Universidade de Aveiro Departamento de Biologia
Ano 2013

RHAUL OLIVEIRA

AVALIAÇÃO DOS EFEITOS DE QUÍMICOS USADOS NA AQUACULTURA

EFFECTS ASSESSMENT OF CHEMICALS USED IN AQUACULTURE

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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 da Doutora Paula Inês Borralho Domingues, Investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Amadeu Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Doutor António Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

Prezo insetos mais que aviões.
Prezo a velocidade
das tartarugas
mais que a dos mísseis.
Tenho em mim
esse atraso de nascença.
Eu fui aparelhado
para gostar de passarinhos.
Tenho abundância
de ser feliz por isso.
Meu quintal
É maior do que o mundo.

Manoel de Barros

Àquela que acompanhou meus passos desde pequenino e que agora já não está aqui para ver-me a dar mais um. Já não posso abraçar-te e levantar-te ao colo, rir junto contigo, e girar-te… até que, aos gritos, você chamasse a Vovó convencendo-me, quase de imediato, a soltar-te. Fica aqui uma singela homenagem, pequenina como tu eras, mas de todo meu coração.

Divina Batista Chagas
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Agradecimentos

Aos meus orientadores, Amadeu Soares, António Nogueira e Inês Domingues, pela orientação científica, disponibilidade, confiança.

A Fundação para a Ciência e Tecnologia pelo apoio financeiro sob a forma de uma bolsa de Doutoramento (SFRH/BD/62605/2009).

Aos Professores Dr. Ram Burjel (*Asian Institute of Tecnology*) e Dr. Paul van den Brink (*University of Wageningen*) pela colaboração mantida e em particular ao Me. Andreu Rico não só pelo sólido contributo no desenvolvimento desse trabalho, mas também pelo companheirismo e amizade nessa jornada.

A Inês Domingues, pela amizade, atenção e carinho, pelo jeito único de ensinar, pela serenidade e honestidade em cada ato, por sempre estender a mão nos momentos em que precisei, por ouvir e aconselhar com ternura e acima por se dispor a caminhar ao meu lado em cada novo desafio que surgiu nesses últimos 6 anos.

Ao António Nogueira por sempre estar disponível a ajudar com dados, papeis, experimentos, problemas de toda ordem! Sempre disponível e cordial! Obrigado, pela amizade e pelos ensinamentos. Daqui levo muito mais do que os modelos matemáticos sobre os quais trabalhamos, levo também um modelo de profissional que és tu professor.

Ao Abel e a Violeta pelo suporte no laboratório.

À Danika, Evinha, Tibo e Nessinha, pois nesses últimos 6 anos o mundo já deu muitas voltas e você estiveram comigo vaquinhaamarela em cada uma delas – no coração! Que venham muitas outras voltas, num copo de vinho, numa conversa, num violão, no Brasil, no mundo, na chuva. Obrigado por tudo!

À minha família da R. Jaime Cortesão: Darlanzito, Fê, Mari e Thata, afinal qual de vocês nunca passou uma/duas/três (…) noites comigo no laboratório? Quantas vezes foram digitar 1 e 0 enquanto eu disparava como um radialista: – ovo vivo, ovo morto, larva viva, larva cauda, larva morta. Obrigado pela amizade, pelo carinho e pelas aventuras (tantas!!!!). Com vocês tudo ficou com gosto de saudades boas!

A Carol, pois sem ela não teria chegado até aqui [já estaria perdido em alguma ilha da Tailândia]. Obrigado pela leitura e re-leitura dos *drafts* dos artigos e pela imensa ajuda no laboratório. Obrigado pela amizade e ensinamentos. Obrigado por ser meu porto seguro por tantos anos. Obrigado pelo carinho e cuidado não só para comigo, mas com a minha família, com meus amigos e com a Kaly. Um grande abraço para você e para sua família! Sucesso!

A Jessiquita, ao Pablito e a Thata, pela amizade incondicional, pela força dada, pelo apoio nas horas de maior necessidade! Pelas batatas no Convívio, pela conversa ao pé da máquina de café, pelos dias de praia e pelos dias de serra da estrela. Fazer o doutorado junto com vocês foi um presente, uma aventura muito muito boa. Desejo muito sucesso para vocês! Já tenho imensa saudade.

A minha família portuguesa que cá vai ficar. Rita, Chico, Avózinha [Alice] e Kalypso aqui fica registrado um abraço bem apertado, um desejo muito muito sincero de toda felicidade do mundo para vós e um até já.

Á Susana e a Fátima pela amizade, pelas conversas, pelos sorrisos e pelos bons momentos. Mais acima de tudo Su e Fátima obrigado pelas cerejas e beringelas.

To the *Thai Dream Time* – Baat, Carla, Chris, Coco, Juju, Luis, Nikki, Roy, Taniaaa – Thank you very much! For all the help in the field work, all the good moments around Thailand. You were an essential *part(y)* of this work!

As universidades federais do nordeste do Brasil, porque ela formam as pessoas mais arretadas desse Brazilzão… Larinha, Ritinha, Lili, Nilin, Janise, Letícia e Mônica [hahaha], um cheiro!

À Nini, Fabi e a Paulinha que também não podiam faltar por aqui. Um abraço bem apertado, muito sucesso e muita luz no vosso doutorado, obrigado pelos dias partilhados, pelas conversas, evaristos, finos e cafés!

À malta do LETAL pelos momentos de distracção, partilha de protocolos e discussão de ideias, conversas de corredor, SETACs, jantares e muitos finos. Também a "*Beer Friday generation"* pelos momentos ébrios e de descontracção que foram essenciais para manter a minha sanidade mental e que me impediram de atirar a Ria (em especial ao Marco e Rui.M).

À minha família, pois mais do que resultado do meu esforço essa tese representa o esforço deles! Mãe, irmão, avó, tios e primos o meu muito obrigado.

Á minha mãe, meu orgulho, minha inspiração! Não há palavras para agradecer o apoio incondicional que você me deu em todos os dias dessa jornada. A caminhada nesse mundo ao teu lado é sagrada. É o teu exemplo que me motiva a seguir sempre em frente. Mais que meu porto seguro, tu és meu *Lar*, meu vilarejo. Te amo e Muito obrigado!

Aos meus irmãos pela amizade e apoio. Pelas risadas, pelo carinho e apoio em todas as horas e por sempre acreditarem em mim. E acima de tudo obrigado pelo simples fato de existirem, pois é na vossa figura que vou buscar os momentos mais bonitos guardados na minha memória.

À minha Vó, meu xodó, minha guerreira! Custou muito estar longe de ti nesses últimos anos. Obrigado pelo apoio, pelas palavras de sabedoria e pelo amor demostrado a cada ligação, cada mensagem e cada abraço! Desculpa a ausência em tantos momentos que você tanto precisou. Te amo!

Palavras-chave

aquacultura, ecotoxicologia, antibióticos, desinfectantes, pesticidas, hormonas, avaliação de risco, ecossistemas aquáticos, biomarcadores, *Danio rerio*, *Daphnia magna*, algas, níveis tróficos, distribuição de sensibilidade das espécies

Resumo

A aquacultura fornece sustento para milhões de famílias, sendo uma actividade em crescimento e com um forte potencial de expansão em todo o mundo. Há um interesse por parte de produtores e consumidores em desenvolver modelos sustentáveis de aquacultura abrangendo aspectos sociais, económicos e ambientais. Tais modelos implicam lidar com os impactos ambientais gerados pela aquacultura. O principal objectivo deste trabalho foi estudar o uso de produtos químicos usados nas aquaculturas e avaliar os seus efeitos nocivos para organismos aquáticos. Uma vez que mais de 80% da produção mundial de organismos aquáticos está concentrada no sudoeste da Ásia, foi dada especial atenção à aquacultura nessa região, particularmente na Tailândia. Três diferentes cenários foram considerados: aquaculturas de camarão, aquaculturas de tilápias cultivadas em gaiolas e em tanques escavados no solo. Em cada tipo foram realizados inquéritos e campanhas de monitoramento em várias aquaculturas nas quais foram averiguados o uso, destino e forma de aplicação de químicos. Os resultados indicaram o uso excessivo/inapropriado de alguns compostos, nomeadamente de antibióticos (em aquaculturas em gaiolas), de desinfectantes (em aquaculturas de camarão) e de 1,7α-metiltestosterona (MT) (em aquacultura de tilápias em tanques escavados). Os resultados das campanhas de monitorização mostraram que o uso excessivo de produtos químicos resulta na contaminação do meio ambiente com antibióticos (oxitetraciclina (OTC) e enrofloxacina (ENR)) nas aquaculturas em gaiola, e com MT nas aquaculturas de tilápias em tanques escavados.

No laboratório, foram realizados ensaios de ecotoxicidade com antibióticos, desinfectantes e pesticidas usando organismos modelo pertencentes a diferentes níveis tróficos.

Foram testados os desinfectantes, cloreto de benzalcónio (BKC), gluconato de clorexidina, e glutaraldeído (GA) sendo que os dois primeiros demonstraram ser muito tóxico para consumidores primários e secundários. Para uma análise integrada foi utilizada uma abordagem baseada nas distribuições de sensibilidade das espécies (DSE) com a qual se estimaram as concentrações perigosas (CP) para 5% e 50% das espécies após e exposição ao BKC e GA. O grupo dos produtores primários foi o grupo mais sensível à exposição ao BKC com CP5% = 10,8 µg/l enquanto para GA não foram encontradas diferenças de sensibilidade entre os grupos tróficos e, assim sendo, um valor geral de CP5% = 300 µg/l foi obtido. Além disso, uma análise probabilística dos riscos ambientais indicou uma fracção de espécies potencialmente afectada (FAPE) pelo BKC > 5% em efluentes e águas superficiais. Os pesticidas estudados foram o trichlorfon (TCF) e a ivermectina (IVM). O TCF foi extremamente tóxico para dafnídeos (48h-LC50 = 0,29 µg/l) e afectou também a actividade de acetilcolinesterase em *D. magna* e *D. rerio*. A IVM foi tóxica para adultos, juvenis e embriões de *D. rerio* (96h-CL50 = 18,5 µg/l para os juvenis) afectando o seu desenvolvimento e actividade de biomarcadores. Em exposições crónicas, IVM causou alterações no comportamento e crescimento em *D. rerio* juvenis (21d-LOEC <0,25 µg/l). Os antibióticos amoxicilina e OTC apresentam baixa toxidade para os organismos aquáticos, mas ambos induziram enzimas relacionadas estresse oxidativo em adultos e embriões de *D. rerio*.

Em geral, a maioria dos químicos testados apresentaram toxicidade para algum grupo de organismo da cadeia trófica ou tiveram efeito a nível de parâmetros sub-letais, sendo portanto recomendados estudos de exposição crónica para que se possa refinar a avaliação de risco ambiental destes compostos. Os efeitos letais e sub-letais observados nos organismos não-alvo sugerem que pode haver uma perda da biodiversidade nos ecossistemas aquáticos, e assim, comprometer os serviços prestados pelos ecossistemas num futuro próximo. Sendo o sucesso da aquacultura dependente directo destes serviços (por exemplo, água de alta qualidade), há uma necessidade urgente de medidas de controlo/educação, tanto no uso de produtos químicos na aquacultura e monitoramento/mitigação dos impactos negativos destes nos ecossistemas naturais.

Keywords

Aquaculture, ecotoxicology, antibiotics, disinfectants, pesticides, hormones, risk assessment, aquatic ecosystems, biomarkers, *Danio rerio*, *Daphnia magna*, algae, trophic levels, species sensitivity distributions

Abstract

Aquaculture provides food and income for millions of families worldwide being an activity with a high growth rate and with a strong potential for further expansion. Both producers and consumers are interested in a sustainable model of aquaculture development covering social, economic and environmental aspects. Such model implies to cope with the environmental impacts generated by aquacultures. The main objective of this work was to evaluate the use of chemicals applied in aquaculture farms and their harmful effects to aquatic organisms. Since more than 80% of global production of aquatic organisms is concentrated in Southwest Asia, special attention was given to the aquaculture in this region, particularly Thailand. Three different types of aquaculture scenarios were studied: shrimp farms and tilapia farms in cages and in earth ponds. Surveys and monitoring campaigns were conducted in several farms and the fate, use and application patterns of chemicals were identified. In cage farms the results indicated overuse/misuse of antibiotics, in shrimp farms the major group of chemicals used were disinfectants whereas in earth pond farms 1,7α-methyltestosterone (MT) was the most used. The results from the monitoring campaigns showed that the excessive use of chemicals was correlated to contamination of the surrounding environment namely with the antibiotics oxytetracycline (OTC) and enrofloxacin (ENR) in the cage farms, and with MT in the earth pond farms.

In the laboratory, ecotoxicity assays were performed with antibiotics, disinfectants and pesticides using organisms belonging to different trophic levels.

The disinfectants benzalkonium chloride (BKC), chlorhexidine gluconate (ChD) and glutaraldehyde (GA) were tested. BKC and ChD demonstrated to be very toxic to primary and secondary consumers. Moreover, an approach based on the species sensitivity distributions (SSD) was used to estimate the hazardous concentrations for 5 and 50 % of the species after BKC and GA exposure highlighting the sensitivity of primary producers to BKC exposure (HC5 = 10.8 µg/l). In the case of GA, different trophic levels showed similar sensitivities and a general HC5 = 300 µg/l was obtained. Additionally, a probabilistic environmental risk assessment was performed indicating a PAF (potentially affected fraction) of species > 5% for adverse effects of BKC in effluents and surface water. Concerning pesticides, both trichlorfon (TCF) and ivermectin (IVM) were studied. TCF showed to be extremely toxic to daphnids (48h-LC50 = 0.29 µg/l) and affected the acetilcolinesterase activity in *D. magna* and *D rerio*. IVM showed to be acutely toxic to *D. rerio* life stages (96h-LC50 = 18.5 µg/l for juveniles) and affected their development and biomarkers. In chronic exposures, IVM led to changes in the behaviour and growth of *D. rerio* juveniles (21d-LOEC < 0.25 µg/l). The antibiotics amoxicillin and OTC were not acutely toxic to aquatic organisms but both induced oxidative stress related enzymes of adults and embryos of *D. rerio*. Globally, most of the tested chemicals showed to compromise at least a particular group of organisms or sub-lethal parameters, requiring further long term studies so that the environmental risk assessment of these compounds can be refined. Lethal and sub lethal effects obtained to non-target organisms suggest a potential biodiversity loss in the aquatic ecosystems which might, thus, compromise the services provided by the ecosystems in a near future. Since the aquaculture relies directly on these services (e.g. high water quality) to succeed, there is an urgent need of control/education measures both in the use of chemicals in aquaculture and monitoring/mitigation of adverse impacts in natural ecosystems.

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Chapter 1

General Introduction







1. General Introduction

1.1 A brief context, aims and outline

In the last century, a fast degradation of freshwater and marine natural resources has been noticed. The growth of human population is associated to an increase of anthropogenic activities including industries, mining, building, agriculture, fishing, among many others. Most of these activities are based on a misconception of development in which natural resources are exploited beyond support capacity of the ecosystems. One of most severe effect of such high pressure on ecosystems is biodiversity loss (Cardinale et al. 2012). Once affected, some ecosystems might take decades to recover their biodiversity. The more biodiversity aquatic ecosystems lose, the longer they will take to recover and, consequently, the alterations on ecosystems structure and functioning are, in some cases, irreversible. Gradually, the ecosystems lose their ability to provide essential goods and services such as food, fertile soil, potable water and clean air to the humanity (Cardinale et al. 2012; Costanza et al. 1997).

One of the most important examples of ecosystem degradation due to unsustainable anthropogenic activities is the rapid depletion of the fish stocks in the seas (Worm et al. 2007). Few years ago Worm *et al.* (2006) raised the question "*Will there be fish in the ocean in 2050?*" Indeed, due to decades of overfishing, a global collapse of several taxa currently fished can be expected to occur by 2048. In this context, we are assisting a decrease of the global ocean food provision and an increase of aquaculture (Bostock et al. 2010).

In a changing world, aquaculture is one of the most promising agricultural activities of the century. With a high growth rate, the modern aquaculture is highly diverse including the cultivation of several plants, algae, crustaceans, mollusks and fish. Globally, aquaculture contributes to reduce the pressure on the wild stocks of fish in the seas and rivers, especially in the tropics where the aquaculture finds the perfect niche to develop. In fact, in the tropical countries, water bodies are abundant and there is potential for several species to be cultivated, which allows the establishment of aquaculture systems

with high productivity. Although aquaculture has several benefits, such as generating employment, improving food security, and promoting local development, it has to follow sustainable models of development; otherwise, no longstanding benefits will be provided by the aquaculture sector (Pauly et al. 2002).

As others anthropogenic activities, the aquaculture also has some drawbacks. Among the most discussed environmental problems related to aquaculture are (i) the habitat degradation, (ii) the spread of diseases, (iii) escapes and introduction of invasive species, (iv) use of genetically modified species, (v) pollution through nutrient input and (vi) chemical contamination of aquatic ecosystems (Cole et al. 2009). Regarding the several impacts of aquaculture in the environment and, at the same time, recognizing the complexity of the environmental risk assessment of aquacultures, in the present thesis three major areas were chosen as central subjects ***– Aquaculture, Chemical Pollution and Ecotoxicology***. Briefly, the present document brings new knowledge on the use, fate and toxicity of chemicals applied in aquacultures worldwide, namely disinfectants, pesticides and antibiotics. Most of the studies presented in the thesis were developed regarding the tropical aquaculture scenarios in South East Asia, particularly in Thailand.

This thesis is composed by ten chapters. The **chapter 1** consists of this contextualization and an introduction with an overview on aquaculture and chemical pollution. **Chapters 2 to 9** are in the format of scientific publications (five published and three in preparation to submitted, all in international peer reviewed scientific journals) and present all results of effects assessment of disinfectants (glutaraldehyde, chlorhexidine, benzalkonium chloride), pesticides (trichlorfon and ivermectin) and antibiotics applied in aquacultures. In the final chapter (**chapter 10**) a thesis overview and final remarks are drawn. In detail:

Chapter 1: General Introduction. In this section is giving a brief definition of aquaculture including principal producing countries, products and systems. A contextualization of aquaculture environmental impacts related to use of chemicals worldwide and especially in Thailand is also addressed.

Chapter 2: From sub cellular to community level: toxicity of glutaraldehyde to several aquatic organisms. The effects of the disinfectant glutaraldehyde on algae, invertebrates and fish were investigated using toxicity tests and biomarkers; the effects to the community were extrapolated using the Species Sensitivity Distribution approach. The results suggest glutaraldehyde as moderately toxic to aquatic organisms with toxicity values ranging from 3.6 mg/l to 31.3 mg/l for the tested species. Moreover, GA had a similar biocide effect on organisms independent of trophic levels.

Chapter 3: Lethal and sub lethal effects of the biocide chlorhexidine on aquatic organisms. The effects of the disinfectant chlorhexidine to algae, bacteria, invertebrates and fish embryos were studied using several toxicity tests and biomarkers. Results suggest that chlorhexidine is highly toxic to algae, daphnia and fish embryos. Moreover, chlorhexidine caused changes in cholinesterase levels and hatching rate of fish embryos.

Chapter 4: Use of species sensitivity distributions to assess the toxicity of benzalkonium chloride to aquatic organisms from different trophic levels. The toxicity of the disinfectant benzalkonium chloride to organisms of four trophic levels, namely decomposers, primary producers, primary consumers and secondary consumers was studied. Data from the toxicity tests performed and from the literature were used to fit a species sensitivity distributions curves. Toxicity tests and SSD analysis indicated a high toxicity of BKC to primary producers and consumers, raising concern about the toxicity of this disinfectant in real scenarios of contamination.

Chapter 5: Assessing lethal and sub lethal effects of trichlorfon on different trophic levels. In this work the acute toxicity of the organophosphate pesticide trichlorfon was evaluated using organisms of different trophic levels. Additionally, the usefulness of using biomarkers in the detection of sub-lethal effects of TCF in daphnids and fish was assed. The results suggest trichlorfon as a very toxic compound to invertebrates with LC50 values < 1µg/l, which are corroborated by the strong inhibition of cholinesterase activity observed in daphnids; a strong inhibition of cholinesterase activity was also observed in fish embryos.

Chapter 6: Effects of ivermectin on zebrafish early life-stages and adults. The toxicity of the parasiticide ivermectin to embryos and adults of *D. rerio* was compared. A higher toxicity of ivermectin was observed in adults rather than embryos. However, a similar pattern of effects on biomarkers and behaviour parameters was obtained for both embryos and adults.

Chapter 7: Ivermectin exposure to *Danio rerio* affects its growth rate, behavior and vitellogenin levels. In this chapter, the chronic toxicity of ivermectin to fish was studied by performing a 21 days assay with *D. rerio* juveniles. The growth rate and feeding behavior of male and female were affected at concentrations ≤ 2.5 µg/l. Moreover, inhibition of biomarkers was observed, namely glutathione-S-transferases, catalase and vitellogenin.

Chapter 8: Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). The sub lethal effects of the antibiotics amoxicillin and oxytetracycline on the development and enzymatic activity of the fish *D. rerio* were studied. Our study shows that exposure to OTC and AMX for 96 h leads to oxidative stress and hatching rate alterations in zebrafish only at much higher doses suggesting that physical and physiological impairment of natural fish populations are unlike to occur.

Chapter 9: Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. In this field study, the use of antibiotics in different tilapia cage farms in Thailand was investigated through surveys and monitoring campaigns. The results indicated a heavy use of several antibiotics in cage farms, which agrees with the concentrations of oxytetracycline and enrofloxacin found in the surrounding environments of the tilapia cage farms.

Chapter 10: Final Remarks. This last chapter consists of a short overview with the principal highlight of the thesis.

Aquaculture is, undoubtedly, an activity with a remarkable potential of expansion but such development must follow a sustainable model covering social, economic and environmental aspects. The present thesis brings to the light useful information, providing support to better cope with problems generated by pollution arising from aquacultures and its environmental impacts.

1.2 What is aquaculture?

One of two fish consumed in our table comes from aquacultures (Hishamunda et al. 2009). It was a long way to aquaculture reaches the status of one of the most important protein producers for humans. The aquaculture is very old activity; evidences suggest that since 2500 B.C the Egyptians grew tilapia in artificial ponds (FAO, 2003). In Asia, the oldest record refers to the culture of carps in China in about 2000 B.C. In the 21st century, the aquaculture settles as one of the most important agricultural activity driven by the global increasing demand for food, especially in the tropics. For decades, the aquaculture has been the only activity growing at a higher rate than the human population (average 5.8% between 2004 and 2008 (Bostock et al. 2010)). Additionally, the modern aquaculture is not limited to produce food supplies but is also dedicated to generate raw material and molecules to be used in biotechnological and pharmaceutical industries (Ibañez and Cifuentes 2013; Mazarrasa et al. 2013).

Many definitions of aquaculture can be found in the literature. Briefly, one could define aquaculture as the reproduction, rearing and harvesting of animal and/or plants in artificial or semi-artificial aquatic environments. Thus, the aquaculture is nothing more than an organized production of an animal/plant in the aquatic systems. Aquaculture can be classified depending on its intensification, systems, species, region or environment chosen to develop the farm. Although, the Food and Agriculture Organization of the United Nations has defined aquaculture as below:

"*Aquaculture is the farming of aquatic organisms: fish, mollusks, crustaceans, aquatic plants, crocodiles, alligators, turtles, and amphibians. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated...*" (FAO, 1990)

Despite being a widespread activity, 88.9 % of world aquaculture production is concentrated in Asia (Bostock et al. 2010). Among the ten biggest producers of aquatic organisms, eight are from Asia (**Figure 1**). The exceptions are Norway, which is the biggest producer of Salmon (mostly, *Salmo salar*

accounting for more than 80 % in Norwegian aquaculture), and Egypt where the production of native tilapia species (*Oreochromis niloticus* and *Oreochromis niloticus*) reached 68049 tons in 2009 (FAO, 2013). In several Southeast Asia countries, aquaculture is a major source of food, employment and income for the population contributing to the economic development of countries. Thus, aquaculture has an important role in the local economy, rural populations, and the global economy since much of the production is exported, mainly shellfish.

A large variety of aquatic organisms can be farmed including fish, shellfish, algae, aquatic plants. The most common types of aquaculture are:

- **Fish farming** is the most traditional form of aquaculture that usually aims at food production. Several species are farmed worldwide being the most common carp, salmon, tilapia and catfish species. The farming can be performed in different types of systems such as tanks, cages, raceways or automatic systems.
- The **mariculture** refers to the cultivation of aquatic organisms in saltwater natural or semi-natural systems with a salinity usually set > 20‰. Several habitats in coastal zones can support a mariculture including mangroves, fjords, open seawaters and inland seas.
- The **algaculture** consist in cultivate of algae species in natural, artificial (bioreactors) or semi-artificial systems. The green microalgae are the majority of algae cultivated being highly used for feeding fish and shellfish in low-scale aquaculture. The macroalgae, red and brown seaweed are between the most produced aquatic organisms in the world (**Table 1**) having many commercial and industrial applications.
- The most advanced aquaculture systems are **multitrophic systems** which are the most efficient and environmental friendly because nutrients are recycled in a trophic chain based model. Aiming at the balance of the system, various organisms with different nutritional needs are combined (e.g. primary producers such as seaweed growing by using the nitrogen and phosphorus excreted by consumers (fish species). In another niche, the shellfish species can feed on the uneaten food pellets and feces of fish and/or shrimps).

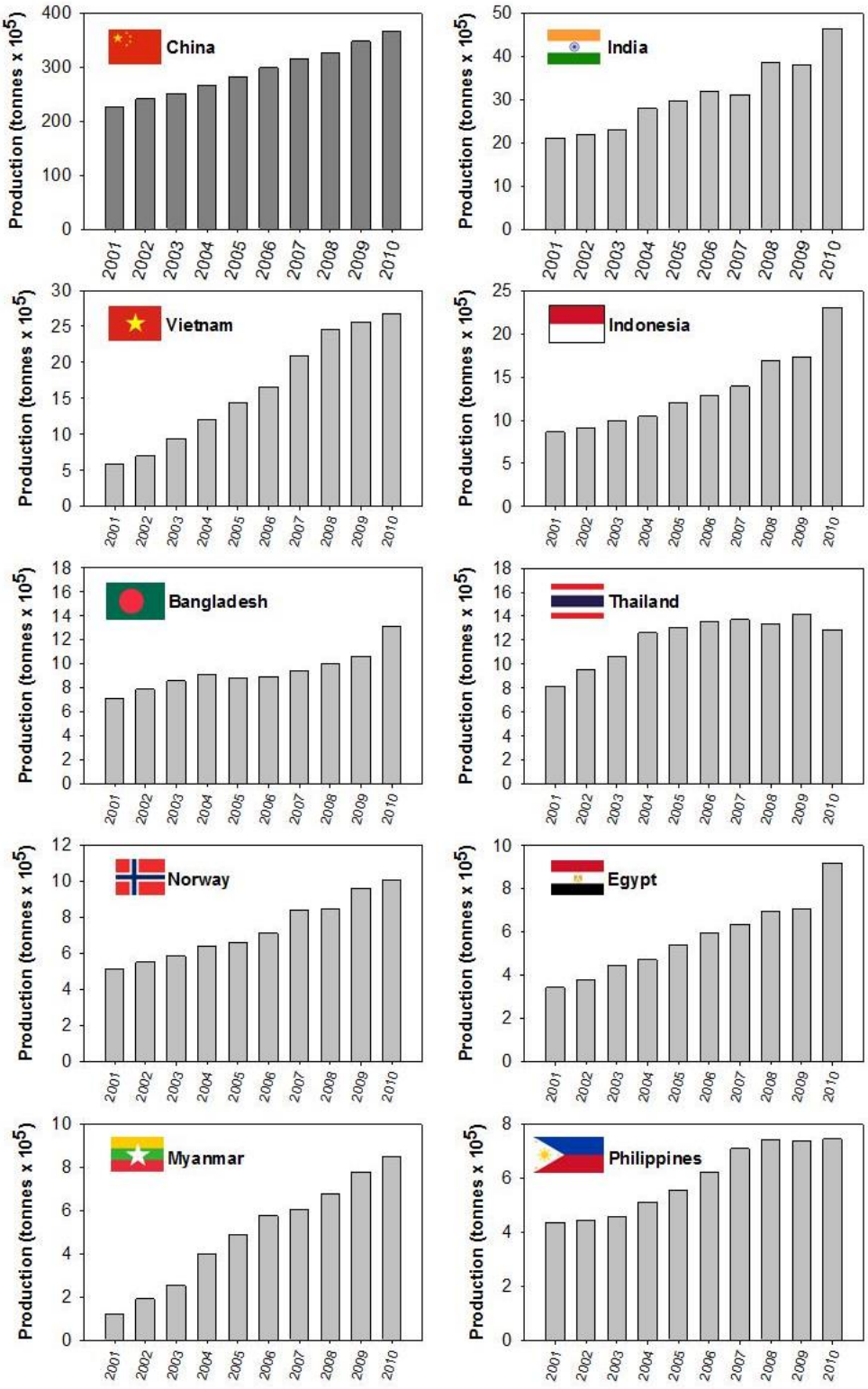

Figure 1 – The ten world aquaculture producers in 2010 (FAO 2012).

