embryos, the experimental model selected for this work, showed to be adequate to achieve the proposed objectives and showed to have advantageous features for the use in ecotoxicology. Particularly relevant is the high sensitivity of the locomotor assay deployed with the high throughput system Zebrabox. The analysis of the individual stress components at sublethal level provided information of effects at different levels of biological organization potentially contributing to the establishment of adverse outcome pathways of the compounds within the organism.

Environmental components studied were selected based on the relevance in the context of the climate changes that have been verified. pH is one of the most determinant parameters for any chemical and biochemical effects and is pointed out as one of the most important factors conditioning survival and fitness of many freshwater species.

As for dissolved oxygen, although hypoxia episodes may occur in the aquatic environments, their frequency have been increasing by anthropogenic activities related to organic and nutrient enrichment in a phenomenon known as eutrophication. Due to rapid

human growth and global warming, the problem of hypoxia is likely to worsen in the upcoming years. An increasing temperature will lead to a considerable reduction in oxygen solubility especially in freshwater systems.

In aquatic systems, organisms may have to face the effects of another important environmental parameter: UV radiation. Projections show that the baseline levels of UV radiation will not be restored in the next decades (Weatherhead and Andersen, 2006). In addition, alterations in dissolved organic matter could affect UV transparency increasing the exposure of aquatic species to UV radiation specially vertebrates such as amphibians and fish that deposit their eggs in shallow surface waters.

The effect of each environmental stressor was evaluated on the survival and development of zebrafish embryos. Changes in environmental conditions showed to greatly impact the performance of embryos by decreasing survival and inducing a series of effects such as hatching and developmental delay, decreased heart beat rate, increase incidence of deformities, reduced body length among others. This is the first comprehensive and detailed analysis on the effects of environmental parameters including a time-course analysis for both lethal and sublethal endpoints. These data give an important contribution in the field of mixture toxicity providing baseline information to study the interaction of environmental stress factors and toxic contaminants in the zebrafish embryo model.

The evaluation of the toxicity of two widely used pesticides (carbaryl and carbendazim) using a multiple endpoint approach allowed the assessment of effects at several levels of biological organization including developmental, biochemical and behavioural. Behaviour and biomarkers were the most sensitive endpoints, been capable of detecting effects in very low concentration of pesticides, contributing to a mechanistic understanding of the effects.

Environmental parameters can interact with chemical pollutants in a variety of ways. They can influence the degradation and/or bioavailability of chemical compounds and change the toxicokinetics. Temperature effects on uptake and detoxification rates are a classical example as reviewed by Heugens et al (2001). Furthermore, environmental factors can also compromise the physiological state of organisms, potentially impairing fitness and the capacity to cope with additional stress. Spurgeon et al., (2010) proposed a framework to investigate mixture effects that can also be applied to the study of multiple

stressors toxicity (chemical and nonchemical stress). The framework takes into account the three above mentioned process: the bioavailability, toxicokinetics and the sensitivity of the organism (toxicodynamics) (Fig 1).