Some of the aquatic organisms with the highest aquaculture production in tons are carp, tilapia and other cichlids, fresh water crustaceans, shrimps, oysters, mussels, scallops, clamps, brown and red sea weeds. All these have shown a growth rate in the last decade ranging from 9.66 % for carps to 0.62 % to scallops (see **Table 2**).

The aquaculture malleability allows the use of a miscellaneous of habitats to install farming systems (e.g. freshwater, brackish water, and saltwater environments) but the drawback is that each niche has its own biological, physical and chemical characteristics. Thus, the carrying capacity and maintenance routine will vary in each niche chosen to install aquaculture systems increasing risks of failure either in family or in large-scale commercial farms. Globally, different systems are used for the cultivation of aquatic organisms: static water ponds, recirculating systems (reconditioned water/closed systems); rice fields; raceways; cages and enclosures; Hanging, 'on-bottom' and stick methods of oyster culture (FAO, 2013).

The intensification of the farming systems is an important characteristic used to classify the aquacultures. The **extensive** aquaculture is based on the adoption of traditional techniques with a high dependence of natural goods and with a much lower control over the farmed stocks. On the other hand, the **intensive** aquaculture combine a wide range of techniques based on scientific pond design, fertilization, supplemental feeding or only feeding without fertilization. Moreover, in intensive systems is observed a higher control of stock, which includes aspects such as manipulation, disease diagnostic and treatment, methodical harvesting, high density and high production volume. **Semi-intensive systems** adopt mid-level technology, partial dependence on natural productivity, fertilization, supplementary feeding, stock manipulation, medium level inputs and medium rate of production (FAO, 2013).

Table 1 – World aquaculture production by species groups (tons)

Year	Carp	Tilapia	Fresh water crustaceans	Shrimps	Oysters	Mussels	Scallops	Clamps	Brown sea weeds	Red sea weeds
2001	14575724	1303310	520932	1310818	3786892	1375080	1102345	2799550	2246971	4268451
2002	14940384	1418814	577055	1467161	3883683	1552254	1113078	3065762	2599014	4515172
2003	15635401	1587046	784817	2050572	4016389	1623298	1102063	3372451	3125495	5983337
2004	16889379	1795089	845978	2364511	4142740	1669844	1052561	3634661	3963301	6402367
2005	17752747	1991697	913640	2667909	4155900	1718513	1146909	3677841	4682477	6926355
2006	18522123	2234342	954603	3111718	4260119	1771475	1261693	3798412	5292952	6644891
2007	18955178	2554145	1271586	3294956	4402914	1598065	1464172	4202069	6071739	6536197
2008	20682222	2826556	1373879	3400136	4147512	1587987	1410899	4364979	6700168	6626914
2009	22230509	3108920	1555218	3532129	4311217	1727638	1583614	4451898	8043542	6725016
2010	24237303	3497391	1681482	3787706	4488544	1812371	1727105	4885179	8978535	6784193
Growth rate per year (%)	**9.66**	**2.19**	**1.16**	**2.48**	**0.7**	**0.44**	**0.62**	**2.09**	**6.73**	**2.52**

Source: FAO statistics (2013)

1.3 Environment, chemical use and impacts

Fish aquaculture is increasing worldwide, driven by the rising demand for food. In Thailand, aquaculture is one of the most important industries with a strategic role not only for the economy but also food security of the country. Highly diverse, the aquaculture sector in Thailand can be artificially divided in two. First, the **freshwater aquaculture** (tilapia, catfish and prawns) covers mainly the domestic consumption demands and it is crucial to provide the rural poor with high quality protein food for home consumption. On the other hand, the **brackish water aquaculture** typically produces shrimps, among other high values species, targeting the global markets such as United States and European Union. Briefly, the main freshwater and brackish water species cultured in Thailand are:

- *Oreochromis niloticus* (Nile tilapia)
- *Clarias macrocephalus* X *C. gariepinus* (Hybrid catfish)
- *Barbodes gonionotus* (Silver barb)
- *Macrobrachium rosenbergii* (Giant river prawn)
- *Trichogaster pectoralis* (Snakeskin gourami)
- *Peneaus monodon* (Giant tiger prawn)
- *Penaeus vanamei* (Whiteleg shrimp)
- *Perna viridis* (Green mussel)
- *Anadara spp.* (Blood cockle)
- *Crassostrea commercialis* (Oyster)

The growth of aquaculture in recent decades is associated with various environmental problems including eutrophication, deforestation of coastal areas, the introduction of invasive species in aquatic ecosystems and environmental pollution. The negative impacts of farms in the ecosystems might compromise natural communities, human health and the success of aquacultures themselves (as they are highly dependent on natural resources) Once the focus of the present thesis is the chemical pollution, the other impacts including its effects and management measures are only shortly summarized in the **Table 2**.

Table 2 – Summary of environmental impact from aquacultures activities

Impact	Description	Effect	Management measure
Nutrients	Input of effluent rich in nutrients, mainly from feces and food pellets	Eutrophication and algal blooms	Multitrophic systems, reuse of wastewater for irrigation, settling tanks etc.
Feed additives and chemicals	Vitamins, probiotics, disinfectants, antibiotics, hormones etc.	Deleterious effects on non-target organisms, effects at the ecosystem levels, trophic chains	Adequate use and replacement of highly toxic chemicals, effluent treatment, control measures
Antibiotic resistance	Acquisition of resistance by disease-causing bacteria, which can reach humans and other organisms	Increased susceptibility of organisms and man to bacterial diseases	Avoiding antibiotics used for human therapy use and prophylactic use of any antibiotic
Over exploitation of wild stocks	Capture of wild organisms for initial stocks and/or for fish oil production applied in carnivores fish aquaculture	Wide population depletion	More research on aquaculture feed with vegetal oil for replacement of the fish oil.
Spread of diseases	Spread of diseases to wild populations including microbes, parasites, virus	Effects on wild populations health	Investment on diseases preventions by vaccines and early diagnostics
Escapements	Exotic species are introduced in different habitats	Potential treat to native species and ecosystems	Farming of native species and preventions of escapements (double net cages)
Habitat losses	Coastal zone degradation (e.g. mangroves)	Degradation of spawning zones of marine species	Establishment of marine protected areas

Several chemicals are used in aquaculture in order to guarantee the production of low-cost food for millions of consumers. The water bodies in the surrounding areas of aquaculture are, very often, subjected to environmental contamination due to the dumping of untreated effluent usually with a heavy load of organic matter (feces, food and other organic wastes) and chemicals (e.g. antibiotics, pesticides, herbicides, antibiotics, disinfectants) (Graslund and Bengtssona 2001; Gräslund et al. 2003; Primavera 2006; Rico et al. 2012; Rico et al. 2013; Rico and Van den Brink 2014; Valiela et al. 2001). Several of these chemicals are applied in different phases of the aquaculture production line. For example in a tilapia cage farm, the most used chemicals are applied mixed with the food or in baths to treat or prevent diseases (e.g. antibiotics) (**Figure 2**). In a shrimp farm, a higher use of disinfectants is expected in the preparation of the ponds, cleaning of materials and treatment of the water. Thus, both shrimp farm and tilapia farms have a high potential to contaminate aquatic ecosystems through chemicals input, especially in the cage systems where chemicals are often applied directly into seas, rivers and lakes.

In aquaculture, the chemicals can be applied in the treatment of diseases but often they are also applied as preventive measures reducing the risk of disease outbreaks. Disinfectants, antibiotics, pesticides, and probiotics may be applied in the pond preparations, sterilization of equipment, elimination of parasites and/or pathogens. However, in order to guarantee the human and environmental safety of animals, the best management practices and biosecurity measures must be followed.

The Disinfectants are used in greatest quantities in intensive cultures, particularly in finfish and shrimp hatcheries and grow-out facilities. They are used in site and equipment preparation, to maintain hygiene throughout the production cycle and, in some cases, to treat diseases. There is little or no use in extensive systems. The classes of compounds listed below are substances used in specific surfaces or areas with the objective of killing microorganisms, although they are usually not capable of killing all kinds of them. According to Block (Block 2001), a disinfectant is an agent that frees from infection, usually a chemical agent but sometimes a physical one that destroys harmful microorganisms but may not kill

bacterial spores. It refers to substances applied to inanimate objects. Disinfection is not as powerful as sterilization, which uses physical and chemical methods to completely destroy microorganisms (CDC 2005). Disinfectants are also different from antibiotics and antiseptic, that destroys microorganisms within the body and in living tissue, respectively.

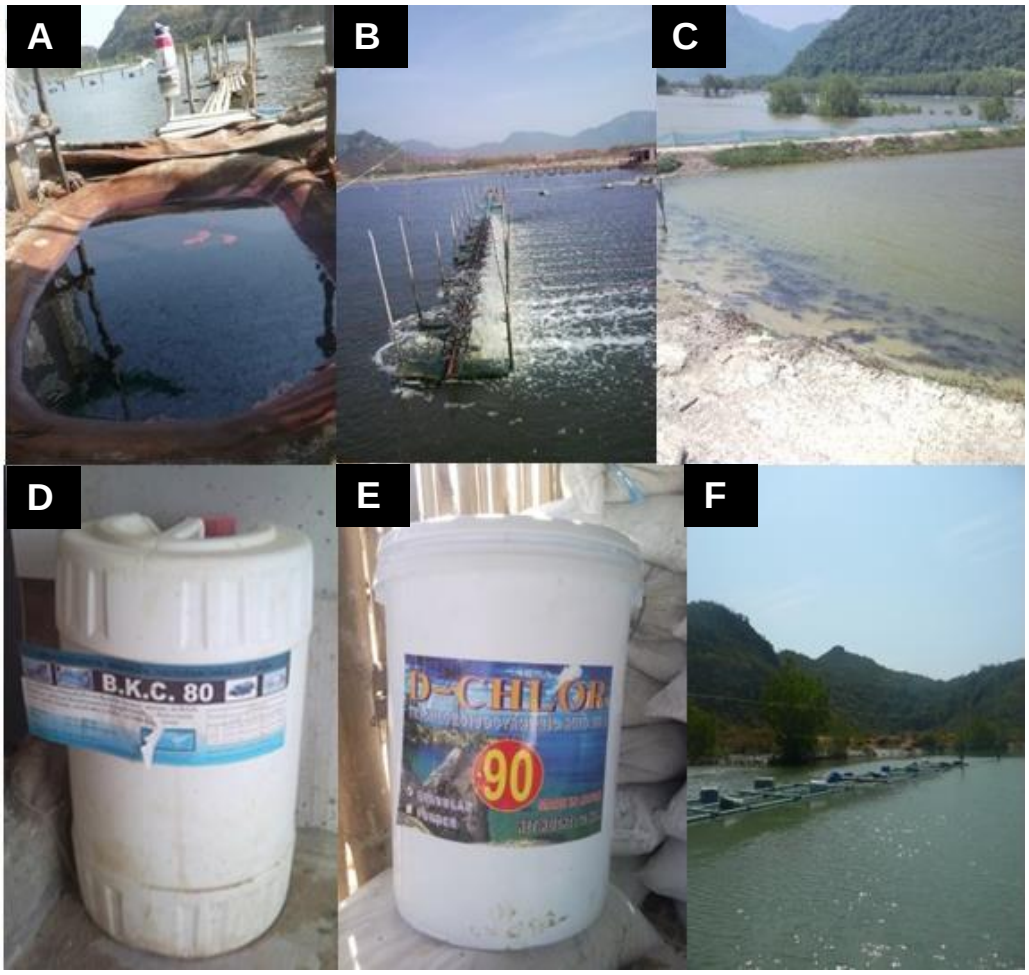

Figure 2 – Shrimp farms at the Marine National Park in Khao Sam Roi Yod, Hua Hin, Thailand. (A) Potassium permanganate puddle in the entrance of the shrimp tanks; (B) shows in detail a shrimp earth pond with aeration; (C) decantation tanks for effluents; (D and E) Benzalkonium Chloride and Sodium Hypochlorite used for pond treatment; (F) Cage farms

Antibiotics are among the most extensively used products worldwide. Antibiotics are natural or synthetic drugs with the capacity to kill or inhibit the growth of microorganisms (Serrano 2005). Antibiotics are mainly used in hospitals, agricultural fields, and livestock production facilities to either treat or prevent diseases (Bondad-Reantaso et al. 2012; Lipsitch et al. 2002; Nathan 2004). In many countries, there is considerable prophylactic use of antibiotics, particularly in shrimp hatcheries. Bacterial diseases occur most frequently and severely in intensive culture systems and thus, high amounts of antibacterial are used. Antibacterial agents are important in human and veterinary medicine. But their use in aquaculture may contribute to increased resistance and cause detrimental effects in medicine more generally. In aquaculture, antibiotics are applied directly into the aquatic compartment and have a high potential to reach the environment. Their high usage associated to the toxicological hazard especially to algae and invertebrates sentenced their inclusion in the priority lists of different countries (Berghmans et al. 2008; Boxall et al. 2003; Boxall 2004; Capleton et al. 2006; Kim et al. 2008). With the number of fish farms and aquaculture activity and the levels of antibiotics and drugs used in fish diseases treatment and prevention rapidly increasing, future research should be directed to the study of chronic effects of the exposure to these drugs in aquatic biota.

In Thailand, The Ministry of Agriculture and Cooperatives allow the use of antibiotics such as oxytetracycline in fish farming or any other medicines that are mixed with the feed according to a recommended dose. However, the misuse of antibiotics to treat diseased fish can have an effect on wild fish populations and other organisms although it is difficult to estimate these secondary effects on the surrounding ecology and environment. OTC is utilized in fish and shrimp farming and for other intensive animal farming systems as a growth promoter or for bacterial disease treatment. Several field studies have showed that OTC can persist in the sediment of aquatic environments for more than 180 days.

Predicting the impacts of the chemicals used in tilapia cage culture on aquatic ecosystems is a task often accomplished by means of laboratory assays, with single chemical exposures to obtain (sub) lethal effects concentrations for aquatic organisms. The EC50 or LC50 values are calculated for standard test

species, which are used to derive safe environmental concentrations. In general, this is a procedure used in the initial stages of risk assessments. As an alternative for the traditional approaches of toxicity assessment under laboratory standard conditions, several methodologies have been developed based on in situ exposure tests or bioassays with field samples. The combination of bioassays and environmental samples can be a useful strategy for a monitoring program or to provide information about priority areas to assess ecological damage. In addition, employing short-term bioassays to screen samples can exclude groups of chemicals or sites before the chemical analysis of environmental samples, which can reduce the cost and time of monitoring.

Many antibiotics are applied in aquaculture in Southeast Asia. In Thailand, protocols have been developed for risk assessment in coastal aquaculture which attempts to eliminate the use of antibiotics in shrimp farms; however, little efforts have been made by Thai authorities to control the use of antibiotics in freshwater aquaculture. After a comprehensive review that includes government reports and scientific papers (articles, thesis, books and minutes of meetings), OTC, an antibiotic widely used in Thailand; permitted by law, was chosen as a chemical model for environmental monitoring. In the second phase, tilapia cage farms located by the Moon River, Ubonrachatani, a tributary of the Mekong River were chosen for this study given the importance for local communities and due to the intensive use of OTC. This area was characterized through a visit where technical managers and employees were interviewed from August to November of 2010.

Hormones are used in aquaculture to promote the spawning, sexual differentiation or growth of cultivated organisms. The 1,7α-metiltestosterona (MT) is the most used androgenic agent in masculinization of tilapia species. In fish farms, MT is mixed to the feed of *Oreochromis niloticus* (tilapia) juveniles to induce sex reversion of females (Clemens and Inslee 1968; Homklin et al. 2011; Pandian and Sheela 1995). These techniques are widely applied in fish-farming to induce monosex males due to commercial interests since all male stock grows faster and homogenously (Johnstone et al. 1983).

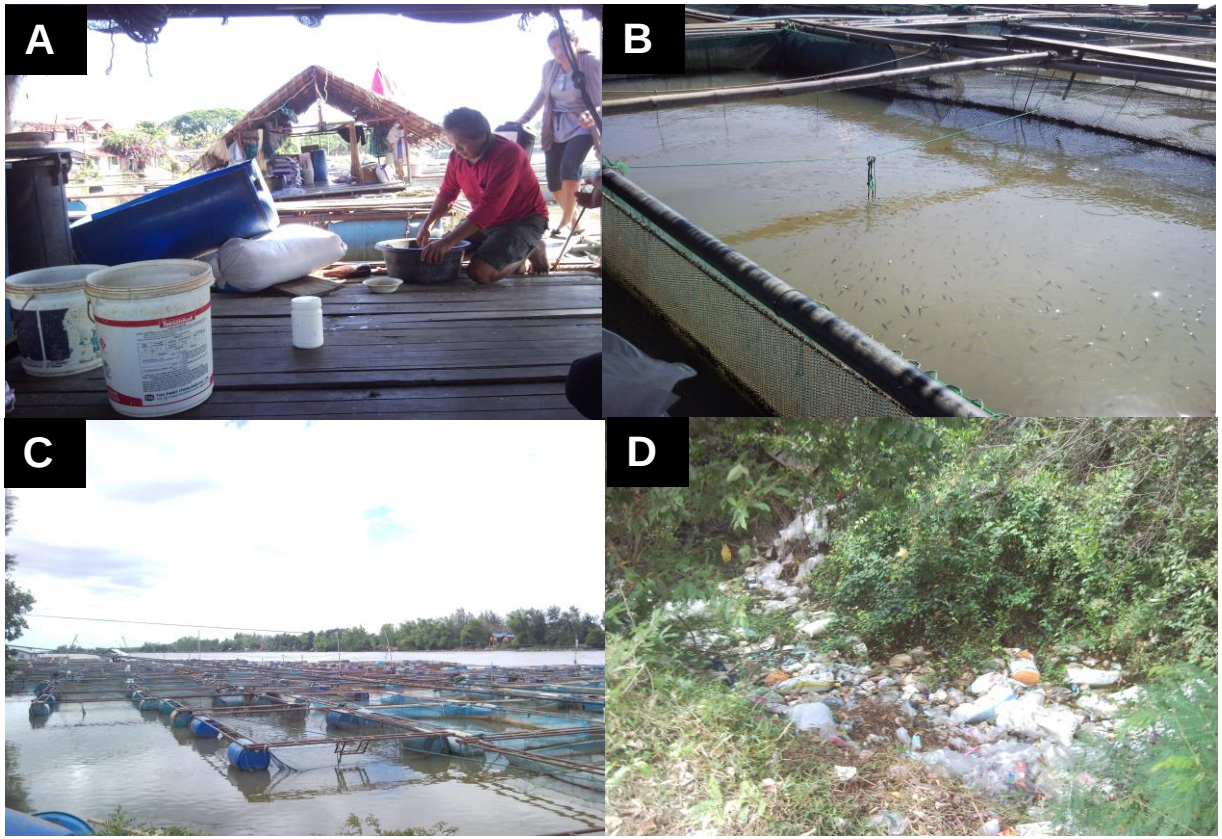

Figure 3 – An example of cage tilapia farm at Moon river, Ubon Ratchathani, Thailand: (A) Shows the fish oxytetracycline and/or enrofloxacin routine of administration mixed with the food; (B) shows in the details the cage for tilapia fingerlings growth; (C) shows an overview of the farm where the cages are placed in the rivers bank; (D) disposal of used food and chemicals packages.

The effects of MT in the target species are well known, but the possible adverse effects to the aquatic environment and different non-target species are not well known. Carp pituitary extracts, natural purified hormones, and synthetic hormones and peptides also have an important role in the aquacultures allowing the control ovulation timing and total control of life cycles of several species, consequently ensuring uninterrupted supply of early life stages organism.

In the earth ponds tilapia farms the input of MT to the environment is a result of untreated of effluent release in the aquatic ecosystem, which contains uneaten or non-metabolized MT-impregnated food, which can remain in the sediments of model ponds for up to three months. Furthermore, pond sediments are use as fertilizer for crop production and further spreading the risk of MT exposure to terrestrial organisms. In addition, currently there are no available studies about the MT effects on the health of employees at fish farms. The workers are exposed to MT in several ways, from the feed preparation to the pond maintenance activities (summarized in the **Table 3**). Despite the evidence of biological effects of MT there is no specific regulation or training program for teach the standard procedures for dealing with chemicals and products such as MT. Therefore, efforts between farmers, researchers, and government workers are essential to generate data on the effects of hormone MT on different aquatic species, to evaluate risks to ecosystems and human health.

The detection of different chemicals in aquaculture surrounding areas have caught the attention of the scientific community which have been pressing the responsible authorities to look for new methodologies to estimate with more accuracy the risk of aquaculture chemicals to the human health and to the environment (Palaniyappan et al. 2013; Zheng et al. 2012).

Table 3 - Routes of exposure of staff to 17-α-methyltestosterone during hormonal sex reversal of tilapia at Nam Sai Farms CO. LTD

1) Feed preparation

- *Preparation of stock solution in which MT is dissolved in 95% ethanol:* 5g of hormone (weighed on a balance by management staff) is dissolved in 10 liters of 95% (or more)food grade ethanol. 10 liters divided into 11 bottles of 900 ml. There is almost no contact with the liquid except for occasional small spillages.

- *Mixing of stock solution with feed:* 15 kg of feed is added to the mixer and 2 bottles of stock solution added. A further 1.8 liters of ethanol is added and the feed thoroughly mixed. There is no contact with the feed during mixing.

- *Drying of feed outside on plastic sheets:* The feed is tipped out of the mixer (mixer can be rotated) onto a plastic sheet and the feed is then funneled into a plastic bag. After preparing 200 kg of feed it is dried on plastic sheets outside. The feed is turned over via the use of a rake. Protective masks are used during this process. There is contact with the feed on the hands to some extent via contact with the bags and plastic that the feed is placed on for drying. Gloves are not worn.

2) Feeding fish

- *Weighing feed into plastic bags:* Feed is scooped into a plastic bag on a balance using a rice spoon. 1 bag is prepared for 1 hapa per day. Each farm has approximately 40 hapas at any one time. There is little contact with the feed during weighing, although inevitably they will get some on their hands. Masks are not always worn during weighing.

- *Feeding fish:* The bags of feed are kept in a basket carried around the pond. One worker puts one fifth of the feed (5 feeds per day) in a feeding pot held by another worker. This is done either without touching the feed using a spoon or simply by pushing the sides of the bag to force the feed out. The worker holding the feeding pot broadcasts the feed over the water surface in the hapa and doesn't touch the feed. There is little contact with the feed, although hands of the person putting the feed in the pot will inevitably come in contact with some.

3) Stocking and harvesting fish

- *Installing hapas:* Workers install hapas and stock swim-up fry in the pond every day for an average of 1 hour. This occurs at the start of the cycle before feeding commences. The time for stocking is quick and hormone levels in the water will be low, so small exposure is predicted.

- *Harvesting fish at end of sex reversal:* Workers spend an average time of 2.5 hours per day in the water harvesting and grading fish that have finished sex reversal. The fish are then transferred to nursery ponds where MT is not used. Hormone levels in the water will be high at the end of the cycle and harvesting and grading takes time. Hence, exposure will be significant.

Persistent chemicals or metabolites that may lead to long-term effects on non-target organisms and chemicals that have a continuous input to aquatic ecosystems (despite their half-life) are likely to cause chronic effects on aquatic biota. Issues of food safety of aquaculture products, which concern public health authorities and affect consumer acceptance in general, are receiving growing attention. It can be expected that there will be increasing concerns with regard to issues of environmental impacts by aquaculture farms affecting the products of neighboring farms, self-pollution, and environmental impacts by non-aqua culturists affecting the quality and safety of both aquaculture products as well as of aquaculture supplies, especially feeds and feed ingredients (FAO, 2005). Effects can also occur at a distance with interchange of living material between farms and a consequent spread of disease (FAO, 2005).

In an attempt to predict and generate regulatory frameworks on the environmental contamination arising from aquaculture are employed traditional methods of risk assessment based on models for predicting environmental concentrations of contaminants and acute toxicity tests with organism's water models. The Lethal Concentrations (LC_x) and Effective Concentrations (EC_x) values obtained by means of laboratory tests are used to derive concentrations of chemicals that are safe for the environment. These procedures are part of the initial stages of risk assessment, however your exclusive use and widespread has been questioned by the scientific community and regulatory authorities. Many studies with aquatic organisms show that, although no lethal effects are detected during the exposure to contaminants, alterations at several biologic levels (enzymatic, genetics, embryologic) are possible to identify, even when these compounds are present at low concentrations. This may result in a negative long-term impact to the ecosystem. In this way, choosing uniquely lethal parameters can lead to an underestimation of the environmental risk of a chemical compound.

The current legislation in Europe and in other countries requires adequate data on risk assessment for the registration of chemicals. The European Medicines Evaluations Agency (EMEA) stipulates acute fish tests in the protocols for evaluation of environmental risk of veterinary products for aquaculture ecosystems. In the EMEA/VICH guidelines, the environmental risk of aquaculture

drugs is assessed in a tiered approach (EMEA 2006, EMEA 2007). Within the first phase, a number of questions concerning the drugs application and its properties is raised. The environmental exposure is estimated based also on the expected amount of the product to be applied. For example, if the predict environmental concentration (PEC) exceeds 10 ng/L in the water, studies on environmental fate and acute effects on selected species (fish, daphnia, algae) have to be performed in the second phase. In phase II the environmental risk is evaluated deterministically by comparing the predicted environmental concentrations (PECs) with the predicted no effect concentrations (PNECs) in several environmental compartments. Thus, intensive experimental testing is required for implementing a phase II ERA. As final steps, risk quotients (RQ) of PECs and PNECs are calculated: a value less than one indicate that no further testing of the veterinary medicines is necessary.

This thesis addressed the environmental toxicology, fate and impacts of chemicals used in three different types of aquaculture in Thailand, namely shrimp farms and tilapia farms in cages and in earth ponds. Surveys and monitoring campaigns were conducted (see Annex I and II). In cage farms the results indicated overuse/misuse of antibiotics, in shrimp farms the major group of chemicals used were disinfectants whereas in earth pond farms 1,7α-methyltestosterone (MT) was the most used. The results from the monitoring campaigns showed that the excessive use of chemicals was correlated to contamination of the surrounding environment namely with the antibiotics oxytetracycline and enrofloxacin in the cage farms (see Chapter 10), and with MT in the earth pond farms (Barbosa et al. 2013). Moreover, the Chapters 2 to 9 present all results and discuss the aquatic toxicity of the disinfectants (glutaraldehyde, chlorhexidine, benzalkonium chloride), pesticides (trichlorfon and ivermectin) and antibiotics (oxytetracycline, amoxicillin and enrofloxacin).

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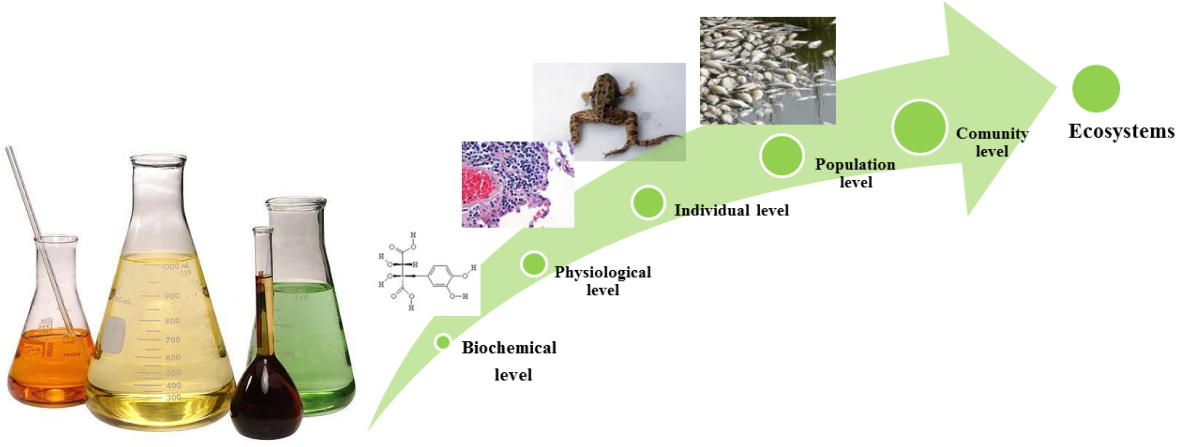
Chapter 2

From sub cellular to community level: toxicity of glutaraldehyde to several aquatic organisms



From sub cellular to community level: toxicity of glutaraldehyde to several aquatic organisms

Rhaul Oliveira†; Susana P.P. Pereira†; Sónia Coelho; Carolina Musso; Amadeu M.V.M. Soares; Inês Domingues & António J.A. Nogueira

† The two authors contributed equally to the study

This chapter has been accepted as an original article in:

Science of total enviroment

Abstract

The biocide glutaraldehyde (GA) is widely used as a disinfectant and sterilizing agent against bacteria and virus in hospital and veterinary facilities. In this work, the GA lethal and sub lethal toxicity to aquatic organisms from different trophic levels was evaluated. A battery of toxicity tests with primary producers, primary consumers and secondary consumers were performed and a species sensitive distribution (SSD) for GA was built. Moreover, effects on biomarkers (catalase, lactate dehydrogenase, glutathione-S-transferase, and cholinesterase) were measured in *Danio rerio* embryos and adults. Primary consumers (*Thamnocephalus platyurus* 24h – EC50 = 3.6 mg/l; *Daphnia magna* 48h – EC50 = 6.6 mg/l) and *D. rerio* adults (96h – LC50 = 5.5 mg/l) were slightly more sensitive to GA than *D. rerio* embryos (96h – LC50 = 22.2 mg/l) and primary producers (*Lemna minor* 168h – EC50= 73.8 mg/l; *Pseudokirchneriella subcapitata* 72h – EC50 = 12.3 mg/l; *Chlamydomonas reinhardtii* 72h – EC50 = 14.6 mg/l; *Chlorella vulgaris* 72h – EC50 = 31.3 mg/l). However, no significant differences between the trophic levels were found and general HC5 and HC50 values of 0.3 and 8.1 mg/l were respectively estimated. Despite the low GA toxicity to *D. rerio* embryos, hatching delay and malformations were found (96h – EC50 = 11.9 mg/l). For biomarkers, an inhibition of lactate dehydrogenase activity was observed in embryos whereas an inhibition in catalase, lactate dehydrogenase and glutathione-S-transferase activities was observed in adults. Thus, GA is moderately toxic (doses >1 mg/l) to aquatic organisms, independently of the trophic level. However, considering the varied range of effects depending on the life stage and organism tested and relatively low HC5 value of 0.5 mg/l, mesocosm and chronic toxicity tests seem to be a next step in direction of more realistic scenarios of GA risk assessment in aquatic ecosystems.

Keywords: primary producers; primary consumers; *Danio rerio*; malformations; biomarkers; species sensitivity distribution; glutaraldehyde; biocides

1. Introduction

The biocide glutaraldehyde (GA) is widely used as disinfectant and sterilizing agent against bacteria and virus. Its biocidal properties are caused by the cross-linking of amine groups in the cell membranes of microorganisms (Boillot, 2007), such as Gram-negative bacteria, fungi, and virus (Simões et al., 2011). GA is mainly used for disinfection in hospital and livestock production facilities (e.g. aquaculture); other applications include hardener in x-ray film processing, biocide in the paper pulp and petroleum industries, embalming agent (e.g. microscopy), preservative for canned food and cosmetics.

Particularly in hospitals, high amounts of GA are daily used for sterilization with concentrations between 0.50 and 3.72 mg/l being already reported in hospital wastewaters (Jolibois et al., 2002). Without a proper treatment these toxic residues might affect organisms on receiving water bodies (Boyd and Massaut, 1999; Hoh and Berry, 2005). The ratio between measured concentrations in hospital wastewaters and predicted non-effect concentration (PNEC) can reach up to 4960 (Orias and Perrodin, 2013). This high risk quotient value suggests that under scenarios of insufficient dilution or major discharge of hospital wastewaters, GA represents a potential risk for the environment (Boillot, 2007; Emmanuel, 2005; Jolibois et al., 2002). In accordance, the aquaculture industry also applies high doses of GA for several disinfection purposes (Mainous et al., 2012). Some examples are found in the literature: in shrimp farms it is applied as a bactericide in a range of 1 to 10 mg/l (Boyd and Massaut, 1999) or for surface disinfectant of eggs of numerous fish species it is used in short-time prophylactic therapies at concentrations between 200 and 400 mg/l (Katharios et al., 2007).

GA has been described as toxic to several aquatic organisms including algae, invertebrates and fish (**Table 1**). However, despite previous studies on the GA toxicity at the species level, little is known about GA effects at the subcellular and community levels. The GA intrinsic characteristic of cross-linking with proteins can affect the activity of some enzymes (Walt and Agayn, 1994). Thus, enzymatic biomarkers from different metabolic pathways (e.g. neuronal, energetic metabolism and oxidative stress) can be used to evaluate

the early stress responses to GA and additionally provide insights on its mechanism of toxicity (Elia et al., 2006; Van der Oost et al., 2003). Among the neuronal enzymes, the cholinesterase (ChE) activity is the most used as biomarker due to its key role in the hydrolysis of acetylcholine in peripheral and central nervous systems (Holth et al., 2008). The glutathione-S-transferase (GST) is an enzyme involved in the detoxification of exogenous and endogenous compounds (Oruç and Üner, 2000). The lactate dehydrogenase (LDH) is a glycolytic enzyme virtually present in all tissues, catalyzing the interconversion of pyruvate and lactate (Diamantino et al., 2001). Catalase (CAT), an antioxidant enzyme that acts against reactive oxygen species (ROS) can be useful to assess the oxidative stress status of organisms (Barata et al., 2005). Regarding the effects at higher organization levels, the estimation of GA risk to natural populations is often based on single species toxicity tests performed in order to assess a PNEC. Nevertheless, to better understand the effects of GA at a community level, approaches such as Species Sensitivity Distributions (SSDs), integrating toxicity tests with several species from different trophic levels, can be employed (Maltby et al., 2005; Mochida et al., 2012). In the SSD approach the responses of different organisms are displayed as cumulative distribution functions allowing the estimation of the affected fraction of the community and the calculation of the hazardous concentrations for 5 and 50 % of species (HC5 and HC50, respectively) (Beaudouin and Péry, 2013).

This work aims to assess GA effects in aquatic organisms at different levels of organization, including the individual, subcellular and community levels. The effects at the subcellular level were evaluated using a battery of biomarkers in *D. rerio* embryos and adults, namely ChE, GST, LDH and CAT. The effects at the individual levels were evaluated using model species from different trophic levels: primary producers (*Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Lemna minor*), primary consumers (*Daphnia magna* and *Thamnocephalus platyurus*) and a secondary consumer (*Danio rerio*, adults and embryos). The toxicity values for the tested species were used to develop a SSD, which allows the estimation of GA concentrations that might affect relevant fractions of the aquatic communities (HC5 and HC50).

Table 1 – Glutaraldehyde effects on different aquatic organisms

Species	Value (mg/l)[1]	Endpoint	Effect	Exposure	Time (days)	Reference
Primary producers						
Pseudokirchneriella subcapitata	3.9	ILm[2]	Biomass	–	4	Vilkas's report (as cited in OECD SIDS, 1998)
	0.81	EC_{50}	Biomass	–	5	WIL's report (as cited in Leung, 2001)
	0.5	NOEC	Growth			
	0.31	IC_{50}	Growth	Static	4	(USEPA, 2000)
	0.042	NOEC				
	0.75	IC_{50}	Growth	Static	5	
	0.5	NOEC				
	3.95	EC_{50}	D.O.P.[3]	Static	2	(Chen et al., 2005)
	13.2	EC_{50}	Growth			
	1	IC_{50}	Growth	Static	4	(Sano et al., 2005)
	0.7	NOEC				

	1.4	LOEC				
	1.8	IC_{50}				
	1.3	NOEC				
	2.1	LOEC				
Scenedesmus subspicatus	0.97	EC_{50}	Biomass	–	4	(RCC, 1990)
	0.31	NOEC				
	1	EC_{50}	Growth	–	4	NICNAS's report (as cited in Boillot, 2007)
Skeletonema costatum	0.17	EC_{50}	Growth	–	3	SINTEF's report (as cited in Leung, 2001)
	0.92	EC_{50}	Growth			
	0.61	EC_{50}	Biomass	–	3	SPL's report (as cited in Leung, 2001)
	0.33	NOEC	Growth			
Primary consumers						
Acartia tonsa	0.11	LC_{50}	–	Static	2	SPL's report (as cited in Leung, 2001)
	0.029	NOEC				

Artemia sp.	27	LC_{50}	Survival	Static	3	(Sano et al., 2004)
Americamysis bahia	7.1	LC_{50}	–	Flow Through	4	SLI's report (as cited in Leung, 2001)
	0.78	NOEC				
	7.1	LC_{50} (early juvenile)	–	Flow Through	4	(USEPA, 2000)
	5.5					
	20.6	LC_{50} (adults)		Static		
Balanus improvisus	7.5	LC_{50}	–	Static	10	SINTEF's report (as cited in Leung, 2001)
Carcinus maenas	1100	LC_{50}	Survival	Static	2	UCC's report (as cited in OECD SIDS, 1998)
	465	LC_{50}			4	
Ceriodaphnia dubia	9	LC_{50}	Survival (neonates)	Static	1	(Sano et al., 2003)
	10	LC_{50}	Survival (adults)		1	
	4.7	LC_{50}	Survival	Semi-static	8	(Sano et al., 2005)
	2.4	NOEC				
	4.9	LOEC				

	4.7	LC_{50}				
	2.4	NOEC	Reproduction			
	4.9	LOEC				
	4.9	NOEC	Growth			
Chaetogammarus marinus	582				1	
	304	LC_{50}			2	Adema and Bakker's paper (as cited in OECD SIDS, 1998)
	208		Survival	Semi-static	3	
	191				4	
	56	NOEC			4	
Crassostrea virginica	0.55	LC_{50}	–	Static	2	UCC's report (as cited in Leung, 2001)
	0.78	LC_{50}	–	Flow Through	4	SLI's report (as cited in Leung, 2001)
	0.16	NOEC				
	0.75	EC_{50}	Intoxication	Flow Through	4	(USEPA, 2000)
	< 0.089	NOEC				
Daphnia magna	>25	LC_{50}	Survival	Static	1	UCC's report (as cited in OECD SIDS, 1998)
	16.3	LC_{50}			2	
	8	NOEC				

0.35	LC_{50}	Survival	Static	2	Vilkas's report (as cited in OECD SIDS, 1998)
5	LC_{50}	Survival	Static	2	UCC's report (as cited in Leung, 2001)
2.1	NOEC	Reproduction	Semi-static	21	CCR's report (as cited in Leung, 2001)
16.3	LC_{50}	Survival	–	2	NICNAS's report (as cited in Boillot, 2007)
4.3	LOEC	Reproduction		21	
0.75	EC_{50}	Intoxication	Static	2	(USEPA, 2000)
3.5	EC_{50}				
6.7	EC_{50}				
14.6	EC_{50}				
8.5	NOEC	–	Semi-static	22	(USEPA, 2000)
4.25	LOEC				
14	LC_{50}	Survival (neonates)	Static	1	(Sano et al., 2003)
56	LC_{50}	Survival (adults)			
21	LC_{50}	Survival (neonates)	Static	1	(Sano et al., 2004)

	20.0	EC_{50}	Immobilization	Static	1	(Boillot, 2007)
Dreissena polymorpha	21	LC_{50}	Survival	Static	2	(Sano et al., 2004)
Hyalella azteca	289	LC_{50}	Survival	Static	1	(Sano et al., 2003)
	189	LC_{50}	Survival	Static	1	(Sano et al., 2004)
Lumbriculus variegatus	11.1	LC_{50}	Survival	Static	1	(Sano et al., 2003)
	6.3[4]	LC_{50}	Survival	Static	1	(Sano et al., 2004)
	16[5]	LC_{50}				
Mytilus edulis	0.2	LC_{50}	–	Flow Through	5	SINTEF's report (as cited in Leung, 2001)
Palaemonetes vulgaris	400	LC_{50}	Survival	Static	2	UCC's report (as cited in OECD SIDS, 1998)
	41	LC_{50}	–	Static	4	SINTEF's report (as cited in Leung, 2001)
Secondary consumers						
Cyprinodon variegatus	32	LC_{50}	–	Static	4	SLI's report (as cited in Leung, 2001)
	24	NOEC				

	31.4	LC_{50}	Survival	Flow Through	4	(USEPA, 2000)
	40	LC_{50}		Static		
Danio rerio	5.8	LC_{50}	–	–	4	(OECD SIDS, 1998)
Lepomis macrochirus	9.4	LC_{50}	–	Static	4	UCC's report (as cited in Leung, 2001)
	2.5	NOEC				
	11	LC_{50}	–	Static	4	UCC's report (as cited in Leung, 2001)
	5	NOEC				
	14.9	LC_{50}	Survival	Static	1	Vilkas's report (as cited in OECD SIDS, 1998)
	11.8	LC_{50}			2	
	11.2	LC_{50}			4	
	10	NOEC				
	12.2	LC_{50}	Survival	Static	4	(USEPA, 2000)
	7.8	NOEC				
	22.6	LC_{50}				
	7.8	NOEC				

Oncorhynchus kisutch	3	LC_{50}	–	Flow through	4	SFU's report (as cited in Leung, 2001)
Oncorhynchus mykiss	11	LC_{50}	–	Static	4	UCC's report (as cited in Leung, 2001)
	8	NOEC	Survival	Flow through		
	12	LC_{50}	–	Static	4	UCC's report (as cited in Leung, 2001)
	9	NOEC	Survival	Flow through		
	3.5	LC_{50}	Survival	Flow through	4	(USEPA, 2000)
	9.5	LC_{50}		Static		
	23.9	LC_{50}				
	18	NOEC				
	1.6	NOEC	Survival (early-life stages)	Flow through	97	
	5.1	LOEC				
	13.6	NOEC	Survival (embryos)	Semi-static	35	(Sano et al., 2005)
	1.8	EC_{50}	Hatch-out rate	Semi-static	62	
	1.3	NOEC				

	2.5	LOEC				
	1	NOEC	Survival (larvae)			
	1	NOEC	Growth (larvae)			
Pimephales promelas	6	LC_{50}	–	Static	4	SLI's report (as cited in Leung, 2001)
	4	NOEC				
	5.4	LC_{50}	–	Static	4	UCC's report (as cited in Leung, 2001)
	2.6	NOEC				
	11.6	LC_{50}				
	5.4	LC_{50}	Survival	Static	4	(USEPA, 2000)
	2.6	NOEC				

[1]All concentration values refer to the active ingredient concentration of GA
[2] Median inhibitory limit
[3] Dissolved oxygen production
[4] Organism were exposed to GA in 19ºC
[5] Organism were exposed to GA in 10ºC
"-" means information not available

2. Materials and Methods

2.1 Test solutions and GA chemical analysis

High purity GA ($C_5H_8O_2$; CAS no 111-30-8), aqueous solution (≈ 50% water; Fluka, St. Louis, MO) was used in all the toxicity tests. For each test, a stock solution was freshly prepared by diluting the GA aqueous solution in the appropriate test medium. Stock solutions were kept refrigerated and protected from light. Test solutions were prepared immediately before the test by successive dilution of the stock. In addition, four vessels containing 100 ml of GA solutions with a nominal concentration of 1 mg/l were conditioned in the climatic chamber of each test. Five ml of each vessel were daily sampled and preserved at -20 ºC to further chemical analysis. The chemical analysis aimed to assess the degradation of GA in the tests conditions of each toxicity test and was conducted spectrophotometrically at 635 nm (Thermo Scientific Multiskan® Spectrum) based on the procedure proposed by Matthews and Howell (1981) adapted for microplate. The results are presented in the **Supplementary Material 1**.

2.2. Microalgae assays

Three species of freshwater unicellular green algae, *C. reinhardtii*, *C. vulgaris* and *P. subcapitata* were obtained from non-axenic batch cultures of Woods Hole MBL medium at 24 ± 1 ºC and continuous light (cool-white fluorescent light, 3400 lux).

The algae growth inhibition tests were based on the OECD guideline 201 (OECD, 2006a) and ran for 72 h. Each species was exposed to control medium and GA concentrations of 3.2, 10, 16, 50 and 100 mg/l, in triplicate. Tests started with 1.0×10^4 cells/ml of algae (log exponential growth phase) in Erlenmeyer flasks (50 ml of exposure solution). The test conditions were similar to the culture conditions, except that the Erlenmeyer flasks were incubated in an orbital shaker at 150 rpm. The absorbance (λ = 440 nm) was daily measured by spectrometry (Thermo Scientific Multiskan® Spectrum). The absorbance values (ABS) were converted to the number of cells using regression models previously developed:

$\text{Cell / ml} = 2.0^7 \times ABS - 2.0^4$ for *C. reinhardtii*

$\text{Cell / ml} = 2.0^4 \times ABS - 8.0^6$ for *C. vulgaris*

$\text{Cell / ml} = 2.0^5 \times ABS - 1.0^7$ for P*. subcapitata*

The average specific growth rate for a specified period was calculated as the logarithmic increase in cell concentration from the following equation:

$$\mu_{i-j} = \frac{\ln Xj - \ln Xi}{tj - ti}$$

Where μ_{i-j} is the average specific growth rate from time *i* to *j* (per day); *Xi* is the cell concentration at time *i*, and *Xj* is the cell concentration at time *j*; *ti* is the initial time of the exposure and *tj* is the final time of the exposure (OECD, 2006a).

2.3 Lemna minor assays

The *L. minor* monoculture was established as an axenic culture using a modified Swedish Standard medium (OECD, 2006b) at 25 ± 1 ºC and continuous light (6,500 – 10,000 lux).

The macrophyte growth inhibition test was based on the OECD guideline 221 (OECD, 2006b) and ran for 168 h. *L. minor* was exposed to control medium and GA concentrations of 1, 1.8, 3.2, 5.6, 18, 32, 100 mg/l, in triplicate. Each replicate consisted in 4 sets of fronds, each one with 3 leaves in a glass beaker (100 ml of exposure solution). The test conditions were similar to the culture conditions. Effects on number of leaves and chlorosis were recorded daily; additionally fresh weight, dry weight and root size were measured after the exposure period. The average specific growth rate per day was calculated as the logarithmic increase in number of leaves from the equation:

$$\mu_{i-j} = \frac{\ln Xj - \ln Xi}{tj - ti}$$

Where μ_{i-j} is the average specific growth rate from time *i* to *j* (per day); *Xi* is the number of leaves at time *i*, and *Xj* is the number of leaves at time *j*; *ti* is

the initial time of the exposure and *tj* is the final time of the exposure (OECD, 2006b).

Doubling time of frond number (*Td*) was calculated as:

$$T_d = \frac{\ln 2}{\mu}$$

Where *μ* is the average specific growth determined.

2.4 Daphnia magna assays

D. magna, clone K6, were cultured in ASTM had water (ASTM, 1998), enriched with seaweed extract (Marinure, Glenside Group, UK) and daily fed with the algae *C. vulgaris* (3.0×10^5 cells/ml). The culture was kept under constant/controlled physical conditions (24 ± 2 °C, 16h : 8h light/dark photoperiod cycle and 3400 lux light intensity) and water physicochemical parameters (total hardness = 175.4 ± 5.5 mg/l $CaCO_3$, pH = 8.2 ± 0.3 and conductivity = 577.6 ± 9.0 µS/cm) . The culture was renewed three times per week. Newly released neonates (<24h old; 3rd to 5th broods) were used in the *Daphnia* immobilization test.

The immobilization test was performed according to the OECD guideline 202 (OECD, 2004) and ran for 48 h. *D. magna* neonates were exposed to control medium and GA concentrations of 4.8, 5.8, 6.9, 8.3, 10.0 and 11.9 mg/l, in triplicate. Each replicate consisted in 5 neonates in a glass beaker (100 ml of exposure solution). The test conditions were similar to the culture conditions, except that no food was provided during the test. Immobilization (defined as the inability to swim or move within 15 s of gentle agitation and taken to indicate lethality) was daily recorded.

2.5 Thamnocephalus platyurus assays

T. platyurus was obtained through the THAMNOTOXKIT™ kits (Microbiotests, Inc., Mariakerke – Gent, Belgium). The cysts of *T. platyurus* were incubated with Toxkit standard freshwater medium at 25 ± 1 °C and fluorescent light (3400 lux) for 20 – 22 h to hatch.

The acute test followed the ISO standard 14380 (ISO, 2011) and ran for 24 h. *T. platyurus* were exposed to control medium and GA concentrations of 4, 8, 12, 16 and 20 mg/l, in triplicate. Each replicate consisted in 10 organisms per treatment which were firstly placed in a *Petri* dish with the respective test solution and then transferred to the one well of a 24-well microplate (1 ml of exposure solution). This procedure was adopted to avoid dilution of the exposure solutions in the microplates. Microplates were incubated in the dark at 25°C ± 1ºC. After the exposure period, mortality was recorded under a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation, Kanagawa, Japan).

2.6 Danio rerio assays

D. rerio facility established at the Department of Biology, University of Aveiro (Portugal) provided all organisms (embryos and adults) used in this study. In the facility, organisms were kept in carbon filtered water, complemented with salt “Instant Ocean Synthetic Sea Salt” (Spectrum Brands, Madison, WI), which was also used in the preparation of all test solutions for *D. rerio* tests. This water was kept at 25.0 ± 2 ºC, conductivity of 750 ± 50 µS/cm, pH of 7.5 ± 0.5 and dissolved oxygen > 95 % saturation. Photoperiod was adjusted to16 h: 8 h light/dark cycle. Adult *D. rerio* were fed twice daily with commercially available artificial diet (ZM 400 Granular, ZM systems, Hampshire, UK) and brine shrimp. *D. rerio* eggs were collected immediately after natural mating. Before the test, eggs were rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope – SMZ 1500, Nikon Corporation, Kanagawa, Japan); those with cleavage irregularities or injuries were discarded.

The embryo test was based on the OECD draft guideline on Fish Embryo Toxicity Test (FET) (OECD, 2012) and in an extended version described in detail in Domingues et al. (2010b) and ran for 120 h. *D. rerio* newly fertilized eggs were exposed to control medium and GA concentrations of 0, 5, 10, 25, 50, 75 and 100 mg/l, in quadruplicate. Each replicate consisted in 10 eggs individually placed in the wells of 24-wells microplates (2 ml of exposure solution). The test conditions were similar to the culture conditions. Embryos

were daily observed under a stereomicroscope (magnification used for observations was 70 x for embryos and 40 x for hatched embryos). Different endpoints were evaluated, observed and reported in the embryo (coagulation of fertilized eggs, lack of somite formation, non-detachment of the tail-bud from the yolk sac, lack of heart-beat, delay in eye and body pigmentation, delay in the absorption of the yolk sac, delay of the otolith formation, lack of tail blood circulation, edemas, spine deformations, abnormal posture, undersize, hatching delay and mortality of the post-hatched embryos. All parameters were assessed in a qualitative way (presence or absence).

For biomarker analysis a sub lethal range of GA (0, 5, 10 and 25 mg/l) was tested using a similar design. . After 96 h of exposure 10 clusters of eight embryos per treatment were snap-frozen in microtubes for posterior biomarkers determinations.

D. rerio adults test followed the OECD guideline 203 (OECD, 1992) and ran for 96 h. Adults of similar length and age (2 ± 1 cm, 6 months old) were exposed to control medium and GA concentrations of 1.2, 2.8, 4.2, 5.8 and 7.2 mg/l, in quadruplicate. Each replicate consisted in 3 adults in aquaria (1000 ml of exposure solution). The test conditions were similar to the culture conditions, except that no food was provided during the test. During the test, mortality and behavior changes were daily recorded. A sub lethal test of GA (0, 1.2, 2.8 and 4.2 mg/l), using a similar design was run to collect organs for biomarker analysis. At the end of the test, the living fish were sacrificed in ice by decapitation. Heads, muscles and gills were isolated and snap-frozen in microtubes for posterior biomarkers determinations.

Each one of the adult tissue and embryos samples were conditioned in adequate buffer (K-phosphate buffer, 0.1 M, pH 7.2 for ChE and CAT; K-phosphate buffer, 0.1 M, pH 6.5 for GST and Tris-NaCl buffer, 0.1 M, pH 7.2 for LDH) and preserved at - 80 ◦C until analysis.

2.7 Biomarkers determinations

In embryos, the determination of the enzymatic activities was performed using clusters of organisms, as mentioned previously. However, in adults, the

determination of the enzymatic activities was carried out in different organs: ChE activity was measured in heads and muscle, LDH activity was measured in the muscle, GST activity was measured in the liver and gills and CAT activity was measured in liver and muscle. On the day of analysis, samples were defrosted on ice, homogenized (Ystral GmbH D – 7801, Dottingen, Germany).

Briefly, ChE activity was determined, using 50 µl of homogenate (4º C, 3000 g, 10 min) and 250 µl acetylthiocholine solution, at 414 nm (20 min) through the conjugation product of thiocoline (a product of the degradation of acetylthiocholine) and 5,5 – dithiobis – 2 – nitrobenzoic acid (absorbance increase), according to the method of Ellman (1961). GST activity test, using 100 µl of homogenate (4 °C, 9000 g, 30 min), was based on the measurement (5 min) of the conjugation product between the 1 – chloro – 2,4 – dinitrobenzene (substrate) and glutathione at 340 nm (absorbance increase) according to the method of Habig and Jakoby (1981). Determination of LDH activity, using 40 µl of homogenate (4 °C, 3000 g, 5 min), was based on the decrease of absorbance (5 min) due to the oxidation of NADH measured at 340 nm, according to the method of Vassault (1983). CAT activity, using 15 µl of homogenate (4º C, 3000 g, 10 min), was determined at 240 nm by monitoring (during 3 minutes) the decrease of absorbance due to degradation of H_2O_2, as described by Clairborne (1985).

Enzyme activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute per mg of protein.

Protein concentration in samples was determined in quadruplicate by the Bradford method (Bradford, 1976), at 595 nm, using γ – globulin as standard. A Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA) reader was used for all biochemical determinations except CAT which was determined using quartz cell and a Jenway 6505 UV/vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK).

2.8 Species sensitivity distribution (SSD)

The SSD was performed with toxicity values (EC50) from the experimental data of the present study and values retrieved from the current

literature (**Table 1**). Multiple toxicity data for the same species were summarized as geometric means. A logistic curve (log) was fitted to the data using nonlinear regression. The predicted toxicity for the 5 % and 50 % most sensitive organisms were estimated (HC5 and HC50, respectively). The SSD plot was generated using the U.S. Environmental Protection Agency spreadsheet build over excel (USEPA, 2005). In order to compare the sensitivity of primary producers, primary consumers and secondary consumers to GA, significant differences between the distributions of toxicity data were evaluated by the two-sample Kolmogorov-Smirnov test.

2.9 Statistical Analysis

Sigma Stat 3.5 statistical package was used for statistical analyses (SPSS, 2004). A one-way ANOVA was used to detect the differences between the groups for normally distributed data sets. When data did not pass the Kolmogorov–Smirnov normality test and the Levene's homogeneity of variance test, a Kruskal–Wallis test was used. However, if significant results were found, the Dunnett or Dunn's test (for parametric or non-parametric tests, respectively) was used to detect significant differences between the tested concentrations and the control. The toxicity values LC50 or EC50 were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel. All statistical analyses based on 0.05 significance level.