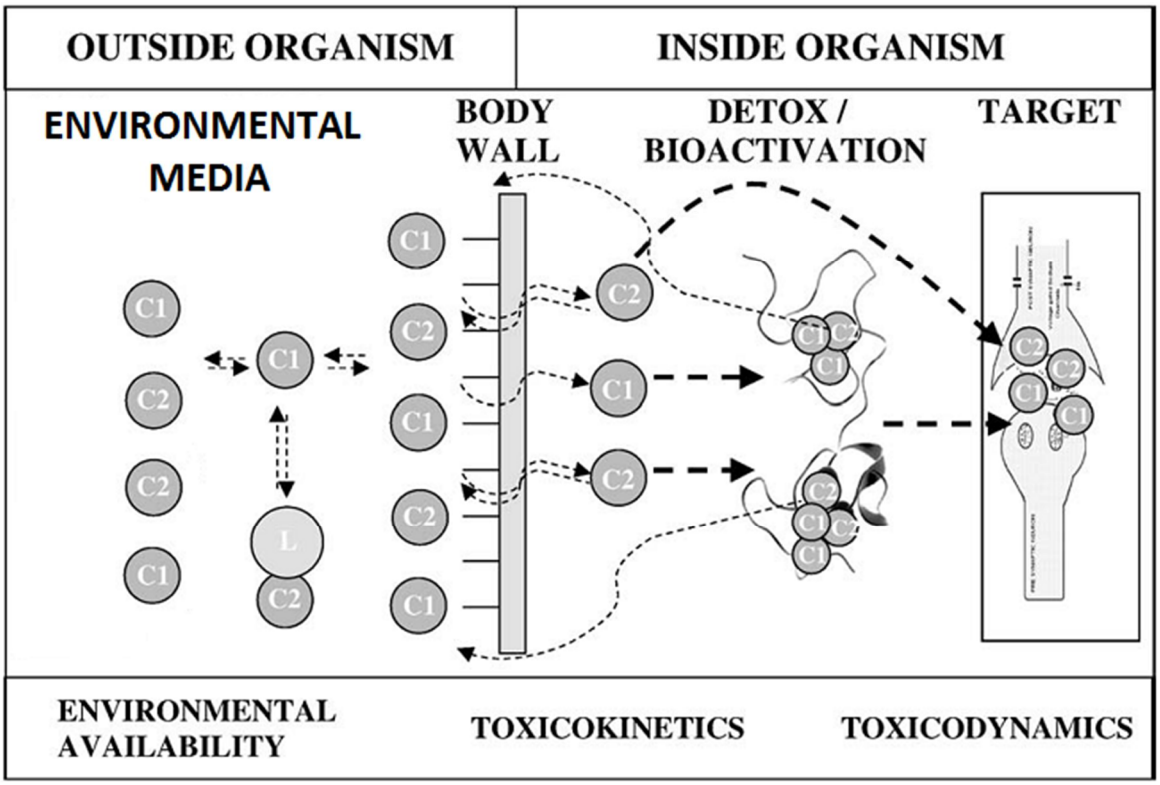

Figure 1: Framework to investigate multiple stressors effects. Adapted from Spurgeon et al 2010.

Understanding how the environmental factors may act in each of the above mentioned processes is essential to interpret the underlying mechanisms of the toxicity of mixtures when the combined effects deviate from either concentration addition (CA) or independent action (IA) (Spurgeon 2010).

In this thesis we evaluated the combined effects of acidic and alkaline pH on the toxicity of the carbamate insecticide carbaryl by applying the well-known predictive models CA and IA. The results showed that concentration addition allows a precise prediction of the toxicity of the combination of acid pH and carbaryl. Nevertheless, for alkaline condition both concepts underestimated the effects, although the inaccuracy of the CA model was smaller than that of the IA model. In the case of alkaline level, high pH values played a crucial role by modifying the toxicity of carbaryl through hydrolysis and consequently increasing its toxicity to zebrafish embryos and may be the main cause of

deviation from CA and IA. Neither CA nor IA considers specific chemical characteristics of the components of the mixture as well as physiological process of the exposed organisms. This can be a drawback of these two models to evaluate combined effects of chemical and nonchemical stressors, but at the same time, the simplicity of these two concepts may allow the establishment of guidelines and frameworks for mixture toxicity assessment in general (e.g. Backhaus & Faust 2012).

Mostly importantly, the results of this thesis demonstrated that pH can significantly affect the toxicity of pesticides to zebrafish embryos. The findings show that natural stressors and/or multiple stressors toxicities should not be neglected in the risk assessment of chemicals in order to avoid underestimation of risk and, therefore adequately protect aquatic ecosystems. Results presented here consist of a first tier assessment focusing at the lethal level only and although may not reflect a realistic environmental exposure scenario (except in extreme cases) highlight the importance of considering environmental variables as co-stressors in aquatic environments. Future approaches comprising also sublethal effects and therefore including realistic exposure scenarios may give a more sound perspective of risk in the context of climate changes.

Although the study of combinations of chemical and nonchemical stressors have progressed in the last few years, further research has to be conducted to evaluate and/or develop new approaches to describe this type of combination in order to detect deviations on the expected toxicity. Future work should focus on the evaluation of the potential impact of other important environmental stressors in the toxicity of chemical pollutants as dissolved oxygen, where a lack of studies has been identified (chapter 2). Moreover, the effects of combinations of more than two stressors should also be considered reflecting a more realistic scenario as in the environment. In addition, given that the assessment of every single pesticide under all exposure scenarios is unfeasible, the challenge for the future will be the identification and therefore prioritization of most relevant exposure conditions to be assessed.

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