3. Results

3.1 Toxicity tests

Among primary producers, GA was more toxic to the microalgae than the macrophyte. The 72 h- EC50 for growth inhibition for *C. reinhardtii*, *C. vulgaris* and *P. subcapitata* was respectively 14.6 mg/l, 31.3 mg/l and 12.3 mg/l, whereas the 168 h – EC50 for *L. minor* was 73.8 mg/l (based on dry weight). Concerning *L. minor*, GA caused toxic effects on various endpoints, namely reduction of the dry weight (One–way ANOVA: $F_{10, 32} = 31.5$, $p < 0.001$), an increase followed by a reduction of the fresh weight (One–way ANOVA: $F_{10, 32} = 50.5$, $p < 0.001$), reduction of the number of leaves (One–way ANOVA: $F_{10, 32} = 58.5$, $p < 0.001$), increase of the doubling time (Kruskal–Wallis H =

29.6, p = 0.001), and reduction of the root size (One–way ANOVA: F 7, 23 = 29.7, p < 0.001). The EC_{50} for each parameter is showed in the **Table 2**.

GA showed to be highly toxic for primary consumers. The 48 h – EC50 for *D. magna* was 6.6 mg/l and the 24h – LC50 for *T. platyurus was* 3.6 mg/l (**Table 3**).

Concerning the fish, for adults a 96h – LC50 value of 5.3 mg/l was estimated (**Figure 1**; **Table 3**). Furthermore, no effects on behavior of adult fish were observed. For embryos a 96 h – LC50 of 22.2 mg/l was calculated. Along the test, the mortality rate rose in most of the GA treatments reaching 100 % at 100 mg/l at 24h, at 75 mg/l at 48h and at 50 mg/l at 96h (**Figure 2**). Concerning embryos development, the control embryos developed normally as described made by Kimmel (1995), while GA exposure augmented the incidence of malformations including delay in the absorption of the yolk sac (24 h: Kruskal–Wallis H = 15.82, p = 0.003; 96 h: Kruskal–Wallis H = 10.2, p = 0.02; 120 h: One–way ANOVA: F 3, 31 = 7.8, p < 0.001), spine deformations (120 h: Kruskal–Wallis H = 9.2, p = 0.03), and pericardial edema (48 h: Kruskal–Wallis H = 17.4, p = 0.002) (**Figure 3** and **4).** All embryos from the control group hatched between the 48 h and 96 h. In opposition, in the exposed embryos hatching delays were observed at 48 h (Kruskal–Wallis H = 22.9, p <0.001), 72 h (Kruskal–Wallis H = 27.7, p <0.001), 96 h (Kruskal–Wallis H = 19.8, p <0.001) and 120 h (Kruskal–Wallis H = 20.8, p <0.001) (**Figure 2**, **Table 4**). Considering all effects of GA on zebrafish embryos a combined 96 – EC_{50} of 11.9 mg/l was attained.

3.2 Biomarkers

Biomarkers, also used as sub lethal endpoints, were measured on post hatched embryos at 96 h. No alterations on GST, ChE and CAT levels were observed (**Figure 5** a – c). Only LDH activity was responsive (One–way ANOVA: F 4, 34 = 5.8, p = 0.001), being inhibited at 25 mg/l (**Figure 5** d).

Table 2 – Glutaraldehyde effective doses (mg/l) to several endpoints of *L. minor* (standard error between brackets)

GA	Dry weight (mg)	Fresh weight (mg)	Leaves Number	Td[§] (day)	Root size (mm)	H_2O (%)	Chlorosis (%)
0	4.4 (0.6)	58.3 (7)	71.0 (1.7)	2.4 (0)	12.7 (0.3)	0.9 (0)	0 (0)
2	4.3 (0.2)	62.7 (3.8)	67.0 (3.7)	2.4 (0.1)	9.9 (0.6)	0.9 (0)	0 (0)
4	5.5 (0.3)	80.9 (4.6)*	74.7 (5.6)	2.3 (0.1)	10.6 (1.2)	0.9 (0)	0 (0)
8	5.1 (0.2)	78.3 (2.8)*	80.7 (3.6)	2.2 (0)	11.5 (0.2)	0.9 (0)	0 (0)
16	4.6 (0)	69.3 (1.3)	66.0 (0.9)	2.4 (0)	10.4 (0.6)	0.9 (0)	0 (0)
32	5.8 (0.1)*	59.9 (3.8)	56.7 (3.8)	2.7 (0.1)	4.4 (0.2)*	0.9 (0)	0 (0)
64	4.1 (0.4)	23.4 (1.1)*	33.3 (2.9)*	3.8 (0.2)*	3.5 (0.6)*	0.8 (0)	0.5 (0)
128	1.6 (0.2)*	16.5 (3.9)*	23.0 (0.9)*	5.2 (0.2)*	2.9 (0.1)*	0.9 (0)	0.8 (0)
256	1.1 (0)*	9.8 (1.1)*	17.3 (1.4)*	8 (1.2)*	—	0.9 (0)	1 (0)
612	1.3 (0.1)*	10.0 (0.5)*	14.3 (0.5)*	10.7 (1)*	—	0.9 (0)	1 (0)
1224	1.5 (0.1)*	9.1 (0.7)*	14.0 (1.2)*	12.6 (2.2)*	—	0.8 (0)	1 (0)
EC_{50}	78.8 (23.2)	46.9 (10.5)	46.9 (10.5)	264.3 (209.2)	23.9 (6.8)	¥	68.6 (3.5)

"§" means doubling time; "*" Mean significantly different from control (Dunnett's test, P<0.05), "—" means root size < 1 mm; "¥" means not calculated

In adults, enzymatic activities were analyzed in different tissues. GST activity was analyzed in both liver and head (**Figure 5** e). No alterations of GST activity were noted in the liver, but GST activity in head was inhibited at 4.2 mg/l (One–way ANOVA: F 3, 30 = 8.4; p <0.01). ChE activity was not affected in both the muscle and head (**Figure 5** f). LDH activity in the muscle was inhibited at 4.2 mg/l (Kruskal–Wallis H = 11.5; p = 0.01) (**Figure 5** g). CAT activity was not affected by GA in muscle but was inhibited in all tested concentrations in liver (1.2, 2.8 and 4.2 mg/l) (One–way ANOVA: F 3, 18 = 9.1; p <0.01) (**Figure 5** h).

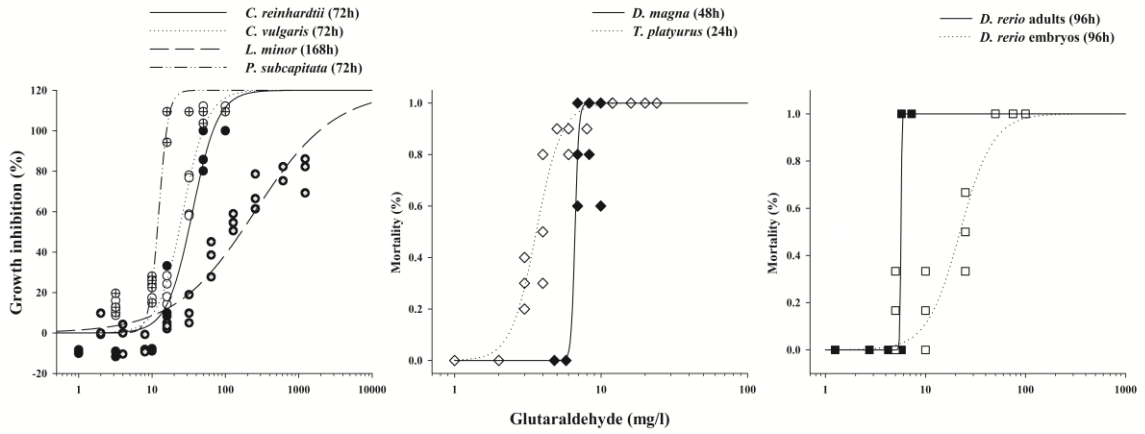


Figure 1 – Comparison of glutaraldehyde toxicity across trophic levels: dose response curves of the primary producers *C. reinhardtii* (black dots), *C. vulgaris* (white dots), *P. subcapitata* (crossed white dots) after 72 h of exposure and *L. minor* (168 h exposure, x-hair black dots); dose response curves of the primary consumers *D. magna* (48 h exposure, black diamonds) and *T. platyurus* (24 h exposure, white diamonds); and dose response curves of the secondary consumer *D. rerio* adults (black squares) and embryos (white squares) after 96 h of exposure.

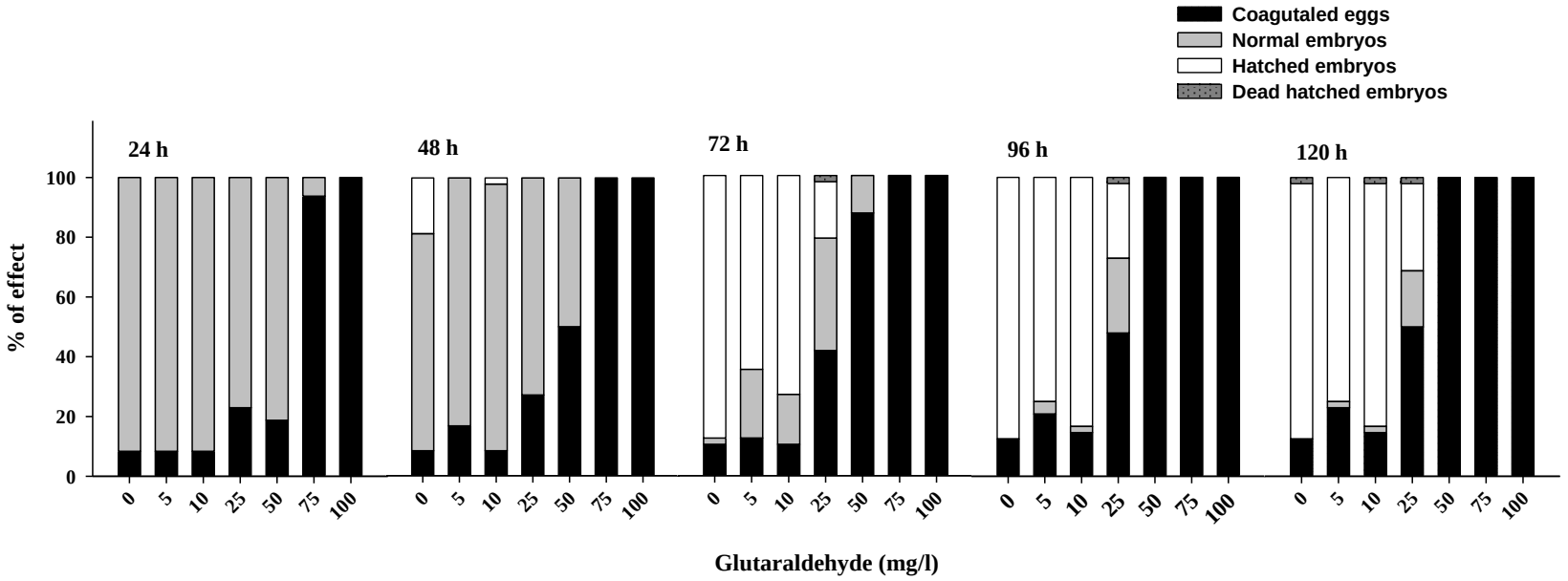


Figure 2 – Overview of glutaraldehyde effects on *D. rerio* embryos during the 120h (5 days) of exposure

3.3 SSD

The SSD plot is shown in Fig. 6. The predicted toxicity for the 5 % and 50 % most sensitive species are, respectively, 0.6 mg/l (upper limit = 1.0 and lower limit = 0.3 mg/l) and 11.4 mg/l (upper limit = 20.6 and lower limit = 6.4 mg/l) of GA (**Figure 6**). A similar toxic effect of GA was found between all the trophic levels. No difference of sensitivity was found between the primary producers and primary consumers (*ks* = 0.25, n1 = 6, n2= 14, p<0.62), primary producers and secondary consumers (*ks* = 0.33, n1= 6, n2 = 6, p<0.51) or between primary and secondary consumers (*ks* = 0.29, n1 = 14, n2 = 6, p<0.50).

Table 3 – Glutaraldehyde toxicity values (mg/l) obtained using a non-linear allosteric decay model.

Test species	Parameter	EC_{10}	EC_{20}	EC_{50}	$p(r^2)$	*p*
C. reinhardtii	growth	11.7 (3.5)	12.7 (3.0)	14.6 (1.5)	0.972	< 0.001
C. vulgaris	growth	24.5 (6.9)	26.8 (5.0)	31.3 (1.2)	0.979	< 0.001
P. subcapitata	growth	9.7 (0.6)	10.6 (0.6)	12.3 (0.6)	0.988	< 0.001
L. minor	growth[¥]	21.1 (48.9)[#]	33.5 (39.6)[#]	73.8 (14.4)	0.969	< 0.001
D. magna	immobility	6.2 (2.0)	6.4 (1.6)	6.6 (0.8)	0.925	< 0.001
T. platyurus	immobility	2.3 (0.3)	2.7 (0.3)	3.6 (0.1)	0.959	< 0.001
D. rerio embryos	mortality	9.6 (2.7)	13.1 (2.7)	22.2 (2.7)	0.917	< 0.001
D. rerio adults	mortality	5.0 (6.5) [#]	5.2 (1.7)	5.5 (4.0)	0.85	< 0.001

Confidence interval (95%) values between brackets; "[¥]" data calculated for dry weight; "[#]"Mean extrapolated data

4. Discussion

Biocides, including GA, can pose risks to the environment due to its intrinsic biocidal properties and/or associated use patterns (European Council,

2012). In the present study, GA toxicity was studied at different levels of organization, including the subcellular, individual and community levels. In general, GA showed to be moderately toxic to aquatic organisms from different trophic levels with toxicity values ranging from 3.6 mg/l (24 h EC50 of *T. platyurus*) to 31.3 mg/l (72 h EC50 of *C. vulgaris*) (**Table 2**). Additionally, GA inhibited the activity of a number of enzymes in both *D. rerio* embryos and adults

GA toxicity to primary producers has been poorly studied. In the literature, only few studies are found reporting toxicity values for green algae species but no studies with macrophytes were found in order to compare with the data here achieved. For instance, the growth inhibition EC50 value obtained in this study for *P. subcapitata* was similar to the value reported by Chen et al. (2005), 12.3 and 13.2 mg/l, respectively. Wil (2000) reported a 72 h EC50 of 0.81 mg/l for algae biomass, which suggests that biomass, might be a more sensitive endpoint to assess GA toxicity to green algae (as cited in Leung, 2001a). *C. reinhardtii* was more sensitive to GA than *C. vulgaris*, which may be explained by differences in the cell wall composition and the presence of other protective barriers (Sano et al., 2005). Indeed, since GA toxicity depends on its interaction with proteins (cross-linking amino and sulfhydryl groups) in the cell wall and intracellular components, differences in the cell wall composition could result in differential sensitivity of algae species to GA. Moreover, GA showed a low toxicity to *L. minor* where the most pronounced effect was the shortening of the roots (168 h EC50 of 23.5 mg/l). The EC50 for growth inhibition was much higher (168 h EC50 of 73.8 mg/l). The presence of protective barriers such as the epidermis in the abaxial part of the leaves might avoid GA damage. Once the roots are submerged in the solutions, they are more vulnerable to GA damage. Interestingly, GA promoted the growth of *L. minor* at low concentrations (NOEC = 4 mg/l) (**Table 4**). No references in the literature were discovered displaying evidences of the GA boosting effect in primary producers, however there are available commercial products containing polymerized isomers of GA (polycycloglutaracetal), which are employed in the water as fertilizer of aquatic plants (e.g. Flourish Excel™, in the United States). The product sheet of Flourish Excel describes the CO_2 rising from GA degradation

(process previously described in detail by Leung (2001b)) as an additional source of carbon that might be used by *L. minor*. With regard to primary consumers, the EC50 value for *D. magna* achieved in the present study (6.62 mg/l) has shown similarities to other values present in some references with an average L(E)C50 of 5 mg/l (**Table 1**). No EC50 values for *T. platyurus* were found in the current scientific database; however, toxicity values obtained are in the same range of values found for other branchiopods microcrustaceans (e.g. daphnids and *Artemia* sp.). Based on literature data the GA toxicity seems to be more pronounced for early life-stages of invertebrates and microinvertebrates. On the other hand, malacostraca crustaceans (e.g. *H. azteca, C. marinus* and *C. maenas*) are extremely resistant to GA exposure with LC50 values greater than 100 mg/l. The invertebrates, in the middle of aquatic food webs, play a critical role in the nutrients/organic matter cycling and simultaneously are a source of food for fish, reptiles, birds and mammals (Covich et al., 1999). However, in spite of their importance in aquatic ecosystems, a gap of knowledge about GA toxicity to invertebrates is observed, particularly to aquatic insects, where not a single study was discovered.

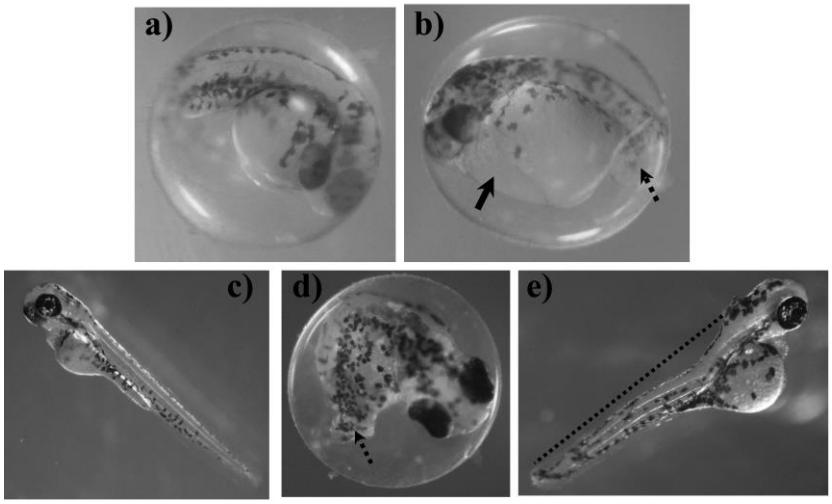


Figure 3 – Embryos and larval development anomalies on *D. rerio* exposed to glutaraldehyde. a) and c) control organisms with normal development after 48 and 72h, respectively; b) 48 h-old embryo exposed to 25 mg/l with pericardial edema (solid arrow) and tail deformities (dashed arrow); d) shows a 72 h-old embryo exposed to 25 mg/l with delay in yolk-sac absorption

(sac) and tail deformities; e) 72 h-old hatched embryo exposed to 25 mg/l with tail deformities.

Studies of the GA effects to fish species reported a wide range of lethal values (**Table 1**). A good agreement of LC50 values for *D. rerio* adults was found between this study and OECD SIDS (1998), 5.3 and 5.8 mg/l, respectively. Other fish species, namely *Pimephales promelas*, *Oncorhynchus mykiss*, and *Oncorhynchus kisutch*, show a similar sensitivity to *D. rerio* whereas *Cyprinodon variegates* and *Lepomis macrochirus* seem to be about 4 to 5 times more resistant than *D. rerio*. For embryos, a well-defined dose response was found and a 96 h – LC50 of 22.17 mg/l was calculated. Similar result was obtained by Sano et al., (2005) that reported NOEC values of 13.6 mg/l for embryos of *O. mykiss*. With regard to the hatching delay was perceived at 5 mg/l only at 48h whereas for higher concentrations (equal or above 25 mg/l) the hatching inhibition last until the end of the test. Similar effects on hatching of *O. mykiss* embryos were observed by Sano et al., (2005) who reported that embryos exposed to 2.5 mg/l of GA were unable to hatch. Different toxicity mechanisms might explain the hatching failure/impairment: increased hardness of chorion (Salvesen and Vadstein, 1995), inhibition of embryo movement and/or possible dysfunction of the hatching enzyme chorionase (Escaffre et al., 2001; Hallare, 2005). In addition, abnormalities as tail deformities, pericardial edemas and delay of yolk sac absorption (**Figure 3**) were obtained after GA exposure. In literature, tail deformities are mostly related with trace metals (e.g. cadmium) (Morelli et al., 2012) which probably reduce the intracellular concentrations of calcium and phosphorus (Muramoto, 1985). Pericardial edemas were also found in *D. rerio* embryos suggesting leaks of endothelial vessels, which are connected to the yolk sac that exhibited delayed absorption. Edemas can result in cardiovascular dysfunction, compromising the survival of embryos (Guiney, 1990). Surviving organisms with malformations lose mobility and consequently the ability to capture food and escape from predators. The detected developmental effects might not allow the embryos to survive at long-term in natural ecosystems; therefore, an interesting approach could be the use of malformations, hatching and mortality in the determination of a more accurate effective concentration of GA (e.g. EC50).

Using malformations and hatching delay a 96 h – EC50 value of 11.9 mg/l was obtained, which reduces the differences of sensitivity between embryos and adults (96 h – LC50 = 5.3 mg/l).

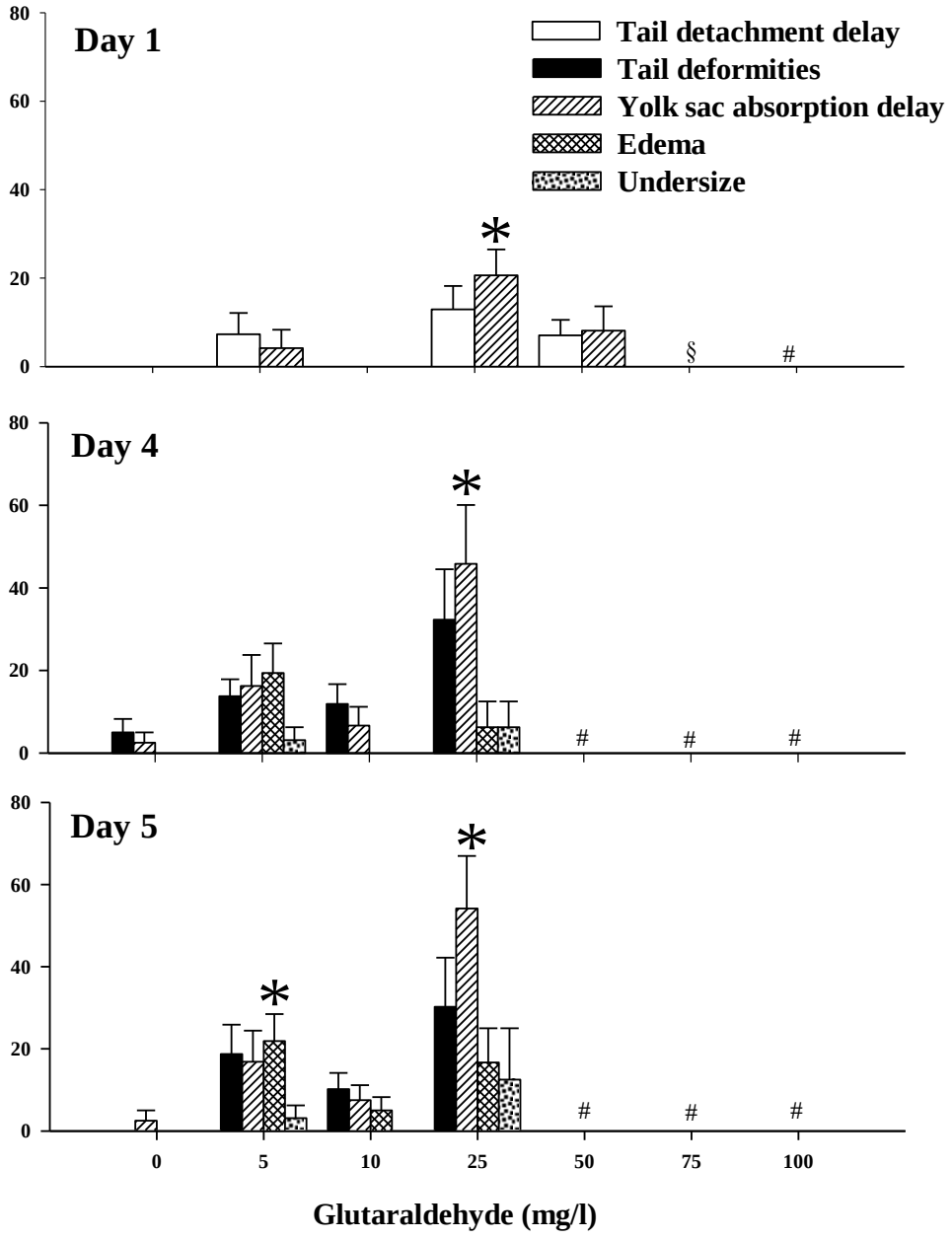


Figure 4 – Incidence of anomalies (mean value ± standard error) during development of embryos exposed to glutaraldehyde. "*" means statistically different from the control group (Dunn's method, $p<0.05$). "§" indicates that the number of surviving organisms was too low to evaluate developmental endpoints. "#" indicates no survival

In summary, the evaluation of several developmental effects and the extension of test duration provided a more realistic and accurate inference of the GA embryo toxicity than a standard 48h FET.

Table 4 – Percentage of zebrafish eggs hatched after exposure of GA during FET Test (standard error between brackets)

GA (mg/L)	Day 2	Day 3	Day 4	Day 5
0	22.1 (10.4)	97.9 (3.4)	100 (0)	100 (0)
5	0 (0)*	74.0 (10.0)	94.4 (6.1)	96.9 (5.1)
10	2.5 (4.1)	80.8 (10.2)	97.5 (4.1)	97.5 (4.1)
25	0 (0)*	32.3 (15.3)*	52 (13.8)*	59.4 (11.9)*
50	0 (0)*	_	_	_
75	_	_	_	_
100	_	_	_	_

*Mean significantly different from control (Dunn's test, $P<0.05$), "-" means that all organisms were dead

The biomarkers have been used as a tool to detect early signs of chemicals effects that can pose risk to aquatic ecosystems (Domingues et al., 2010a; Van der Oost et al., 2003). In the present study, a consistent inhibition of LDH activity was identified in adults muscle samples and embryos homogenate. LDH is involved in cellular respiration, namely in the glycolysis, and its activity has been used as indicator of the metabolic state of organisms under chemical or natural stress (Almeida et al., 2010; Osswald et al., 2013). Nevertheless, since LDH may play different roles in the metabolism of embryos and adults, the effects observed in the present study on these life stages may not be directly comparable. For adults, inhibitions of enzymatic activities were also noticed for GST in gill tissues and CAT in muscle tissues. Those outcomes suggest that GA might modulate antioxidant defensive systems of *D. rerio*. One hypothesis focuses on the inhibition of GST and CAT which can lead to a rise of ROS and,

consequently, to an increase of oxidative damage such as DNA mutations and breaks or lipid peroxidation but such hypothesis needs further investigation.

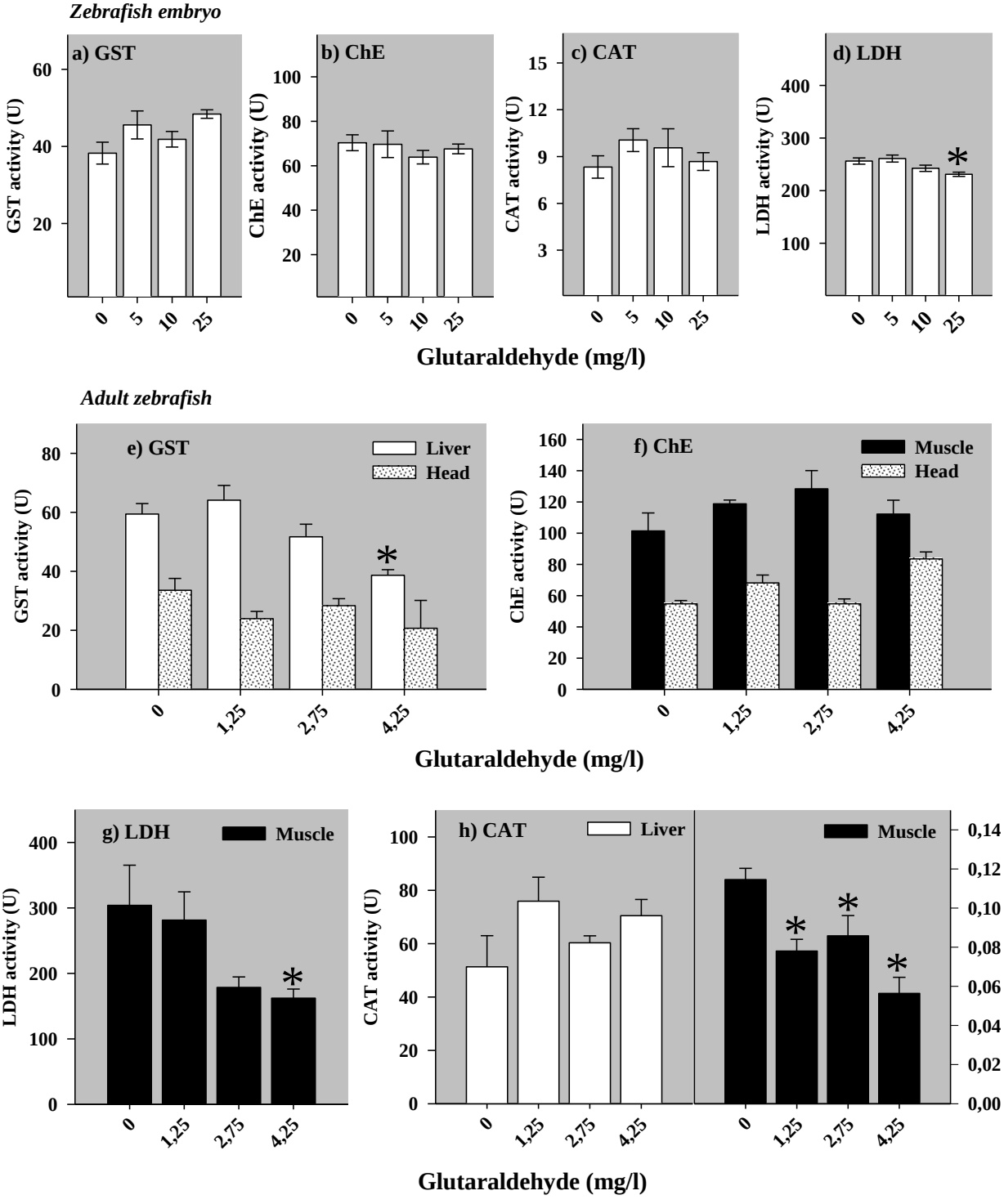
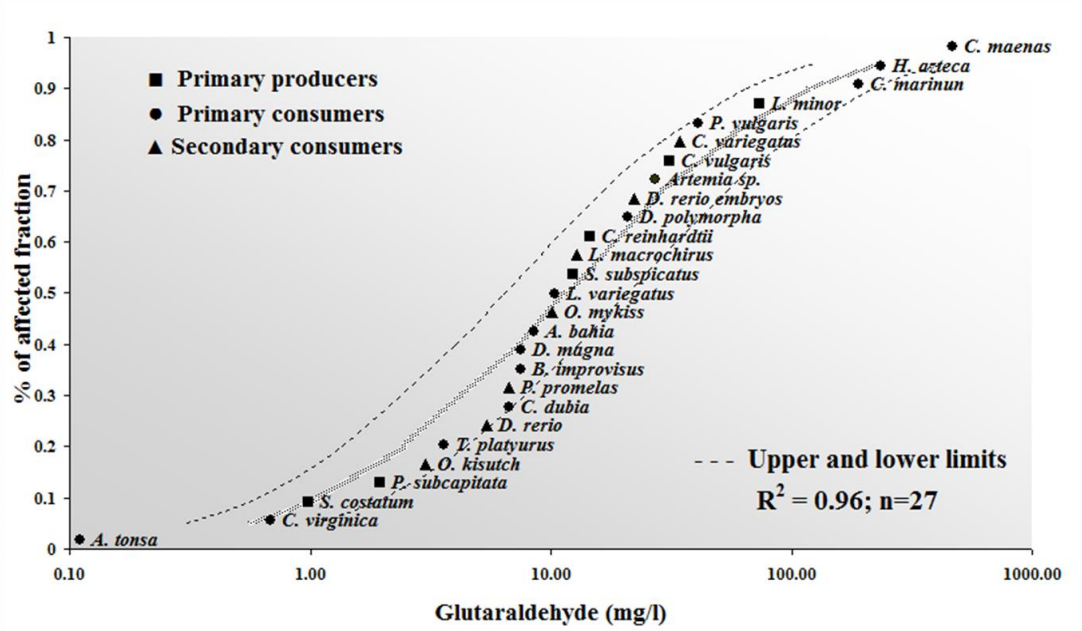


Figure 5 – Enzymatic activities (mean value ± standard error) on *D. rerio* embryos and adults after exposure to glutaraldehyde. U = nmol min^{-1} ml^{-1} mg of $protein^{-1}$; "*" means significantly different from the respective control ($p<0.05$)

To consent a better comparison of GA toxicity across different trophic levels and to predict GA toxicity at the community level, an SSD was developed (**Figure 6**). The data obtained in the present study together with the data collected from the literature provided a wide diversity in terms of taxonomy, of the sensitivity and ecological traits. In the comparison of sensitivity were not found differences between the organisms from different trophic levels. Still, a trend of higher sensitivity of smaller organisms was noticed. The algaecide affect of GA could cause a pressure on algae and microinvertebrates triggering a bottom-up effect. Concentrations up to 3 mg/l of GA values were reported for hospitals wastewaters in Europe (Jolibois et al., 2002). Such values are 10 times higher than the HC5 of 0.3 mg/l located in the present study, recommending that such an elevated input of GA might cause harm to numerous aquatic species. However, to draw a more accurate analysis we propose that future contributions for GA risk assessment should take into account derivations of species sensitivity from a higher number of toxicity data



particularly from chronic toxicity allowing a better understanding of the effects of GA at an aquatic community level.

Figure 6 – SSD plot of the affected fraction of species versus glutaraldehyde concentration. The squares represent the primary producers, the circles the primary consumers and the triangles secondary consumers.

5. Conclusions

GA is moderately toxic to aquatic organisms with toxicity values ranging from 3.6 mg/l to 31.3 mg/l for the tested species. Moreover, GA had a similar biocide effect on organisms independent of trophic levels. Several sub lethal endpoints were measured in *D. rerio* embryos and adults. For embryos, malformations and hatching delay can be highlighted as sensitive endpoints, while for adults the CAT activity showed to be the most sensitive biomarker to GA exposure. Furthermore, the sub lethal effects in *D. rerio* should be further investigated and linked with population fitness. Considering the wide-ranging of effects varying with the life stage and organism tested and the relatively low HC5 value of 0.5 mg/l, mesocosm and chronic toxicity tests seem to be a next step in direction to more realistic scenarios of GA risk assessment in aquatic ecosystems.

Acknowledgements

Authors acknowledge to University of Aveiro, Department of Biology & CESAM by the institutional support; Fundação para Ciência e Tecnologia (FCT) by the financial support through the grants for the authors (SFRH/BPD/31752/2006, SFRH/BD/78168/2011, SFRH/BD/64938/2009, SFRH/BD/62605/2009 and BI/CESAM/PTDC/AAC – AMB/113649/2009).

Ethical considerations

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

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SUPPORTING INFORMATION

Effects of the biocide glutaraldehyde to aquatic organisms from different trophic levels

Susana P.P. Pereira [a, †, *], Rhaul Oliveira [a, †, *], Sónia Coelho [a], Carolina Musso [a], Amadeu M.V.M. Soares [a], Inês Domingues [a], António J.A. Nogueira [a]

[a]*Department of Biology & CESAM, Campus Universitário de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal*

† The two authors contributed equally to the study

*Corresponding author - e-mail: *rhaul.oliveira@gmail.com; *susanapereira@ua.pt; Fax: +35234372587; Phone: +351234370 790

Solutions of 1 mg/l of glutaraldehyde (GA) were kept under the same experimental conditions as the toxicity tests and analysed daily. The determinations of GA were performed according to the spectrophotometric method described by Matthews and Howell (1981). The standard curve showing the relationship between the amount of GA in the sample and the absorbance at 635 nm is depicted in Figure 1. The concentrations and percentage of recovery from the stock solutions are shown in Table 1.

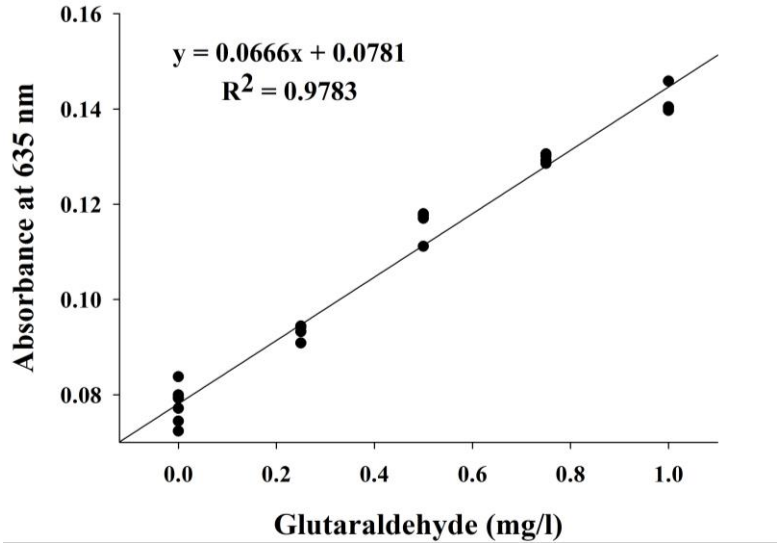

Figure 1 - Standard curve showing the relationship between the amount of GA and the absorbance at 635 nm.

Table 1 – Concentrations of glutaraldehyde and percentage of recovery from the stock solutions (concentrations followed by standard deviation in brackets)

Time	**Thamnocephalus conditions***		**Fish conditions***		**Algae/Lemna conditions***	
	Concentration (mg/l)	*% of recovery*	*Concentration (mg/l)*	*Concentration (mg/l)*	*Concentration (mg/l)*	*% of recovery*
Day 0[#]	0.95 (0.16)	**94.89**	0.95 (0.16)	**94.89**	0.95 (0.16)	**94.89**
Day 1	0.96 (0.25)	**95.46**	1.00 (0.23)	**100.38**	0.98 (0.25)	**97.60**
Day 2	-	-	0.73 (0.08)	**73.45**	0.77 (0.10)	**76.91**
Day 3	-	-	0.50 (0.15)	**49.83**	0.61 (0.15)	**60.60**
Day 4	-	-	0.59 (0.13)	**58.70**	0.42 (0.33)	**42.41**
Day 5	-	-	0.43 (0.07)	**42.86**	0.63 (0.32)	**63.19**
Day 6	-	-	-	**-**	0.01 (0.01)	**00.06**
Day 7	-	-	-	**-**	0.01 (0.01)	**00.01**

[*] Thamnocephalus conditions: temperature of 25 ± 1ºC, photoperiod of 24 h dark; Algae/Lemna conditions: 24 ± 1 ºC, continuous light (3400 lux); Daphinia/fish conditions: 25.0 ± 2ºC, photoperiod 16 h light: 8 h dark, light intensity of 3400 lux; [#] Nominal concentrations at Day 0 is 1 mg/l;

Reference

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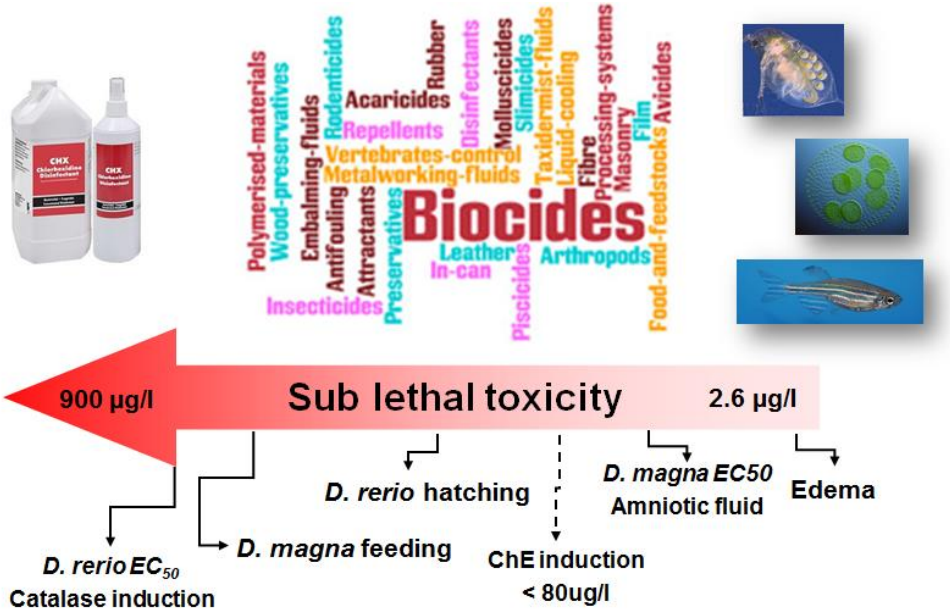
Chapter 3

Lethal and sub lethal effects of the biocide chlorhexidine on aquatic organisms



Lethal and sub lethal effects of the biocide chlorhexidine on aquatic organisms

Rhaul Oliveira†; Fátima T. Jesus†; Andreia Silva; Ana L. Catarino; Amadeu M.V.M. Soares; António J.A. Nogueira & Inês Domingues

This chapter has been published as an original article in:

Ecotoxicology (*In press*)

Abstract

Chlorhexidine is among the most used biocides in Europe, however its toxicity to aquatic organisms is scarcely known. The main objective of this study was to assess the lethal and sub lethal effects of chlorhexidine digluconate (ChD) on four aquatic model organisms: the bacteria *Vibrio fischeri*, the algae *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the embryos of the fish *Danio rerio*. ChD was very toxic to algae and crustaceans, with a 72 h-EC50 of 62.2 µg/l and a 48 h-EC50 of 45.0 µg/l, respectively. Toxicity to fish embryos and the bacteria was lower, with a 96 h-EC50 of 804.0 µg/l and a 15 min-EC50 of 1694.0 µg/l, respectively. Concerning sub lethal effects on *D. magna* (feeding inhibition) a 6 h-EC50 of 503.7 µg/l was obtained. In fish, ChD caused developmental abnormalities, namely alterations in the amniotic fluid (48 h-EC20 of 753.6 µg/l) and early hatching. Moreover, enzymatic biomarkers on fish embryos showed an induction of cholinesterase activity in all ChD tested concentrations (80 – 900 µg/l). The catalase activity was also induced at the highest concentration tested (900 µg/l) whereas no changes were observed for glutathione-S-transferase and lactate dehydrogenase activities. The toxicity of ChD to the algae and crustacean raises concerns about its potential effects in aquatic food webs, since these organisms are in the base of trophic chains, and highlights the need for further studies on ChD toxicity to aquatic organisms.

Keywords: zebrafish embryos; daphnia; algae; bacteria; biomarkers; feeding inhibition

1. Introduction

Biocides are used worldwide in daily activities to control organisms that are harmful to human or animal health or that cause damage to materials (e.g. biofouling). Nonetheless, they can pose risks to humans, animals and the environment due to both their intrinsic biocidal properties and associated use patterns (European Council 2012). Facing the increasing use of biocides, the European Commission recently implemented new rules on biocidal products, which concern their commercialization, in order to improve consumers and environmental safety (European Council 2012).

Chlorhexidine and its salts (acetate, digluconate, and hydrochloride) are among the most used biocides in Europe (Boxall et al. 2006; Garvey et al. 2001). Chlorhexidine digluconate (ChD) is a disinfectant with antimicrobial activity against Gram-positive bacteria and, to a less extent, to Gram-negative bacteria, fungi and certain types of virus (Fiorentino et al. 2010). ChD leads to cell wall/membrane disruption, causing the loss of lipid components (Cheung et al. 2012). Due to its high antimicrobial activity, ChD is used in hospital, veterinary and cosmetic products (Fiorentino et al. 2010; Hebert et al. 2003). In particular, ChD can be used in surgical scrubs, preoperative skin preparations, skin antiseptics and cleansers, wound protectors, burn ointments, acne creams, ophthalmic solutions, soaps, and oral products such as mouth rinses and toothpaste (Ranganathan 1996).

In spite of the widespread use of ChD, there is a lack of knowledge concerning its toxicity to aquatic organisms. ChD was reported to be toxic to bacteria (Guimarães et al. 2012). Moreover, two studies reported bioaccumulation of ChD on river microbial biofilms and a significant effect of ChD on the structure of microorganism communities (increase in the proportion of archaea, bacteria and cyanobacteria when compared to algae) at doses of 10 µg/1 (Lawrence et al. 2008; Dynes et al. 2006). Such a scarcity of data on ChD toxicity led

environmental agencies, such as USEPA, to rely on the few acute toxicity data of chlorhexidine diacetate to assess the environmental risk of ChD (USEPA 1996).

The present study intends to contribute to fulfil the gap on ChD toxicity to aquatic organisms by assessing lethal and sub lethal effects on different model organisms, namely *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio* embryos. Since they have been considered representative of primary producers (*P. subcapitata*), first consumers (*D. magna*), secondary consumers (*D. rerio*) and microbial decomposers (*V. fischeri*), this study might give insight on the potential effects of ChD on these trophic levels.

Lethal toxicity to *V. fischeri*, *P. subcapitata*, *D. magna* and *D. rerio* was assessed based on bioluminescence inhibition, growth rate, immobilization and mortality, respectively. Sub lethal effects on *D. magna* were evaluated based on feeding inhibition, whereas to fish embryos they were assessed based on enzymatic biomarkers and embryo development. Feeding inhibition of *D. magna* has been used as a sensitive indicator of toxicity and is an ecologically relevant endpoint since food intake determines energy acquisition and further allocation and, thus, growth, reproduction and survival of individuals (Nogueira et al. 2004). Moreover, the early stress responses caused by ChD on zebrafish embryos were assessed based on a battery of enzymatic biomarkers from different metabolic pathways. Since ChD affects the nervous system of several organisms (Röed 1973; Henschen and Olson 1984; Shaihutdinova et al. 2009) the cholinesterase (ChE) was selected due to its key role in the hydrolysis of acetylcholine in peripheral and central nervous systems (Holth et al. 2008). In addition, intending to give a first insight on the oxidative stress effects caused by ChD, the enzyme catalase (CAT), an antioxidant enzyme that acts against reactive oxygen species (ROS) (Oost et al. 2003), was also selected. Furthermore, the glutathione-S-transferase (GST), an enzyme involved in detoxification processes (Oost et al. 2003), and the lactate dehydrogenase (LDH), a glycolytic enzyme present in all fish tissues, catalyzing the interconversion of pyruvate and lactate (De Almeida-Val et al. 2005) were also included in the battery of biomarkers.

2. Materials and methods

2.1 Stock preparation and chemical analysis

Chlorhexidine digluconate (ChD, CAS No. 18472-51-0) was bought from Sigma Aldrich as a solution in water at 20 % (v/v). For each assay, a ChD stock solution was freshly prepared by diluting the aqueous solution in ultrapure water. The test solutions were prepared immediately before the test by successive dilution of the respective ChD stock solution. At the beginning of each test, stock solution was placed at the same experimental conditions of the respective test and sampled daily, including at the beginning of the exposure (5 ml in quadruplicate) to analyse stability of ChD during the test period. A standard curve was obtained by including a dilution series prepared directly from the original compound (ChD 20 % in water from Sigma Aldrich). ChD in samples and standards was quantified following the procedure described by Jensen and Christensen (1971) which includes an extraction by an organic solvent, re-extraction with an acid buffer solution and then measurement by ultraviolet spectrophotometry. The extraction of ChD (1 ml) was performed with 2 ml of NaOH (2 M) and 20 ml of chloroform. The re-extraction was performed using 15 ml of the organic phase to which 4 ml of glycine buffer (0.1 M, pH 2.10 ± 0.05) was added. ChD was analysed by determining the absorbance of the buffer phase at 250 nm (Thermo Scientific Multiskan® Spectrum).

2.2 *Vibrio fischeri*

The bacteria *V. fischeri* was used in the Microtox® Test, an acute assay that measures the effect of toxicants and environmental samples on organisms light production. The bacteria were purchased as dried kits (Modernwater, Cambridge, United Kingdom). They were stored at − 20 °C and rehydrated prior to testing.

2.2.1 Bioluminescence inhibition assay (Microtox)

Microtox assays were carried out following the 81.9 % Basic test protocol (AZUR Environmental 1998), using the Microtox 500 Analyzer. Testing was conducted using 100 µl of bacterial suspension mixed with 500 µl of test solution

to give ChD final concentrations of 6, 18, 56, 167, 500, 1500, 4500, 13500, and 40500 µg/l. Bioluminescence was measured after 0, 5 and 15 minutes of incubation.

2.3 *Pseudokirchneriella subcapitata*

The freshwater unicellular green algae *P. subcapitata* were cultured in batch cultures of Woods Hole MBL medium (Nichols 1973) at 20 ± 2°C under continuous and uniform cool-white light and continuous aeration.

2.3.1 Growth inhibition assay

The algae growth inhibition assay followed the OECD guideline 201 (OECD 2006b). The assay was initiated with 1.0 x10^4 cells/ml of *P. subcapitata* in the log exponential growth phase and was carried out in triplicate. Erlenmeyer flasks containing 50 ml of sterilized MBL medium with the desired ChD concentration (0, 10, 16, 25, 40, 63, 100, 160, 250 and 400 µg/l) and the inoculums were randomly incubated in an orbital shaker (100 rpm). The temperature was kept at 24 ± 1 ºC, with continuous light (cool-white fluorescent light, 3400 lux). The absorbance at 440 nm was measured after 24, 48 and 72 h by spectrophotometry (Jenway 6505 UV/Visible spectrophotometer UK) and converted to cell concentration (algae cells per ml) using the following equation:

$$Cell\ concentration = -171075 + Absorbance \times 79253500$$

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

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

Where $\mu i - j$ is the average specific growth rate from time i to j (per day); X_i is the cell concentration at time i, and X_j is the cell concentration at time j; t_i is the initial time of the exposure and t_j is the final time of the exposure.

2.4 *Daphnia magna*

Parent animals originated from a single clone of *D. magna* Straus (1820)

(clone F, sensu Baird et al (1991)) and were cultured in synthetic ASTM hard water (ASTM (1998)) with a standard organic additive (Marinure seaweed extract, Glenside Organics Ltd.). Organisms were fed *P. subcapitata* (3 x10^5 cell/ml) daily. Photoperiod was 16 h light: 8 h dark and temperature was 20 ± 1ºC. The culture medium was renewed three times a week.

2.4.1 Acute immobilization tests

The acute immobilization test with *D. magna* followed the OECD guideline 202 (OECD 2004b). Two acute immobilization tests were performed, independently, to guarantee repeatability. Only newly released neonates (> 6 h and < 24 h) from the third to sixth clutches were used in bioassays. Five replicates were used per treatment. Each replicate consisted of 5 organisms exposed to 50 ml of ASTM hard water (OECD 2004a) with the desired ChD concentrations (0, 20, 30, 40, 60, 80, 90, 100, 120, 140 and 150 µg/l). No food was provided; photoperiod and temperature were as for cultures, except light intensity, which was set to a low value: 34.3 ± 3.3 lux (mean ± standard deviation). After 24 and 48 h of exposure, daphnids were observed against a bright light and the number of immobilized daphnids was recorded. Immobilization was defined as the inability to swim or move within 15 s of gentle agitation.

2.4.2 Feeding inhibition tests

Tests were carried out with fourth instar juveniles (96 h old) using three replicates per treatment. Each replicate consisted of three juveniles in a glass vials containing 50 ml of ASTM hard water, algae (*P. subcapitata*, 3 x10^5 cells/ml), and the desired ChD concentrations (0, 100, 200, 300 and 400 µg/l). Two blanks having no animals were added to the experimental setup. The vials were kept in the dark (to avoid growth of algae) at 20 ± 1 ºC for 6 hours. The vials were vigorously shaken before the measurement of the absorbance by spectrophotometry at 440 nm. The absorbance was converted to cell concentration and these values were used to determine the feeding rates using the equation by Allen et al. (1995):

$$F = \frac{V \times (C_j - C_i)}{n \times (t_j - t_i)}$$

where *F* is the feeding rate (cells/animal × h); *V* is the volume of medium in the test vial (ml); C_i is the cell concentration at time *i*, and C_j is the cell concentration at time *j*; *n* is the number of daphnids; t_i is the initial time of the exposure and t_j is the final time of the exposure.

2.5 *Danio rerio*

D. rerio embryos were obtained in the facility established at the Department of Biology, University of Aveiro (Portugal). In the facility, adults are kept in carbon-filtered water complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA), at 27.0 ± 1ºC and under a 14 h light: 10 h dark photoperiod cycle (conductivity 750 ± 50 µS/cm, pH at 7.5 ±0.5 and dissolved oxygen > 95 % saturation). This water was used as dilution water in the preparation of test solutions in all assays performed with fish. Adult *D. rerio* were fed twice daily with commercially available artificial diet (ZM 400 Granular, ZM systems, Hampshire, UK) and brine shrimp. *D. rerio* eggs were collected immediately after natural mating. Before the test, eggs were rinsed with water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation, Japan); those with cleavage irregularities or injuries were discarded.

2.5.1 Fish embryo toxicity assay

The assay was based on the OECD draft guideline on Fish Embryo Toxicity Test (FET) (OECD 2006a) and is described in detail in Oliveira *et al.* (2009). Two tests were performed, independently, to guarantee repeatability. In each test, 3 replicates of six eggs per treatment were used. Tests started with newly fertilized eggs exposed to ChD concentrations of 0, 40, 80, 160, 320, 640, 1050, 1280, 1470, 2040 and 2400 µg/l. Tests were carried out in 24-wells microplates at 26 ± 1 ºC and photoperiod of 14 h light: 10 h dark under low light intensity (32.0 ± 8.2

lux, mean ± standard deviation) and run for 96 hours. Embryos were daily observed under a stereomicroscope, using 70 x magnification for embryos and 40 x for hatched embryos. As defined by the FET guideline the coagulation of fertilized eggs, lack of somite formation, non-detachment of the tail-bud from the yolk sac and lack of heart-beat were assessed as apical endpoints. Other parameters taken as complementary endpoints were: delay in eye and body pigmentation, delay in the absorption of the yolk sac, alterations in the amniotic fluid, oedemas, spine deformations and mortality of the post-hatched embryos. All parameters were assessed in a qualitative way (observed or not observed).

2.5.2 Enzymatic biomarkers determinations

A similar test to the described above was performed for collection of embryos for biomarker analyses, using sub lethal ChD concentrations (0, 80, 160, 320, 640, and 900 µg/l). Test was finished after 96 hours of exposure and 10 clusters of eight embryos per treatment were snap-frozen in microtubes containing K-phosphate buffer (0.1 M, pH 7.4) and stored at – 80 ºC for further biomarker analyses.

Enzymatic assays were performed to analyse cholinesterase (ChE), glutathione-S-transferase (GST), catalase (CAT) and lactate dehydrogenase (LDH) in *D. rerio* embryos. On the day of enzymatic analysis, samples were defrosted on ice, homogenised using a sonicator (KIKA Labortechnik U2005 Control^{TM}) and centrifuged during 20 minutes at 11.500 rpm in order to isolate the post-mitochondrial supernatant (PMS) (Howcroft et al. 2011). Enzymatic determinations were made spectrophotometrically (Thermo Scientific Multiskan® Spectrum) using 96 wells microplates.

ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm, every 20s, during 5 minutes the conjugation product between thiocoline (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase) in K-phosphate buffer (0.1 M, pH 7.2), according to the method of Ellman et al. (1961). Activity determinations were made using 40 µl of PMS of the sample, 250 µl of reaction

mixture (acetylthiocholine (75 mM) and DTNB (10 mM)) in K-phosphate buffer (0.1 M, pH 7.2)).

LDH activity was measured at 340 nm, by continuously monitoring (every 20s, during 5 minutes) the decrease in absorbance due to the oxidation of NADH, following the methodology described by Vassault et al. (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 (0.1 M, pH 7.2).

GST activity was determined at 340 nm by monitoring the increase in absorbance every 20s, during 5 minutes, following the general methodology described by Habig and Jakoby (1981) with modifications introduced by Frasco and Guilhermino (2002). Activity determinations were made using 100 µl of PMS of the sample and 200 µl of reaction mixture (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 by monitoring (every 10s, during 2 minutes) the decrease of absorbance due to degradation of H_2O_2, as described by Clairborne (1985). Fifteen µl of PMS were 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).

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration in samples was determined in quadruplicate by the Bradford Method (Bradford 1976), at 595 nm, using γ-globulin as a standard.

2.6 Statistical Analyses

Sigma Stat 3.5 statistical package was used for statistical analyses (SPSS, 2004). To test whether ChD caused significant differences on test organisms compared to control, a one-way ANOVA was used, followed by the multiple comparisons Dunnett test. In case data did not pass the Kolmogorov–Smirnov normality test and the Levene's homogeneity of variance test, the non-parametric Kruskal–Wallis test was used, followed by the Dunn's test. The effective

concentrations (EC20 and EC50) for *D. magna* and *P. subcapitata* and lethal concentrations (LC20 and LC50) for *D. rerio* were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel. For *V. fischeri* the effective concentrations causing 20 % and 50 % of bioluminescence inhibition (EC20 and EC50, respectively) were calculated by using the Software for MicrotoxOmni Azur (AZUR Environmental 1998). All statistical analyses based on 0.05 significance level.

3. Results

Our experiments showed no changes in ChD concentrations of stock solutions during the exposure to the same experimental conditions as the toxicity tests (remaining within 80-120% of nominal concentrations; see **Online resource 1**).

ChD was toxic to the test organisms. The EC20 and EC50 values for the test species are presented in Table 1.

Table 1 – Effect concentrations of chlorhexidine digluconate (µg/l) to *V. fischeri*, *P. subcapitata*, *D. magna* and *D. rerio* embryos (95% confidence interval between brackets); p(r^2): goodness of fit measure; p (F): test for regression

Test species	Time	* EC_{20}	* EC_{50}	*p* (r^2)	*p* (F)
V. fischeri	5 min	961.3 (208.8 - 4424.5)	3675.2 (722.8 - 18688.1)	0.96	< 0.05
	15 min	434.5 (29.1 - 6494.1)	1694.0 (300.0 - 9567.6)	0.97	< 0.05
P. subcapitata	24 h	20.6 (10.1 - 31.3)	41.3 (34.1 – 48.5)	0.84	< 0.001
	48 h	33.3 (27.4 – 39.2)	43.9 (42.5 – 45.3)	0.98	< 0.001
	72 h	31.1 (16.0 – 46.2)	62.2 (55.4 – 69.0)	0.93	< 0.001
D. magna	24 h	80.0 (54.0 - 106.0)	105.6 (92.6 - 118.6)	0.55	< 0.001
	48 h	22.5 (10.6 - 34.4)	45.0 (39.8 - 50.2)	0.73	< 0.001
D. rerio	24 h	488.6 (125.7 - 851.5)	977.2 (840.2 - 1114.2)	0.92	< 0.001
	48 h	468.9 (296.9 - 640.9)	937.8 (864.6 - 1010.9)	0.91	< 0.001
	72 h	705.0 (618.4 - 791.5)	930.2 (902.6 - 957.8)	0.98	< 0.001
	96 h	609.6 (512.2 - 706.6)	804.0 (773.8 - 834.3)	0.97	< 0.001

3.1 Vibrio fischeri

The toxicity values calculated for *V. fischeri* were 5 min-EC50 of 3675.2 µg/l and 15 min-EC50 of 1694.0 µg/l. The EC20 values were 961.3 µg/l and 434.5 µg/l, respectively after 5 and 15 min of exposure (**Table 1**).

3.2 *Pseudokirchneriella subcapitata*

Exposure of algae to ChD caused a dose-dependent growth inhibition (**Fig. 1**). The EC50 of ChD to the algae varied between 41.3 and 62.2 µg/l after 24 and 72 h of exposure, respectively (**Table 1**). The EC20 varied between 20.6 and 33.3 µg/l after 24 and 48 h of exposure, respectively.

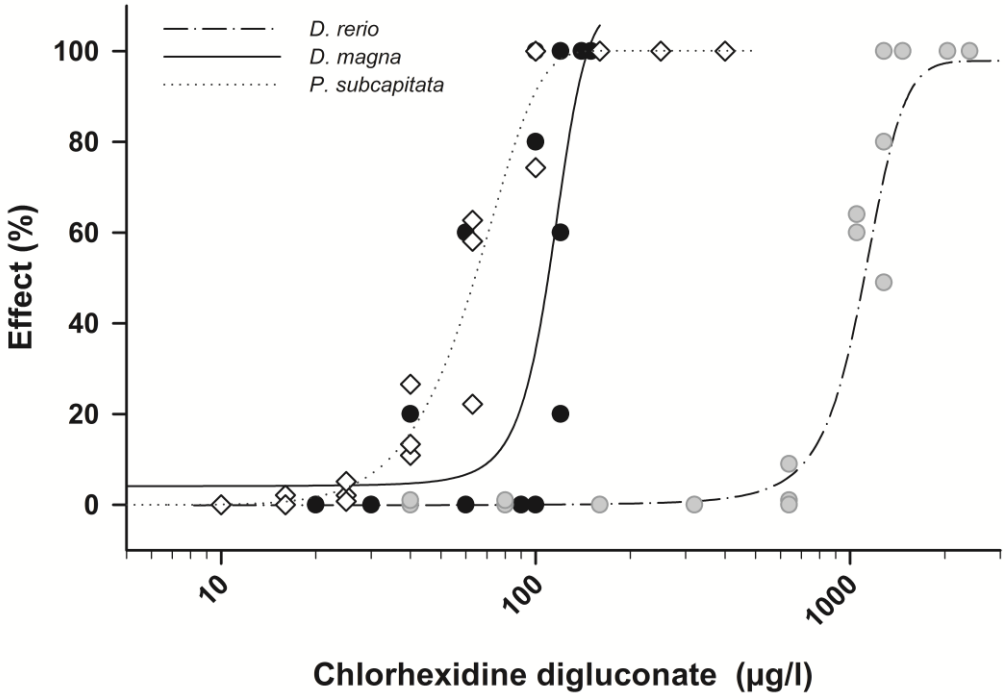


Fig 1 Effect of chlorhexidine digluconate on tested species: (◊) growth inhibition of *P. subcapitata* at 72h, (•) percentage of mortality of *D. magna* at 48h, and (•) percentage of mortality of *D. rerio* embryos at 96h.

3.3 *Daphnia magna*

In the acute immobilization test, no mortality was observed in the control group. Effect of ChD on *D. magna* followed a dose response curve (**Fig. 1**), with

EC50 values ranging between 105.6 and 45.0 µg/l, respectively after 24 and 48 h of exposure. Concerning the feeding inhibition test a 6 h-EC50 of 503.7 µg/l (95 % CI= 237.7 -769.7) was obtained.

3.4 *D. rerio* embryos

No significant mortality was observed in the control group (1.3 % at 96 h). A dose-dependent response to ChD was observed (**Fig. 1**) with a 96 h-LC50 of 804.0 µg/l (Table 1). The development of embryos in the control group was normal as described by Kimmel *et al.* (1995) (**Fig. 2a and c**). No teratogenic effects were found in embryos exposed to ChD but some embryos showed alterations in the amniotic fluid, which presented a non-homogeneous and opaque aspect containing agglomerates of particles (**Fig. 2b and d**). For the alterations in the amniotic fluid, a 48 h-EC50 of 1020.9 µg/l (standard error: 78.8) was calculated. Moreover, ChD exposure increased the hatching rate in lower concentrations tested (**Table 2**). At 48 h no hatching was observed in control group but in the ChD treatments from 40 to 640 µg/l a premature hatching rate over 20% was verified (H = 37.270). At higher concentrations (1050 and 1280 µg/l), above 60 % of the exposed organisms died and no premature hatching was observed in the surviving organisms.

Table 2 - Percentage of *D. rerio* eggs hatched during exposure to chlorhexidine digluconate (standard error between brackets)

ChD (µg/l)	48 h	72 h	96 h
0	0 (0)	97.22 (2.8)	100 (0)
40	23.7 (8.5)	100 (0)	100 (0)
80	31.8 (5.2)	100 (0)	100 (0)
160	56.67 (3.3) *	96.7 (3.3)	96.7 (3.3)
320	40 (5.8)	100 (0)	100 (0)
640	28 (9.2)	100 (0)	100 (0)
1050	0 (0)	66.67 (3.3)	100 (0)
1280	0 (0)	75 (21.4)	100 (0)
1470	0 (0)	-	-
2400	0 (0)	-	-

"*" means significantly different from control (Dunn's test, $p < 0.05$)

"-" all organisms died.

Exposure of *D. rerio* embryos to ChD caused significant changes in ChE (F= 5.842) and CAT (F= 2.967) activities but not in LDH (H = 9.760) or GST (F= 1.591) activities (**Fig. 3**).

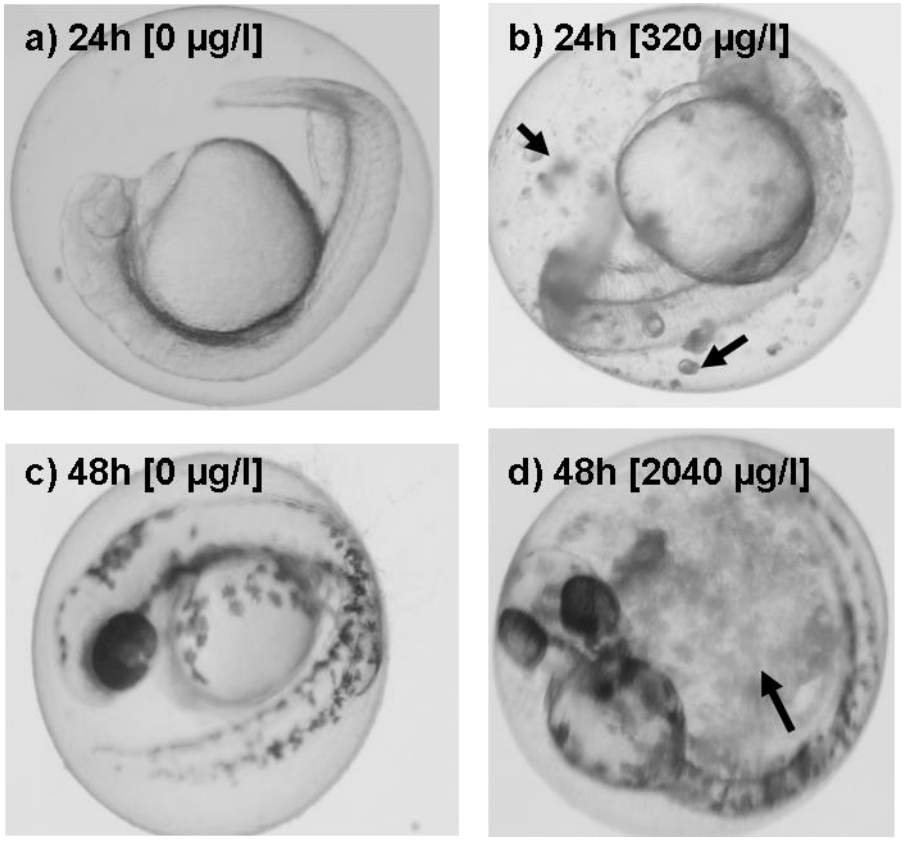


Fig 2 *D. rerio* embryos abnormalities after chlorhexidine digluconate exposure: a) control organism after 24 h; b) embryo after 24 h of exposure to 320 µg/l with alterations in amniotic fluid (black arrows); c) control organism after 48 h; d) embryo after 48 h of exposure to 2040 µg/l with alterations in amniotic fluid (black arrows).

4. Discussion

In the present study, lethal and sub lethal effects of ChD on several aquatic organisms were assessed. ChD was more toxic to the algae *P. subcapitata* and the crustacean *D. magna*, when compared with the bacteria *V. fischeri* and the *D.*

rerio embryos. These results agree with those of previous studies performed with chlorhexidine compounds that also showed higher sensitivity of *D. magna* when compared to bacteria and fish (**Table 3**).

ChD toxicity to *V. fischeri* is similar to the toxicity to *Klebsiella oxytoca* (EC50 of 1550 µg/l) but lower than the toxicity to *Escherichia coli* (EC50 of 320 µg/l) (Guimarães et al. 2012). ChD was highly toxic to the algae *P. subcapitata*, with values as low as 31.1 µg/l causing a 20 % reduction in algae growth rate (i.e. 72 h-EC20). The high toxicity of ChD to the algae observed in the present study is in line with the high toxicity of ChD to river biofilms, as found by Lawrence et al. (2008). These authors reported significant changes in the algae and cyanobacteria biomass, and river biofilms community structure after exposure to 10 µg/l of ChD. However, they could not determine EC50 values for algae and cyanobacteria species (EC50 > 100 µg/l), using 24 wells microplates for carrying out the growth inhibition assays. Similarly to the algae, ChD was very toxic to *D. magna*, with a 48 h-EC50 of 45.0 µg/l. This value is slightly lower than the toxicity value of 63 µg/l reported previously for *D. magna* exposed to chlorhexidine diacetate (USEPA 1996) (**Table 3**). Feeding inhibition of *D. magna* (sub lethal endpoint) was much less sensitive than immobilization (lethal endpoint). Allen et al. (1995) showed that non-polar or negatively charged chemicals in the water (such as ChD) are less likely adsorbed by the surface of negatively charged particles (e.g. algae cells) than will positively charged species and, thus, very high concentrations will be required to exert toxic effects through feeding. This, allied to the lower exposure period, might explain the lower sensitivity of the feeding endpoint compared to the immobilization endpoint.

Table 3 – Toxicity values of chlorhexidine compounds to bacteria, algae, invertebrates and fish

Species	Compound	Value (µg/l)[1]	Endpoint	Effect	Time	Reference
Klebsiella oxytoca	Chlorhexidine digluconate	1550	Respiration	EC50	29.5 min	(Guimarães et al. 2012)
Escherichia coli	Chlorhexidine digluconate	320	Respiration	EC50	33.9 min	(Guimarães et al. 2012)
Vibrio fischeri	Chlorhexidine digluconate	1694	Bioluminescence	EC50	15 min	present study
Pseudokirchneriella subcapitata	Chlorhexidine digluconate	62	Growth inhibition	EC50	72 h	present study
Daphnia magna	Chlorhexidine diacetate	63	Immobilization	EC50	48 h	(USEPA 2000)
Daphnia magna	Chlorhexidine digluconate	45	Immobilization	EC50	48 h	present study
Danio rerio embryos	Chlorhexidine digluconate	804	Mortality	LC50	96 h	present study
Danio rerio	Chlorhexidine	1400	Mortality	LC50	96 h	(EC 2000)
Lepomis macrochirus	Chlorhexidine diacetate	600	Mortality	LC50	96 h	(USEPA 2000)
Oncorhynchus mykiss	Chlorhexidine diacetate	1871	Mortality	LC50	96 h	(USEPA 2000)

[1]All values refer to mean effective concentration of the active ingredient concentration

ChD also showed high toxicity to *D. rerio* embryos with a 96 h-LC50 of 804 µg/l. This value is about 2 -fold lower than the value reported for adult *D. rerio* exposed to a chlorhexidine compound and is in the same range of the 96 h-LC50 values reported for other fish species exposed to chlorhexidine diacetate (**Table 3**). The Fish Embryo Toxicity test allowed the evaluation of ChD effects on hatching and amniotic fluid during the embryonic development. Early hatching of embryos observed in this study has also been described previously but as a result of exposure to crude oil (Boudreau et al. 2009; Carls et al. 1999). Despite no consequences of the premature hatching were found during the exposure period subsequent effects in the development should not be excluded. At high doses the

hatching is not stimulated probably due to the severe toxic effects of ChD that lead to a mortality rate above 60 % at 72 h. Alterations in the amniotic fluid of embryos (48 h-EC20 of 753.6 µg/l) occur at concentrations close to the 96 h-LC50 to *D. rerio*. To our knowledge, no study has been published describing alterations in the amniotic fluid of *D. rerio* embryos caused by biocides.

Among the tested enzymatic biomarkers in fish embryos, ChE appears to be the most sensitive to ChD, being significantly induced in all tested concentrations (80 to 900 µg/l). The induction of ChE in fish exposed to chlorhexidine is not fully understood. In mammals and amphibians, chlorhexidine has been shown to affect the nervous system, including nerve degeneration (Röed 1973; Henschen and Olson 1984) and inhibition of nicotinic acetylcholine receptors (Shaihutdinova et al. 2009). One could speculate that the inhibition of nicotinic acetylcholine receptors may be related to the enhancement of ChE activity since this would lead to an increase of acetylcholine in the synaptic cleft and, consequently, increase ChE activity in an attempt to promote the degradation of the excess of acetylcholine. However, to confirm a specific effect of ChD on acetylcholinesterase activity of zebrafish embryos further studies are required, aiming to distinguish the non-specific activity of other enzymes than acetylcholinesterase (e.g., butyrylcholinesterase). This fact is important since tissues of zebrafish embryos may contain significant amounts of non-specific esterases, which can contribute to the measured activity and may show different sensitivity towards chemical compounds (Kuster, 2005). On the other hand, cytotoxic effects of ChD, namely apoptosis, were observed in different cell types (Giannelli et al., 2008). Since apoptosis is related to upregulation of acetylcholinesterase genes in different cells types one could suggest that the observed induction of ChE activity in zebrafish embryos is linked to the cytotoxic effects of ChD (Zhang et al. 2002).

Concerning CAT, a significant effect was observed only at 900 µg/l. Several studies have reported CAT induction in fish exposed to several toxicants that act modulating the antioxidant defensive systems (Jemec et al. 2010; Oost et al. 2003). The induction of CAT observed in the present study might be related to the

metabolism of reactive oxygen species (ROS). Such hypothesis is supported by a previous study reporting the generation of ROS by chlorhexidine aqueous preparations (Barbin et al. 2008).

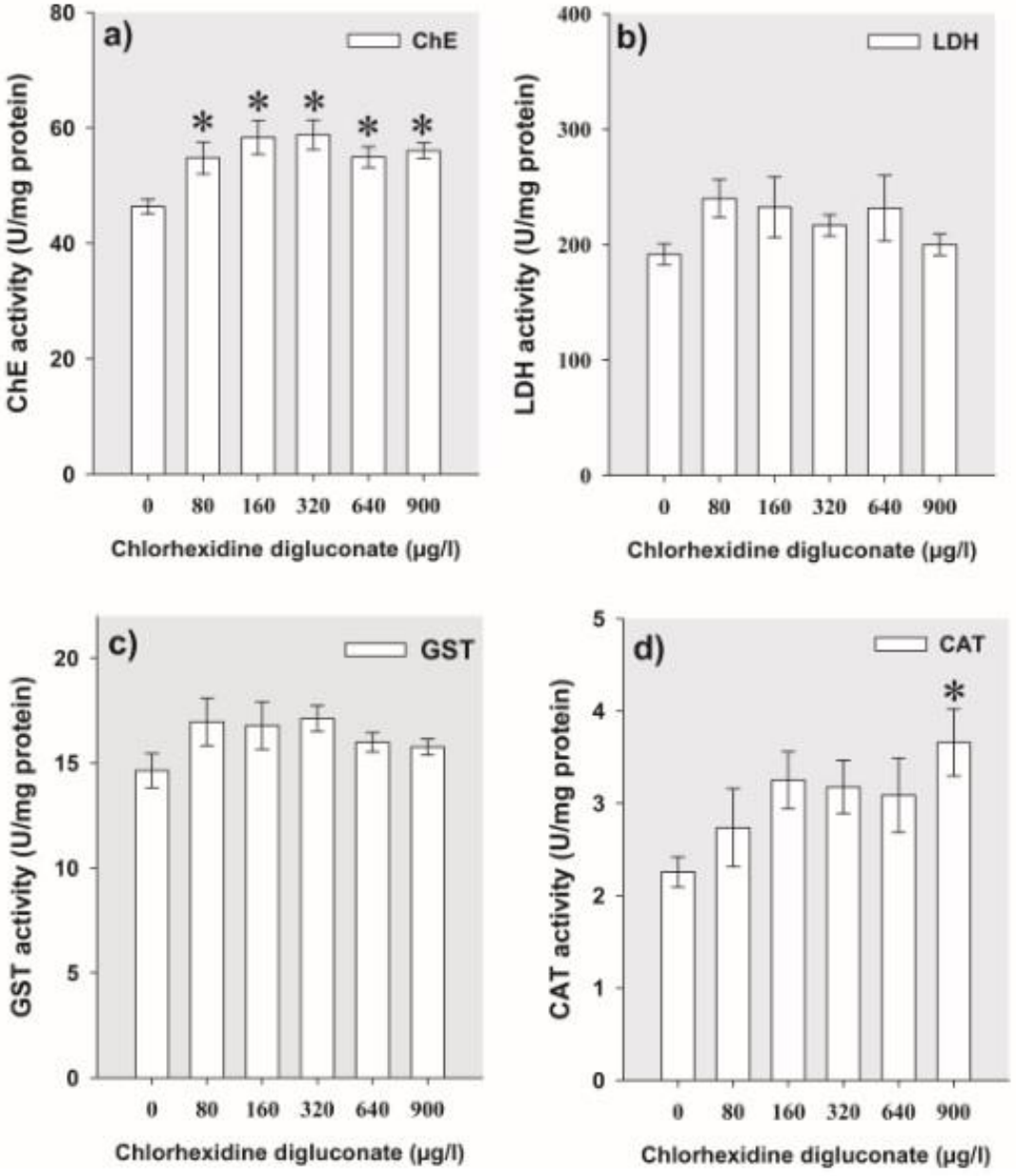


Figure 3 - Biomarkers activities on *D. rerio* embryos after 96 h of exposure to chlorhexidine digluconate (mean values ± standard error). "*" means significantly different from the respective control treatment (Dunett's or Dunn's test, $p < 0.05$)

The current ChD concentrations in aquatic systems remain unknown,

despite recent studies on concentrations of environmental contaminants in aquatic systems (Kolpin et al. 2002; Loos et al. 2012). However, two studies from the 1980's reported concentrations of 10.3 µg/1 in domestic wastewater (Kodama et al. 1988) and up to 1940 µg/1 in hospital wastewater (Matsushima and Sakurai 1984). The high ChD concentration in hospital wastewater is concordant with its use as disinfectant in health care facilities and this value is higher than any EC50 value of ChD to aquatic organisms reported in this study. However, the hospital effluent is not OR likely to be representative of ChD concentration in aquatic systems and, thus, does not an environmental relevant scenario of ChD contamination. On the other hand, the concentration of 10.3 µg/1 reported in domestic wastewater is about half of the 24 h-EC20 of *P. subcapitata* and the 48 h-EC20 of *D. magna*, which suggests deleterious effects to these organisms, especially considering that in the environment these organisms will be exposed to the toxicant for longer periods. The lacking of data on ChD environmental concentrations together with lacking of data on its ecotoxicity, physical, and chemical properties prevents a risk characterization (Boxall et al. 2006).

ChD toxicity, allied to its stability (Jensen and Christensen 1971; Richardson and Bowron 1985) and pattern of use raises concerns about the potential toxicity of ChD to aquatic organisms. Considering a daily application routine of ChD products that can lead to continuous exposure of aquatic ecosystems, long-term adverse effects can be expected particularly under unfavourable conditions that increase ChD half-life. Moreover, after ChD degradation organisms might be exposed to *p*-chloroaniline, a degradation sub-product that also showed to be toxic to aquatic organisms (Boehncke et al. 2003).

The high toxicity of ChD to *P. subcapitata* and *D. magna* suggests that this compound may cause adverse effects on aquatic ecosystems. *P. subcapitata* is an abundant green algae species in freshwater ecosystems (Blaise 1986). Likewise, *Daphnia* species are amongst the most abundant zooplankton in many lake systems (Jeziorski et al. 2008). Thus, the high toxicity of ChD to these organisms might have adverse consequences in the abundance of these species. Moreover, if other algae and crustaceans are equally sensitive to ChD, this might suggest a

high potential for adverse effects of ChD on the structure of aquatic food webs, disturbing aquatic ecosystems.

5. Conclusions

The present study shows a high toxicity of ChD to aquatic organisms. Species sensitivity to ChD decreased in the following order: *P. subcapitata* > *D. magna* > *D. rerio* > *V. fischeri*, with toxicity values ranging from 41.3µg/L (24h-EC_{50} of *P. subcapitata*) to 3675.2 µg/L (5 min-EC_{50} of *V. fischeri*). The toxicity to the algae and the crustacean raises a special concern about its potential effects in aquatic food webs, since these organisms are in the base of trophic chains. Moreover, considering the continuous input of ChD residues driven by the generalized daily use of products containing ChD and the high toxicity to aquatic organisms, long-term adverse effects on aquatic ecosystem can be expected. Future studies aiming to assess ChD toxicity under an ecologically relevant scenario should consider ChD toxicity in complex mixtures, since ChD is usually used simultaneously with other chemicals.

Acknowledgements

Authors acknowledge *Fundação para Ciência e Tecnologia* (FCT – Portugal) by the financial support through the grants SFRH/BPD/31752/2006, SFRH/BD/27637/2006 and SFRH/BD/62605/2009, University of Aveiro.

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

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SUPPORTING INFORMATION

Lethal and sub lethal effects of the biocide chlorhexidine on aquatic organisms

Fátima T. Jesus[1†], Rhaul Oliveira[1†*], Andreia Silva[1], Ana L. Catarino[1], Amadeu M.V.M. Soares[1], António J.A. Nogueira[1], Inês Domingues[1]

[1]*Department of Biology & CESAM, Campus Universitário de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal*

*Corresponding author - e-mail: rhaul.oliveira@ua.pt; Fax: +35234372587; Phone: +351234370 790

Stock solutions of chlorhexidine digluconate (ChD) prepared for each test were analysed daily (including at the beginning of the tests). During this period, they were kept under the same experimental conditions as the toxicity tests. The determinations of ChD were performed according to the spectrophotometric method described by Jensen and Christensen (1971). The standard curve showing the relationship between the amount of ChD in the sample and the absorbance at 250 nm is depicted in Figure 1. The concentrations and percentage of recovery from the stock solutions are shown in Table 1.

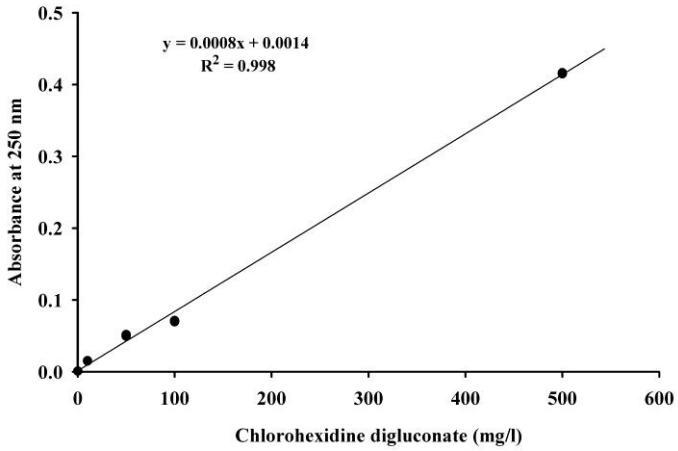

Figure 1 - Standard curve showing the relationship between the amount of ChD and the absorbance at 250 nm.

Table 1 – Concentrations of chlorhexidine digluconate and percentage of recovery from the stock solutions (concentrations followed by standard deviation in brackets)

Time	***Daphnia* conditions***		**Algae conditions***		**Fish conditions***	
	Concentration (mg/l)	*% of recovery*	*Concentration (mg/l)*	*% of recovery*	*Concentration (mg/l)*	*% of recovery*
Day 0 nominal concentration	20		200		20	
Day 0	20.1 (5.3)	**101.0**	232.2 (5.9)	**116.1**	20.1 (5.3)	**101**
Day 1	16.4 (2.8)	**82.0**	230.2 (42.1)	**115.1**	15.9 (2.8)	**79.5**
Day 2	22.5 (2.6)	**112.5**	221.2 (28.8)	**110.6**	17.2 (2.2)	**89.5**
Day 3	-		198.5 (46.1)	**110.7**	16.1 (2.7)	**80.5**
Day 4	-		-		20.3 (2.9)	**101.5**

"*" *Daphnia* conditions: temperature of 20 ± 1ºC, photoperiod 16 h light: 8 h dark, light intensity of 34 lux; algae conditions: 24 ± 1 ºC, continuous light (3400 lux); fish conditions: 26.0 ± 1ºC, photoperiod 14 h light: 10 h dark, light intensity of 32 lux.

Reference

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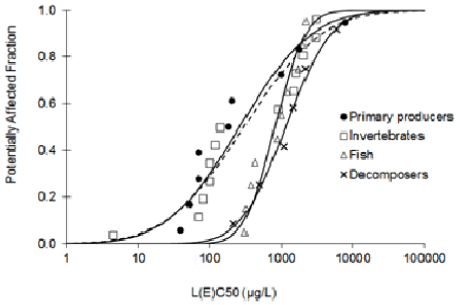
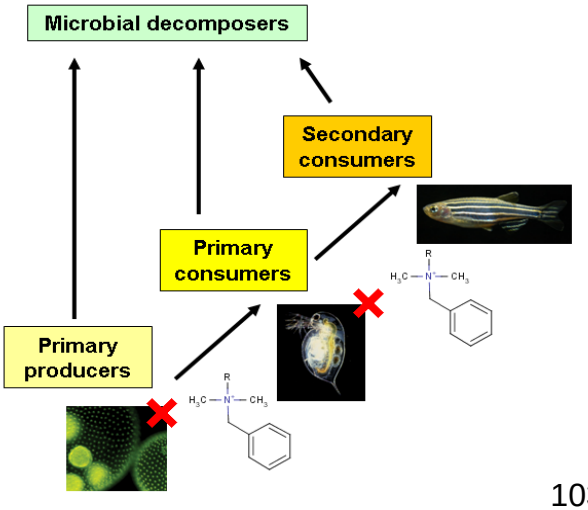
Chapter 4

Use of species sensitivity distributions to assess the toxicity of benzalkonium chloride to aquatic organisms from different trophic levels





Use of species sensitivity distributions to assess the toxicity of benzalkonium chloride to aquatic organisms from different trophic levels

Rhaul Oliveira; Andreu Rico; Susana Pereira; Darlan Quinta; Sakchai McDonough; Amadeu M.V.M. Soares; António J.A. Nogueira & Inês Domingues

This chapter is submitted as an original article to:

Science of Total Environment

Abstract

Benzalkonium Chloride (BKC) is a quaternary ammonium biocide widely used in personal care products, hospitals, wood and food industries (e.g. aquaculture). However, the environmental risk of BKC to aquatic ecosystems has been poorly studied. The main goal of this study was to assess the toxicity of BKC to aquatic organisms through a battery of toxicity tests that allowed a preliminary risk assessment of BKC for four different trophic levels of the aquatic ecosystems. Toxicity tests were performed for decomposers (*Vibrio fischeri*), primary producers (*Lemna minor, Chlorella vulgaris* and *Pseudokirchneriella subcapitata*), primary consumers (*Daphnia magna* and *Thamnocephalus platyurus*), and secondary consumers (*Danio rerio* and *Oreochromis niloticus*). The results from the toxicity tests were used to build a Species Sensitivity Distributions (SSD) and to calculate the BKC median Hazardous Concentrations (HC 5% and 50%), separately to each trophic level. Moreover, using Measured Environmental Concentrations (MEC) of BKC, the Potentially Affected Fractions of species (PAFs) were estimated for four different scenarios (raw and wastewater treatment plants effluents and surface water) by joining SSD results and distributions of MEC. Among primary producers, *P. subcapitata* was the most sensitive (72h-EC50=69 µg/l) followed by *C. vulgaris* (72h-EC50=94 µg/l) and *L. minor* (168h-EC50=1340 µg/l). Primary consumers were highly sensitive to BKC (*D. magna*: 48h-EC50=52 µg/l and *T. platyurus*: 24h-EC50=84 µg/l) whereas fish species showed to be slightly sensitive (*O. niloticus* embryos: 96h-LC50=370 µg/l, *D. rerio* adults: 96h-LC50=2350 µg/l and embryos: 2060 µg/l). The SSD analysis indicate the primary consumers (HC5=10.83 µg/l) and the primary producers (HC5=11.40 µg/l) as the most sensitive groups followed by decomposers (HC5=152.21 µg/l) and secondary consumers (HC5=217.07 µg/l). Due to the high concentrations of BKC in hospital, laundry and industrial effluents the PAFs values obtained were > 5% to the four trophic levels studied suggesting high toxicity of this effluent to aquatic biota. Even after treatment, the concentration of BKC in sewage treatment plant effluents and surface water are high enough to harm primary producers and consumers (PAFs > 5%) raising a high concern on the environmental effects of BKC.

Keywords: species sensitivity distribution, decomposers, primary producers, primary consumers, secondary consumers, affected fraction of species, biocide

1. Introduction

The intensification of human activities is associated with the increasing use of chemicals for different purposes. The benzalkonium chloride (BKC), is a cationic surfactant commonly found in many pharmaceutical and cosmetic formulations, commercial disinfectants, industrial sanitizers, and wood preservatives (Kreuzinger et al., 2007). Most domestic and industrial applications of BKC produce toxic residues typically discharged into wastewater treatment systems. Consequently, aquatic systems are subject to noxious effects of these residues, particularly in scenarios of insufficient dilution or major discharges of effluents. As other quaternary ammonium compounds, BKC has been detected in the surface water and sediments of aquatic ecosystems. Due to the extensive use, high concentrations of BKC are frequently found in effluents of hospitals (1.05 to 3928.5 µg/l) and Wastewater Treatment Plants (WTP's) (0.14 – 145.1 µg/l) (Kümmerer et al., 1997; Ferrer and Furlong, 2001; Martínez-Carballo et al., 2007a; Martínez-Carballo et al., 2007b). Moreover, due to the insufficient BKC removal rate in the WTPs, several studies have found this compound in the surface waters (0.014 – 65.3 µg/l) and sediments (1.39 – 21300 µg/kg) of receiving water bodies (Ferrer and Furlong, 2002). In some aquaculture farms, untreated effluents containing BKC are released directly into rivers and coastal ecosystems (Rico et al., 2012). However, in order to estimate the environmental risk of BKC used in aquacultures more studies are needed, principally with invertebrates and primary producers (Rico et al., 2013; Rico and Van den Brink, 2014).

BKC is reported as highly toxic for various aquatic organisms at environmentally relevant concentrations (Kümmerer et al., 1997; Ding and Liao, 2000; Kreuzinger et al., 2007) (**Table 1**). Up to now, the risk assessment of BKC to natural populations has been based in the ratio between estimated Predicted Environmental Concentrations (PEC) and Predicted No-Effect Concentration (PNEC) derived from single-species toxicity tests (Kreuzinger et al., 2007). However, by using PEC/PNEC ratios, the risk of BKC can be misestimated.

Predicting the risk of chemicals on aquatic ecosystems is a complex task. Effects might differ according to the scenario of exposure, species sensitivity and trophic levels. Thus, to better understand the risk of BKC, different approaches can be employed, namely the Species Sensitivity Distributions (SSDs), integrating data from several species and considering different trophic levels (Maltby et al., 2005; Mochida et al., 2012). In the SSD approach the responses of different organisms are displayed as cumulative distribution functions allowing the estimation of the hazardous concentrations for 5 and 50 % of the species (HC5 and HC50, respectively) (Beaudouin and Péry, 2013). Aditionally, a probabilistic environmental risk assessment can be perfomed to estimate the likelihood and the extent of adverse effects of BKC in different scenarios of exposure. A simple comparison of exposure concentrations and species sensitivity derived from toxicity data gives information on the Potentially Affected Fraction of species (PAFs) by BKC (Verdonck et al., 2003).

The main goal of this study was to assess the toxicity of BKC to aquatic organisms through a battery of toxicity tests that allowed a preliminary risk assessment of BKC for four different trophic levels of the aquatic ecosystems. Primarily, the short-term toxicity of BKC was assessed using bioassays with different species of microbial decomposers (*Vibrio Fischeri*), primary producers (*Lemna minor*, *Chlorella vulgaris* and *Pseudokirchneriella subcapitata*), primary consumers (*Daphnia magna* and *Thamnocephalus platyurus*) and secondary consumers (*Danio rerio* and *Oreochromis niloticus*). In a second step the toxicity data obtained in this study were pooled with data from the literature in order to perform a SSD analysis to the four trophic levels studied and the Potentially Affected Fraction of species (PAFs) for different scenarios of contamination were estimated.

Table 1 – Short-term effects of Benzalkonium Chloride on aquatic organisms namely decomposers, primary producers, primary consumers and secondary consumers (up to 168h of exposure)

Species	Endpoint	Effect	Time (h)	Value (mg/L)	References
Decomposers					
Bacillus cereus	EC50	Physiology	2	1.1	Heise na Ahlf et al. (2005)
Pseudomonas putida	EC50	Growth	16	6	Sütterlin et al. (2008)
Spirostomun ambiguum	EC50	Deformities	24	1.32	Nacz-Jawecki et al. (2003)
Spirostomun ambiguum	EC50	Deformities	24	0.36	Nacz-Jawecki et al. (2003)
Spirostomun ambiguum	EC50	Deformities	24	0.26	Nacz-Jawecki et al. (2003)
Tetrahymena thermophile	EC50	Growth	24	2.94	Kreuzinger *et al.* (2007)
Tetrahymena thermophile	EC50	Growth	24	1.75	Nacz-Jawecki et al. (2003)
Tetrahymena thermophile	EC50	Growth	24	2.12	Nacz-Jawecki et al. (2003)
Vibrio fischeri	EC50	Luminescense	0.25	0.1	Nacz-Jawecki et al. (2003)
Vibrio fischeri	EC50	Luminescense	0.25	0.17	Nacz-Jawecki et al. (2003)
Vibrio fischeri	EC50	Luminescense	0.25	0.08	Nacz-Jawecki et al. (2003)
Vibrio fischeri	EC50	Luminescense	0.25	0.3	Our Study
Vibrio fischeri	EC50	Luminescense	0.25	0.5	Sütterlin et al. (2008)
Vibrio fischeri	EC50	Luminescense	0.25	0.14	Tezel et al. (2009)
Vibrio fischeri	EC50	Luminescense	0.25	0.27	Tezel et al. (2009)
Vibrio fischeri	EC50	Luminescense	0.25	0.66	Tezel et al. (2009)
Vibrio proteolyticus	EC50	Physiology	2	1.12	Heise na Ahlf et al. (2005)
Vibrio proteolyticus	EC50	Physiology	2	1.97	Heise na Ahlf et al. (2005)

Primary producers					
Chaetoceros gracilis	EC50	Growth	120	0.07	Pérez et al. 2009
Chlorella pyrenoidosa	EC50	Growth	24	0.16	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	24	0.26	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	24	0.45	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	48	0.28	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	48	0.51	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	48	0.09	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	72	0.26	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	72	0.54	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	72	0.09	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	96	0.1	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	96	0.28	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	96	0.54	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	24	0.18	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	48	0.19	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	72	0.2	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	96	0.17	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	24	0.06	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	48	0.06	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	72	0.06	USEPA (2013)
Chlorella pyrenoidosa	EC50	Population	96	0.06	USEPA (2013)
Chlorella pyrenoidosa	EC50	Growth	96	0.67	Utsunomiya et al.(1997)
Chlorella vulgaris	EC50	Growth	72	0.07	Our Study
Dunaliella sp.	EC50	Growth	24	1.8	Utsunomiya et al.(1997)

Isochrysis galbana	EC50	Growth	120	0.04	Pérez et al. (2009)
Lemna minor	EC50	Growth	72	7.87	Our Study
Macrocystis pyrifera	EC50	Physiology	96	1	USEPA (1959)
Pseudokirchneriella subcapitata	EC50	Growth	72	0.04	Kreuzinger *et al.* (2007)
Pseudokirchneriella subcapitata	EC50	Growth	72	0.07	Our Study
Scenedesmus pannonicus	EC50	Growth	72	0.12	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	96	0.14	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	24	0.08	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	24	0.75	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	24	0.85	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	48	0.28	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	48	0.32	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	48	0.08	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	72	0.26	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	72	0.33	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	72	0.08	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	96	0.27	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	96	0.32	USEPA (2013)
Scenedesmus pannonicus	EC50	Growth	96	0.09	USEPA (2013)
Scenedesmus pannonicus	EC50	Population	24	0.2	USEPA (2013)
Scenedesmus pannonicus	EC50	Population	48	0.15	USEPA (2013)
Primary consumers					
Artemia fransciscana	LC50	Mortality	24	0.03	Bartolomé and Sánchez-Fortún (2005)
Artemia fransciscana	LC50	Mortality	24	0.01	Bartolomé and Sánchez-Fortún (2005)
Artemia franciscana	LC50	Mortality	24	0.0000007	Bartolomé and Sánchez-Fortún (2005)

Artemia franciscana	EC50	Phototaxis	24	0.00096	Bartolomé and Sánchez-Fortún (2005)
Artemia franciscana	LC50	Mortality	24	0.18	Nacz-Jawecki *et al.* (2003)
Artemia franciscana	LC50	Mortality	24	0.03	Nacz-Jawecki *et al.* (2003)
Artemia franciscana	LC50	Mortality	24	0.03	Nacz-Jawecki *et al.* (2003)
Brachionus calyciflorus	EC50	Mortality	48	0.12	Kreuzinger *et al.* (2007)
Cassostrea gigas	EC50	Embryotoxicity	24	0.14	His *et al.* (1996)
Caenorhabditis elegans	EC50	Growth	96	15.1	Höss *et al.* (2012)
Daphnia magna	LC50	Mortality	48	0.02	Dobbs *et al.* (1995)
Daphnia magna	LC50	Mortality	48	0.04	Kreuzinger *et al.* (2007)
Daphnia magna	LC50	Mortality	48	0.05	Our study
Daphnia magna	EC50	Behaviour	24	0.02	USEPA (2013)
Daphnia magna	EC50	Behaviour	24	0.18	USEPA (2013)
Daphnia magna	EC50	Behaviour	24	0.23	USEPA (2013)
Daphnia magna	EC50	Behaviour	24	0.06	USEPA (2013)
Daphnia magna	EC50	Behaviour	48	0.12	USEPA (2013)
Daphnia magna	EC50	Behaviour	48	0.02	USEPA (2013)
Daphnia magna	EC50	Behaviour	48	0.04	USEPA (2013)
Daphnia magna	EC50	Behaviour	48	0.06	USEPA (2013)
Daphnia magna	LC50	Mortality	24	0.12	USEPA (2013)
Daphnia magna	LC50	Mortality	24	0.16	USEPA (2013)
Daphnia magna	LC50	Mortality	24	0.21	USEPA (2013)
Daphnia magna	LC50	Mortality	24	0.26	USEPA (2013)
Daphnia magna	LC50	Mortality	48	0.11	USEPA (2013)
Daphnia magna	LC50	Mortality	48	0.16	USEPA (2013)
Daphnia magna	LC50	Mortality	48	0.0059	APVMA (2012)

Daphnia magna	LC50	Mortality	48	0.04	USEPA (2013)
Daphnia magna	LC50	Mortality	48	0.1	USEPA (2013)
Galeolaria caespitose	EC50	Fertilization	48	0.1	APVMA (2012)
Fenneropenaeus penicillatus	LC50	Mortality	24	1.6	USEPA (2013)
Macrobrachium rosenbergii	LC50	Mortality	24	2	USEPA (2013)
Metapenaeus ensis	LC50	Mortality	24	3	USEPA (2013)
Mysidopsis bahia	LC50	Mortality	96	0.08	Dobbs *et al.* (1995)
Nitocra spinipes	LC50	Mortality	96	0.9	Liden *et al.* (1979)
Penaeus japonicas	LC50	Mortality	24	3.3	USEPA (2013)
Penaeus monodon	LC50	Mortality	24	3.1	USEPA (2013)
Penaeus semisulcatus	LC50	Mortality	24	1.5	USEPA (2013)
Thamnocephalus platyurus	LC50	Mortality	24	0.1	Our study
Secondary consumers					
Cyprinodon variegatus	LC50	Mortality	96	0.88	Dobbs *et al.* (1995)
Danio rerio	LC50	Embryotoxicity	48	2.06	Our study
Danio rerio	LC50	Mortality	96	2.35	Our study
Lepomis macrochirus	LC50	Mortality	24	0.58	USEPA (2013)
Lepomis macrochirus	LC50	Mortality	96	0.32	USEPA (2013)
Menidia beryllina	LC50	Mortality	96	0.31	Dobbs *et al.* (1995)
Oncorhynchus mykiss	LC50	Mortality	96	1.01	Dobbs *et al.* (1995)
Oncorhynchus mykiss	LC50	Mortality	24	1.3	USEPA (2013)
Oncorhynchus mykiss	LC50	Mortality	96	1.15	USEPA (2013)
Oreochromis niloticus	LC50	Embryotoxicity	96	0.37	Our study
Oryzias latipes	EC50	Behaviour	24	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	24	0.75	USEPA (2013)

Oryzias latipes	EC50	Behaviour	24	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	24	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	48	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	48	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	48	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	48	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	72	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	72	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	72	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	72	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	96	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	96	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	96	0.75	USEPA (2013)
Oryzias latipes	EC50	Behaviour	96	0.75	USEPA (2013)
Oryzias latipes	LC50	Mortality	24	2.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	24	4	USEPA (2013)
Oryzias latipes	LC50	Mortality	24	4.2	USEPA (2013)
Oryzias latipes	LC50	Mortality	24	7	USEPA (2013)
Oryzias latipes	LC50	Mortality	48	2.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	48	3.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	48	4.2	USEPA (2013)
Oryzias latipes	LC50	Mortality	48	6.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	72	2.5	USEPA (2013)
Oryzias latipes	LC50	Mortality	72	3.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	72	4	USEPA (2013)

Oryzias latipes	LC50	Mortality	72	6.3	USEPA (2013)
Oryzias latipes	LC50	Mortality	96	2.4	USEPA (2013)
Oryzias latipes	LC50	Mortality	96	3.8	USEPA (2013)
Oryzias latipes	LC50	Mortality	96	3.9	USEPA (2013)
Oryzias latipes	LC50	Mortality	96	6.1	USEPA (2013)
Pimephales promelas	LC50	Mortality	96	0.28	APVMA (2012)
Pimephales promelas	LC50	Mortality	96	0.36	Dobbs *et al.* (1995)
Poecilia reticulata	EC50	Behaviour	24	1.3	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	24	2.4	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	24	4.2	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	24	0.75	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	48	1.3	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	48	2.4	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	48	4.2	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	48	0.75	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	72	1.3	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	72	2.4	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	72	0.75	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	72	0.75	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	96	1.3	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	96	2.4	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	96	0.75	USEPA (2013)
Poecilia reticulata	EC50	Behaviour	96	0.75	USEPA (2013)
Poecilia reticulata	LC50	Mortality	24	1.8	USEPA (2013)
Poecilia reticulata	LC50	Mortality	24	2.4	USEPA (2013)

Poecilia reticulata	LC50	Mortality	24	4.5	USEPA (2013)
Poecilia reticulata	LC50	Mortality	24	5.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	48	1.6	USEPA (2013)
Poecilia reticulata	LC50	Mortality	48	2.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	48	4.2	USEPA (2013)
Poecilia reticulata	LC50	Mortality	48	4.5	USEPA (2013)
Poecilia reticulata	LC50	Mortality	72	1.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	72	2.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	72	3.8	USEPA (2013)
Poecilia reticulata	LC50	Mortality	72	4.2	USEPA (2013)
Poecilia reticulata	LC50	Mortality	96	1.3	USEPA (2013)
Poecilia reticulata	LC50	Mortality	96	2.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	96	3.4	USEPA (2013)
Poecilia reticulata	LC50	Mortality	96	4.2	USEPA (2013)

2. Materials and methods

2.1 Chemical

High purity Benzalkonium chloride ~50% solution in water (CAS Number: 8001-54-5) was purchased from Fluka (St. Louis, MO, USA).

2.2 *Vibrio fischeri* assays

The bacteria V. fischeri was used in the Microtox® Test, an acute assay that measures the effect of toxicants and environmental samples on organisms light production. The bacteria were purchased as dried kits (Modernwater, Cambridge, United Kingdom). They were stored at − 20 °C and rehydrated prior to testing.

The Bioluminescence inhibition assays (Microtox) were carried out following the 81.9 % Basic test protocol (AZUR Environmental 1998), using the Microtox 500 Analyzer. Testing was conducted using 100 µl of bacterial suspension mixed with 500 µl of test solution to give BKC final concentrations of 10, 20, 70, 210, 620, 1850, 5560, 16670 and 50000 µg/l. Bioluminescence was measured after 0, 5 and 15 minutes of incubation.

2.3. Microalgae assays

Two species of freshwater unicellular green algae *C. vulgaris* and *P. subcapitata* were obtained from axenic batch cultures of Woods Hole MBL medium at 20 ± 1 ºC and continuous light (cool-white fluorescent light, 3400 lux).

The algae growth inhibition tests were based on the OECD guideline 201 (OECD, 2006a) and ran for 72 h. Each species was exposed to control medium and BKC concentrations of 20, 30, 100, 160, 320 and 1000 µg/l, in triplicate. Tests were carried out in 24-well microplates; each well contained the exposure solution and the algae at a density of 1.0 × 104 cells/ml (log exponential growth phase) in a volume of 1 ml. The test conditions were similar to the culture conditions. The absorbance (λ = 440 nm) was daily measured by spectrometry (Thermo Scientific

Multiskan® Spectrum). The absorbance values (ABS) were converted to the cell density (cell / ml) using regression models previously developed:

$\text{Cell / ml} = 2.0^4 \times ABS - 8.0^6$ for *C. vulgaris*

$\text{Cell / ml} = 2.0^5 \times ABS - 1.0^7$ for P*. subcapitata*

The average specific growth rate for a specified period was calculated as the logarithmic increase in cell density from the following equation:

$$\mu_{i-j} = \frac{\ln Xj - \ln Xi}{tj - ti}$$

Where μ_{i-j} is the average specific growth rate from time *i* to *j* (per day); *Xi* is the cell density at time *i*, and *Xj* is the cell density at time *j*; *ti* is the initial time of the exposure and *tj* is the final time of the exposure (OECD, 2006a).

2.4 *Lemna minor* assays

The *L. minor* monoculture was established as a non-axenic culture using a modified Swedish Standard medium at 25 ± 1 ºC and continuous light (6,500 – 10,000 lux).

The macrophytes growth inhibition test was based on the OECD guideline 221 (OECD, 2006b) and ran for 168 h. *L. minor* was exposed to control medium and BKC concentrations of 1000, 1800, 3200, 5600, 18000, and 32000 µg/l, in triplicate. Each replicate consisted in 4 sets of fronds, each one with 3 fronds in a glass beaker (100 ml of exposure solution). The test conditions were similar to the culture conditions. Effects on the number of fronds and chlorosis were recorded daily; additionally fresh weight, dry weight and root size were measured after the exposure period. The average specific growth rate per day was calculated as the logarithmic increase in the number of fronds from the equation:

$$\mu_{i-j} = \frac{\ln Xj - \ln Xi}{tj - ti}$$

Where μ_{i-j} is the average specific growth rate from time *i* to *j* (per day); *Xi* is the number of fronds at time *i*, and *Xj* is the number of fronds at time *j*; *ti* is the initial time of the exposure and *tj* is the final time of the exposure(OECD, 2006b).

Doubling time of frond number (T_d) was calculated as:

$$T_d = \frac{\ln 2}{\mu}$$

Where μ is the average specific growth determined.

On the last day of the exposure the macrophytes of each replicate was weighted (Sartorius, CPA225D, Weighing Technology GmbH) and the roots of each frond were measured using an electronic caliper rule.

2.5 *Daphnia magna* assays

D. magna, clone K6, were cultured in ASTM had water (ASTM, 1998), enriched with seaweed extract (Marinure, Glenside Group, UK) and daily fed with the algae *C. vulgaris* (3.0×10^5 cells/ml). The culture was kept under constant/controlled physical conditions (24 ± 2 °C, 16 h : 8h light/dark photoperiod cycle and 3400 lux light intensity) and water physicochemical parameters (total hardness = 175.4 ± 5.5 mg/l $CaCO_3$, pH = 8.2 ± 0.3 and conductivity = 577.6 ± 9.0 µS/cm). The culture was renewed three times per week. Newly released neonates (<24h old; 3rd to 5th broods) were used in the immobilization test.

The immobilization test was performed according to the OECD guideline 202 (OECD, 2004) and ran for 48 h. *D. magna* neonates were exposed to control medium and BKC concentrations of 10, 20, 50, 80, 100 and 120 µg/l, in triplicate. Each replicate consisted in 5 neonates in a glass beaker (100 ml of exposure solution). The test conditions were similar to the culture conditions, except that no food and seaweed extract were provided during the test. Immobilization (defined as the inability to swim or move within 15 s of gentle agitation and taken to indicate lethality) was recorded daily.

2.6 *Thamnocephalus platyurus* assays

T. platyurus was obtained through the THAMNOTOXKIT^{TM} kits (Microbiotests, *Inc.*, Mariakerke – Gent, Belgium). The cysts of *T. platyurus* were incubated with Toxkit standard freshwater medium at 25 ± 1 °C and fluorescent light (3400 lux) for 20 – 22 h to hatch.

The acute test followed the ISO standard 14380 (ISO, 2011) and ran for 24 h. *T. platyurus* were exposed to control medium and BKC concentrations of 420, 860, 1770, 3620 and 7420 µg/l, in triplicate. Each replicate consisted in 10 organisms per treatment which were firstly placed in a *Petri* dish with the respective test solution and then transferred to one well of a 24-well microplate (1 ml of exposure solution). This procedure was adopted to avoid dilution of the exposure solutions in the microplates. Microplates were incubated in the dark at 25°C ± 1ºC. After the exposure period, mortality was recorded under a stereomicroscope (Stereoscopic Zoom Microscope–SMZ 1500, Nikon Corporation, Kanagawa, Japan).

2.7 *Danio rerio* assays

D. rerio (embryos and adults) were obtained in the facilities established at the Department of Biology, University of Aveiro (Portugal). Organisms were kept in carbon filtered water, complemented with salt “Instant Ocean Synthetic Sea Salt” (Spectrum Brands, Madison, WI), which was also used in the preparation of all test solutions for *D. rerio* tests. This water was kept at 25.0 ± 2 ºC, conductivity of 750 ± 50 µS/cm, pH of 7.5 ± 0.5 and dissolved oxygen > 95 % saturation. Photoperiod was adjusted to 16 h: 8 h light/dark cycle. The adult fish were fed twice daily with commercially available artificial diet (ZM 400 Granular, ZM systems, Hampshire, UK) and brine shrimp. Fish eggs were collected immediately after natural mating. Before the test, fish eggs were rinsed in water and checked under a stereomicroscope; those with cleavage irregularities or injuries were discarded.

2.7.1 *Danio rerio* embryos toxicity test

The embryo test was based on the OECD guideline on Fish Embryo Toxicity Test (FET) (OECD, 2012) and in an extended version described in detail

in Jesus et al. (2013) and ran for 120 h. *D. rerio* newly fertilized eggs were exposed to control medium and BKC concentrations of 0, 1700, 2100, 2500, 3000, 3600, 4300 and 5200 µg/l, in quadruplicate. Each replicate consisted in 10 eggs individually placed in the wells of 24-wells microplates (2 ml of exposure solution). Embryos were daily observed under a stereomicroscope (magnification used for observations was 70 x for embryos and 40 x for hatched embryos). The test conditions were similar to the culture condition. During the test, embryos mortality was recorded daily.

2.7.2 *Danio rerio* adult toxicity test

D. rerio adults test were based on the OECD guideline 203 (OECD, 1992) and ran for 96 h. Adults of similar length and age (2 ± 1 cm, 6 months old) were exposed to BKC concentrations of 0, 200, 350, 610, 1070, 1880 and 3280 µg/l, in quadruplicate. Each replicate consisted in 3 adults in aquaria (1000 ml of exposure solution). The test conditions were similar to the culture conditions, except that no food was provided during the test. During the test, mortality and behavior changes were recorded daily.

2.8 *Oreochromis niloticus* embryos assays

Oreochromis niloticus embryos were provided by the tilapia hatchery facility at the Asian Institute of Technology (Thailand). Adult fish were maintained under flow-through conditions. The water was purified by activated carbon filters and was kept at 26.0 ± 5 ºC, conductivity of 550 ± 50 mS/cm, pH of 7.5 ± 0.5 and dissolved oxygen > 95 % saturation. Photoperiod was adjusted to 12 h : 12 h light/dark cycle. This water was also used in the preparation of all test solutions for O. niloticus tests. Eggs were collected on the same day of the experiment, after natural spawning and they were sterilised in water and maintained in the conditions referred above.

The assay was based on the Fish Embryo Toxicity (FET) Test (OECD, 2012) and on the embryo test described by Oliveira et al. (2009). Prior to the assay, the eggs were checked under a stereomicroscope and unfertilized eggs

and those with cleavage irregularities or injuries were discarded. The test was initiated immediately after fertilization and was continued for 48 hours. *O. niloticus* embryos were individually exposed in 6-well microplates. A total of 3 replicates, contained 10 ml of test solution each with 5 individual eggs, were used in six different BKC treatments: control 200, 350, 610, 1070, 1880 and 3280 μg/ L. Test solutions were prepared by successive dilution of stock solution in water. The test conditions were similar to the culture condition. During the test, embryos mortality was recorded daily.

2.9 Species sensitivity distributions

The inherent sensitivity of decomposers, primary producers, primary consumers and secondary consumers to BKC was assessed by using the SSD approach (Posthuma et al., 2010). Single-species toxicity data (LC50 and EC50 values) calculated with exposure durations of 1-7 days were collected from the present study, peer-reviewed literature and available toxicity databases (e.g. www.epa.gov/ecotox) (see **Table 1**). Multiple toxicity data for the same species or a genus without specific species name were summarized as geometric means. This toxicity data selection yielded a number of 6, 9, 15 and 10 data points for microbial decomposers, primary producers, primary consumers and secondary consumers, respectively. SSDs were generated for each trophic level using the ETX software, version 2.0 (van Vlaardingen, 2004). The fit of the toxicity datasets to a log-normal distribution was assessed using the Anderson-Darling goodness-of-fit test at $p = 0.05$. The median Hazardous Concentration for the 5 and 50% of species (HC5 and HC50, respectively) and their lower to upper 95% confidence limits were calculated with the ETX software package based on the methodology described in Aldenberg and Jaworska (2000).

2.10 Potentially Affected Fraction of species

A probabilistic risk assessment was performed using the SSDs for the four trophic levels and measured concentrations of BKC collected from results of environmental monitoring studies published in peer-review journals and in the "grey" literature in the last twelve years. The total concentrations of BKC were

obtained by the sum of different alkyl chain lengths in the samples, mainly from C12 to C18. Average and worst-case ecological risk assessment scenarios were built based on the 50th and the 95th percentiles of the distributions of measured concentrations, respectively. Subsequently, the median Potentially Affected Fraction of species (PAFs) and its lower and upper estimates (5% and 95% confidence) for each of the scenarios were calculated with the ETX 2.0 software (van Vlaardingen, 2004) using the SSDs built with toxicity data for decomposers, primary producers, primary consumers and secondary consumers, as described above.

2.11 Statistical analyses

Sigma Stat 3.1 statistical package (SPSS, 2004) was used for statistical analyses. For sub lethal data One-way ANOVA was performed, except for data that did not pass the Kolmogorov Smirnov normality test, which was analyzed using the Kruskall-Wallis test. If significant differences were observed, Dunnet or Dunn's tests, respectively for normal and non-normal data, were used to assess which treatments differed from the respective control. Lethal concentrations at 50% (LC50) and effect concentrations at 50% (EC50) for all organisms were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel. All statistical analyses were performed with a significance level of 0.05. Significant differences between the distributions of toxicity data for the four trophic groups in the SSD analysis were assessed by the two-sample Kolmogorov-Smirnov test (p = 0.05), calculated with GenStat 15th Edition software (VSN International Ltd, Hemel Hempstead, UK) (VSN-International, 2011).

3. Results

3.1 *Vibrio fischeri*

After BKC exposure, the bacteria *V. fischeri* showed a decrease in the bioluminescence with a 15min-EC20 = 150 µg/l and a 15min EC50 = 300 µg/l.

3.2 Primary producers

The two algae species tested in the present study, *P. subcapitata* and *C. vulgaris*, showed a very similar sensitivity to BKC exposure both with a 72h-EC50 = 70 µg/l (**Table 2**). For treatments ≥ 100 µg/l of BKC both species showed a significant decrease in the growth rate (*P. subcapitata*: one-way ANOVA: $F_{6, 35}$ = 146.9, p < 0.001; *C. vulgaris*: one-way ANOVA: $F_{6, 35}$ = 55.92, p < 0.001).

The BKC was moderately toxic for *L. minor*. The EC_{50} values for the tested endpoints are summarized in **Table 3**. The number of fronds was affected in treatments ≥ 1000 µg/l (ANOVA: $F_{7, 23}$ = 24.18, p = 0.001) whereas the growth rate, dry and fresh weight were significantly affected at 18000 and 32000 µg/l, the highest concentrations (growth rate: ANOVA: $F_{7, 23}$ = 9.33, p = 0.001; dry weight: ANOVA: $F_{7, 23}$ = 9.33, p < 0.001; fresh weight: ANOVA: $F_{7, 23}$ = 11.52, p < 0.001). The most sensitive endpoint to BKC exposure was the root length, with a 168h-EC50 = 5.8 mg/l. In addition to the shortening of the roots, chlorosis was also observed in the macrophytes exposed to BKC.

Table 2 – Effect concentrations of benzalkonium chloride (mg/l) to aquatic organisms (95% confidence interval between brackets); p(r^2): goodness of fit measure

Test species	Parameter	Time	EC_{20}	EC_{50}	*p* (r^2)
V. fischeri	bioluminescence	15min	0.15 (0.14)	0.3 (0.04)	0.98
C. vulgaris	Growth	72h	0.05 (0.03)	0.07 (0.02)	0.89
P. subcapitata	Growth	72h	0.05 (0.03)	0.07 (0.01)	0.95
L. minor	growth¥	168h	4.16 (3.05)	7.87 (2.46)	0.74
D. magna	immobility	48h	0.04 (1.2)	0.05 (0.03)	0.99
T. platyurus	immobility	24h	0.69 (0.35)	0.1 (0.06)	0.99
D. rerio embryos	Mortality	96h	1.28 (2.37)	2.06 (0.22)	0.75
D. rerio adults	Mortality	96h	2.17 (0.24)	2.35 (0.1)	0.80?
O. niloticus	Mortality	48h	0.03 (0.72)	0.37 (0.15)	0.92

All *p* values < 0.001

¥ Based on fresh weight

3.3 Primary consumers

The crustacean *D. magna* showed high sensitivity to BKC exposure with a 48h-EC_{50} = 50 µg/l. For the freshwater shrimp *T. platyurus*, a 24h-LC_{50} = 100 µg/l was obtained (**Table 2**).

3.4 Secondary consumers

The BKC was moderately toxic for *D. rerio* and *O. niloticus*. For *D. rerio* adults a 96h-LC50 = 2350 µg/l was found whereas for embryos the 96h-LC50 was 2060 µg/l. *O. niloticus* embryos showed to be more sensitive to BKC, with a 96h-LC_{50} = 370 µg/l (**Table 2**).

Table 3 – Effects of Benzalkonium chloride (BKC, mg/l) on several endpoints of *L. minor* after 168h of exposure; the EC50 values are also presented.

BKC	Dry weight (mg)	Fresh weight (mg)	Frond number	Td[§] (day)	Root size (mm)	H_2O (%)	Chlorosis (%)
0	4.4 (0.5)	46.9 (2.8)	58.3 (3.8)	2.5 (0.2)	9.8 (0.8)	0.9 (0)	0 (0)
1	4.3 (0.8)	61.4 (12.4)	79.0 (9.0)	2.3 (0.1)	10.4 (2.2)	0.9 (0)	0 (0)
1.8	4.6 (0.4)	67.4 (7.6)	75.0 (5.9)	2.3 (0.1)	10.4 (0.5)	0.9 (0)	0 (0)
3.2	3.4 (0.3)	44.7 (6.9)	61.7 (5.4)	2.5 (0.1)	8.4 (1.3)	0.9 (0)	0 (0)
5.6	3.4 (0.4)	40.4 (4.9)	55.7 (2.3)	2.7 (0.1)	5.6 (0.6)	0.9 (0)	0 (0)
10	2.7 (0.2)	28.5 (1.3)	48.0 (2.5) *	2.9 (0.1)	7.3 (2.4)	0.9 (0)	0 (0)
18	1.9 (0.2) *	14.7 (0.3) *	23.0(1.2)*	5.2 (0.3)	0 (0)	0.9 (0)	0.9 (0)
32	1.1 (0.2) *	9.8 (0.4) *	14.7 (1.2)*	10.6 (1.6)	0 (0)	0.9 (0)	1.0 (0)
EC_{50}	13.1 **5.7**	7.9 **5.1**	12.0 **7.9**	28.4 **49.0**	5.8 **0.9**	¥	15.8 **10.9**

Standard error between brackets
Confidence interval 95% in bold
"§" Means doubling time
"*" Means significantly different from control (Dunnett's test, P<0.05)
"¥" Means not calculated

3.5 SSD

The HC5 and PNEC values for BKC are presented in **Table 4**. Data from **Table 1** were used for the SSD and PNEC estimations. The SSDs analysis indicated the primary consumers (HC5 = 10.83 µg/l; HC50= 300.44 µg/l) and the primary producers (HC5 = 11.40 µg/l; HC50= 260.93 µg/l) as the most sensitive groups followed by decomposers (HC5 = 152.21 µg/l; HC50= 1153.83 µg/l) and secondary consumers (HC5 = 217.07 µg/l; HC50 = 816.84 µg/l) (**figure 1**).

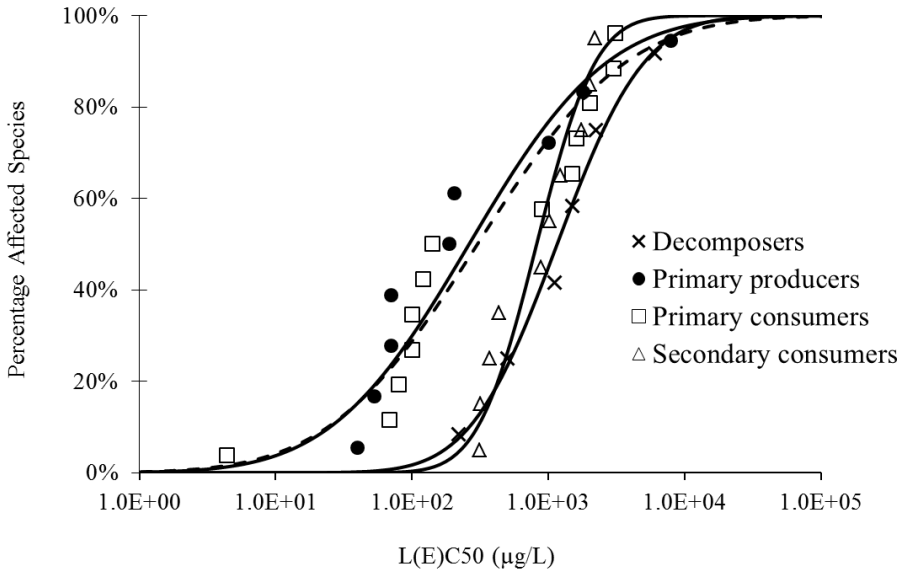


Figure 1 – SSD plot of the benzalkonium chloride concentrations versus percentage of affected of species; (×) represents the decomposers, (●) the primary producers, (□) the primary consumers and (Δ) the secondary consumers.

3.4 PAFs

The PAFs values for decomposers, primary producers, primary consumers, and secondary consumers in raw effluents, wastewater treatment effluents and surface water scenarios are shown in **Table 5**. The ecological risk assessment based on the best and worst case scenarios (50th and 90th percentile MEC, respectively) and the SSDs indicated high risk mostly for primary producers and consumers in all the studied scenarios (PAFs > 5%).

5. Discussion

The effectiveness of BKC to control organisms that are harmful to human/animal health (e.g. bacteria) and to avoid biofouling is well-known but the results from our study highlight some drawbacks of BKC application. After the intended uses, BKC will ultimately end up in rivers, lakes, reservoirs, estuaries and oceans. Once in aquatic ecosystems BKC might be acutely toxic to several aquatic organisms at concentrations as low as 50 µg/L. In the present study, a broad set of data on aquatic toxicity of BKC to several non-target organisms is provided suggesting that this compound is highly toxic to aquatic organisms in concentrations often found in effluents and water bodies.

Based on the results of short-term toxicity tests the species sensitivity to BKC decreased in the following order: *D. magna* > *C. vulgaris* = *P. subcapitata* > *T. platyurus* > *V. fischeri* > *O. niloticus* > *D. rerio embryos* > *D. rerio adults* > *L. minor*, with toxicity values ranging from 50 µg/L (48 h-EC50 of *D. magna*) to 787 µg/L (168 min-EC50 of *L. minor*). Concerning the effects of BKC in different trophic groups primary consumers (HC5=10.83 µg/l) and the primary producers (HC5=11.40 µg/l) were the most sensitive groups followed by decomposers (HC5=152.21 µg/l) and secondary consumers (HC5=217.07 µg/l).

The EC50 value obtained for the microbial decomposer *V. fischeri* (300 µg/L) showed a good agreement with data reported by other authors where the mean value of EC50 is 278 µg/L (Table 1). Moreover, when compared with other bacteria and protozoans, *V. fischeri* showed to be the most sensitive microbial species to BKC exposure (see **Table 1**). In the bacteria *P. aeruginosa* the mechanism of toxicity of BKC involves the damage of the cell wall and membrane

similar effect might occur in the *V. fischeri* compromising its integrity and causing the death of the organism (Richards and Cavill, 1976). Together with some invertebrates, the microorganisms (e.g. protozoans, fungi, bacteria) play a central role in aquatic ecosystems being responsible for the transference of carbon and energy from plant litter to higher trophic levels in aquatic ecosystems (Robarts, 1986). Incoherently with the importance of decomposers, a very limited amount of data was found in the literature for this group. Indeed, values of effective concentration were found for only six microbial species. Thus, more research is needed to accurately drawn the possible effects of BKC on the structure and function of decomposers communities in natural ecosystems (e.g. no studies with Fungi). Other biocides, namely metal based biocides, such as copper, and organic biocides, such as chlorhexidine, have been reported to affect the structure of microbial communities in freshwaters systems (Dynes et al., 2006; Lawrence et al., 2008; Pradhan et al., 2011). Having in consideration the moderate to high toxicity of BKC to microorganisms, similar effects on the structure of microbial communities might be expected in BKC-contaminated environments.

The short-term toxicity tests and SSDs analysis suggested the primary producers as one of the most sensitive group to BKC. Among microalgae species, *C. vulgaris* and *P. subcapitata*, a very similar sensitivity to BKC exposure was obtained. The value obtained to *P. subcapitata* is in accordance with the 72h-EC50 = 42 µg/l reported by Kreuzinger et al. (2007). On the other hand, the macrophyte *L. minor* was the most tolerant specie to BKC exposure, with a value EC20 of 4160 µg/l that are approximately 80-fold higher than the algae species 72h-EC20 of 50 µg/l for both algae. No toxicity values for *L. minor* have been reported previously in the literature. The *L. minor* individuals were only affected at concentrations above 1000 µg/L that may be explained by the high complexity of their fronds. Each frond has an aerial part that is not directly exposed to BKC. In addition, tissues and structures such as the cuticle and the root cap might protect the macrophyte from BKC effects. These characteristics turn macrophytes intrinsically more resistant to chemicals exposure (Pereira et al., 2013). Nevertheless, the noticeably decline in the overall growth, specially the root shortening, may compromise plant fitness in longer exposure periods. Since

primary producers are in the base of trophic chains, a bottom-up effect is expected starting with the reduction of carbon fixation and leading to a direct reduction of herbivore population (Solomon et al., 1996).

D. magna was the most sensitive invertebrate tested with a 48h-EC50 = 50 µg/L. Our result is in line with data from the current literature, in which the average effective concentration is 100 µg/L (**Table 1**). Although no toxicity values were found in the literature to compare with the LC50 obtained to *T. platyurus,* the 24h-LC50 = 100 µg/L is similar with the values previously obtained for daphnids and the crustacean *Mysidopsis bahia* (96h-LC50 = 80 µg/L) (Dobbs et al., 1995). The primary consumers are in the middle of aquatic trophic chains being a source of food for invertebrates and vertebrates (e.g. fish, reptiles, birds and mammals) (Covich et al., 1999). The EC50 and HC5 values obtained for this group fall within the range of BKC concentrations reported in the literature for different effluents and environmental samples (**Table 4**) raising a serious concern about the effects of BKC on zooplanktonic communities.

Among the tested fish species, *O. niloticus* was the most sensitive to BKC exposure with a LC50 = 300 µg/L, whereas both *D. rerio* adults and embryos had a similar responses with a LC50 > 1000 µg/L. The HC5 = 217.07 µg/L for secondary consumers indicates that fish species are the most tolerant group to BKC exposure. In spite of the ability of fish species to cope with high doses of BKC, this group might be compromised due to the reduction of primary producers and consumers in natural ecosystems impacted by BKC (especially fish early life-stages that feed on phytoplankton and zooplankton).

Table 4 – Effects and exposure assessment for benzalkonium chloride in three different scenarios, namely raw effluent, sewage treatment plant effluent and surface water

	Effects assessment			Exposure assessment		
	PNEC (µg/l)	†HC5 (µg/l)	†HC50 (µg/l)	*MEC $_{max}$ Raw effluents (µg/l)	*MEC $_{max}$ WTP effluents (µg/l)	*MEC $_{max}$ Surface water (µg/l)
Decomposers	0.22[a]	152.21 (15.81 - 419.31)	1153.83 (445.37 – 2989.22)			
Primary producers	0.4[b]	11.40 (1.01 – 42.57)	260.93 (83.84 – 812.04)	3928.5	145.1	65.3
Primary consumers	0.004[c]	10.83 (1.56 – 35.71)	300.44 (113.49 – 795.38)	(49.53)	(1.91)	(1.02)
Secondary consumers	0.31[d]	217.07 (84.64 – 369.88)	816.84 (520.08 – 1282.91)			

†Upper and lower limits between brackets

** Average of measured environmental concentrations between brackets*

[a] Based on the geometric mean of the EC50 values reported in our study and by Nałęcz-Jawecki (2003), Sütterlin et al. (2008), Tezel et al. (2009) for V. fischeri and an assessment factor of 1000.

[b] Based on the EC50 value reported by Pérez et al. (2009) for Isochrysis galbana and an assessment factor of 100

[c] Based on the geometric mean of the EC50 values reported in our study and by Nałęcz-Jawecki (2003) and Bartolomé and Sánchez-Fortún (2005) for A. franciscana and an assessment factor of 1000

[d] Based on the EC50 value reported by Dobbs et al. (1995) for Menidia beryllina and an assessment factor of 1000

The approach commonly applied to estimate the risk of BKC for aquatic ecosystem is the ratio between predicted or measured environmental concentrations and predicted non-effective concentrations (PNEC) with a safety factor ranging from 100 to 1000 (**Table 4**). Based on this approach several authors have indicated a risk quotient much higher than 1, which may possibly jeopardize the entire ecosystem for different scenarios of BKC-contamination (Kreuzinger et al., 2007; Rico et al., 2013; Rico and Van den Brink, 2014).

To date, no study reporting the affected fraction of a species assemblage due to BKC exposure in aquatic ecosystems was available. In the present study, an approach based on the calculation of the PAFs values was applied in order to estimate risk of BKC in specific scenarios providing a more accurate interpretation on the ecological risks for each sample and location. The PAFs values presented in **Table 5** are the cumulative probabilities on the SSD curve corresponding to a 50^{th} and 90^{th} percentile of the BKC concentrations in raw effluents, WTPs effluents and surface waters. The estimated PAFs values represent a measure of the percentage of species being affected under this concentration and reflect the toxic risk probability of BKC in each trophic group studied. Values of PAFs higher than 80% were estimated for hospital effluents where high concentrations of BKC are frequently found (1.05 to 3928.5 µg/l) suggesting that this effluent is extremely toxic for aquatic organisms. PAFs values higher than 25% were also obtained for laundry and industrial effluents containing BKC. These results highlight the need of adequate treatment of BKC containing effluents. The environmental concentrations of BKC released by WTP's are reported in only two studies. The work of Martínez-Carballo et al. (2007b) reported values for a Swiss WTP and suggested that no risk due to BKC was expected to occur in any trophic group. On the other hand, BKC levels found by Ferrer and Furlong (2001) in a WTP in the United States indicated risk to primary producers and consumers (PAFs >5%). Similar risk is expected for the surface water in two rivers one in China and another in Taiwan (Ding and Liao, 2001; Liu and Ding, 2008) whereas the BKC concentrations found in rivers from

US and Swiss might not harm the non-target organism of aquatic ecosystems. The high values of PAFs from raw effluents to surface water scenarios suggests a potential biodiversity loss in the aquatic ecosystems which might, thus, compromise the services provided by the ecosystems in a near future.

Following the increasing use of biocides, the European Commission recently implemented new rules concerning their commercialization and use in order to guarantee the safety of consumers and the environment (European Council, 2012). Despite this directive, only few studies have monitored BKC concentrations in effluents and surface waters. Studies focusing on the long term effects of BKC in the reproduction, development and growth of aquatic organisms are not available. Moreover, few studies have been published on possible interactions between BKC and others chemicals, for instance those used simultaneously with BKC in aquacultures and hospitals, such as antibiotics, aldehydes and other chlorine based disinfectants (e.g. sodium and calcium hypochlorite) (Sütterlin et al., 2008). Higher tier studies, such as mesocosms, are needed in order to refine the risk assessment of BKC by answering question such as if photosynthetic tolerant species (e.g. algae) are able to maintain the levels of primary productivity under different scenarios of exposure and also to assess the ability of primary producers and consumers communities to recover after a pulse of BKC contamination. Considering the high toxicity of BKC, its consistent detection in surface and wastewaters and the lack of monitoring studies on the environmental concentrations associated with activities in which BKC is directly applied to the water (such as aquaculture), we strongly recommend a special attention of regulatory authorities and further studies involving different approaches to a more accurate risk estimation for BKC in aquatic ecosystems.

Table 5 – Maximum measured exposure concentrations (MECs), hazardous concentrations calculated from the species sensitivity distributions and median potentially affected fractions (PAFs) based on acute toxicity data.

Monitored water	Country	MEC (µg/l) (50th / 90th percentile)	PAF decomposers	PAF primary Producers	PAF primary consumers	PAF secondary consumers	Reference
Raw effluents							
Hospital	Austria	2788.95 / 3700.59	76.49 / 82.94	89.38 / 91.85	79.38 / 82.83	93.64 / 96.96	Kreuzinger *et al.* (2007)
Laundry	Austria	517.36 / 2185.12	25.55 / 69.97	64.12 /86.85	52.33 /76.01	28.44 / 88.94	Kreuzinger *et al.* (2007)
Industrial	Taiwan	71.45 / 104.53	1.23 / 2.60	24.71 / 31.44	20.16 / 25.32	0.13 / 0.54	Ding and Liao (2000)
WTP effluent							
WTP	US	19.34 / 36.65	0.05 / 0.54	8.55 / 15.05	7.71 / 15.85	0.0 / 0.02	Ferrer and Furlong (2001)
WTP	Austria	0.54 / 2.5	n.e.	0.06 / 0.74	0.12 / 0.95	n.e.	Martínez/Carballo et al. (2007);
Surface water							
River	China	26.40 / 27.12	0.11 / 0.12	11.39 / 11.67	9.95 / 10.16	n.e	Liu and Ding *et al.* (2008)
River	Taiwan	19.15 / 49.60	0.05 / 0.54	8.47 / 19.07	7.65 / 15.85	0.0 / 0.23	Ding and Liao (2000)
River	US	2.47 / 3.47	n.e.	0.73 / 1.17	0.94 / 1.40	n.e.	Ferrer and Furlong (2001)
River	Austria	0.09 / 0.49	n.e.	0.0 / 0.05	0.12 / 0.95	n.e.	Kreuzinger et al. (2007); Grillitsch et al. (2006)

6. Conclusions

The results presented in this work highlight the potential environmental risk of BKC to aquatic organisms, especially for primary consumers and producers. The EC values calculated for *C. vulgaris* and *D. magna* are in the same magnitude or even lower than BKC concentrations measured in municipal WTPs. Moreover, the concentrations of BKC in surface waters are high enough to harm primary producers and consumers (PAFs > 5%) raising a high concern of the environmental safety of BKC. Since primary producers and consumers are in the basis of trophic chains indirect effects of BKC are expected for other species at higher levels of the trophic chain (bottom-up effect). However, higher tier studies such as long-term exposures and mesocosms are strongly recommended in order to refine the risk assessment of BKC.

Acknowledgments

Authors acknowledge the University of Aveiro, Department of Biology & CESAM for the institutional support and *Fundação para Ciência e Tecnologia* (FCT) for the financial support through the grants for the authors (SFRH/BPD/90521/2012, SFRH/BD/62605/2009 and BI/CESAM/PTDC/AAC – AMB/113649/2009).

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee – CREBEA guidelines for ethical principles for animal welfare.

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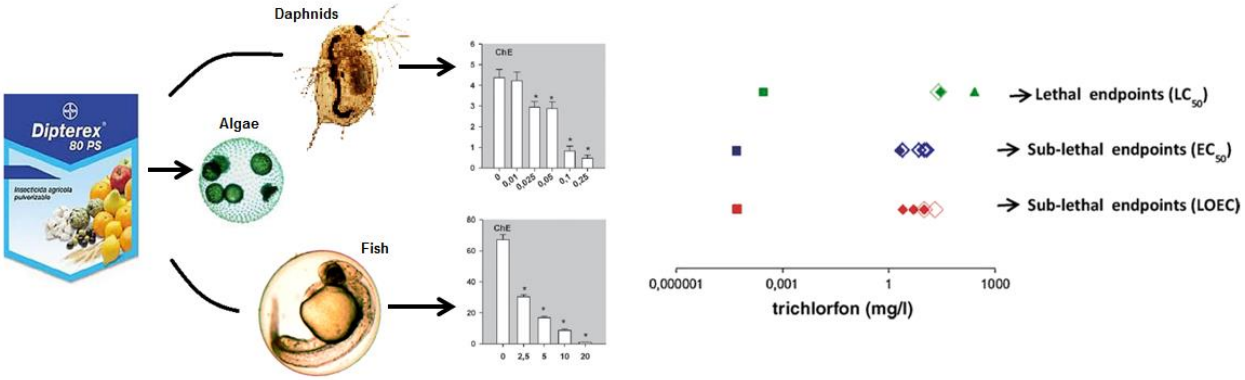
Chapter 5

Assessing lethal and sub-lethal effects of trichlorfon on different trophic levels



Assessing lethal and sub-lethal effects of trichlorfon on different trophic levels

Sónia Coelho; Rhaul Oliveira; Susana Pereira; Carolina Musso; Inês Domingues; Ram C. Bhujel; Amadeu M.V.M. Soares & António J.A. Nogueira

This chapter has been published as an original article in:

Aquatic toxicology v. 103 pp. 191–198 (2011)

Abstract

Worldwide trichlorfon (TCF) is one of the most used veterinary pharmaceuticals not only to fight infestations but also as a preventive measure. The high concentrations used generate concerns about environmental and human health. In this work we assessed the acute toxicity of this compound to non-target organisms belonging to different trophic levels: *Danio rerio* (early life stages and adults), *Daphnia magna* and algae *(Pseudokirchneriella subcapitata* and *Chlorella vulgaris*), and studied the potential of the biomarkers cholinesterase (ChE), glutathione- S- transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) to assess trichlorfon sub-lethal effects in zebrafish and daphnids. The fish embryo test followed the OECD draft guideline FET and was based on the exposure of newly fertilized eggs to 0, 2.5, 5.0, 10, 20, 40, 80 and 160 mg/L of TCF for 5 days; the fish acute test followed the OECD guideline 203 and was based on the exposure of adult fish to 0, 2.5, 5, 10, 20, 40, 60 and 80 mg/L of TCF for 4 days; *Daphnia* sp. immobilization assay followed the OECD guideline 202 and was based on the exposure of juvenile daphnids to 0, 0.1, 0.3, 0.5, 0.7, 0.9, 1 and 2 µg/L of TCF for 2 days and the algae growth inhibition assay followed the OECD guideline 201 and was based on the exposure of the two species to 0, 1, 3.2, 10, 32, 100 and 300 mg/L of TCF for 4 days. Biomarkers levels were measured after 96 h exposure to TCF in zebrafish early life stages and adults and in *D. magna.* Tested organisms seem to have dissimilar sensitivities towards TCF exposure. *D. magna* (48 h-LC_{50} =0.29 µg/L) showed to be the most sensitive organism, followed by zebrafish early life stages and adults (96 h-LC_{50} =25.4 and 28.8 mg/L respectively) and finally by the algae *P. subcapitata* (96 h-LC_{50} =274.5 mg/L) and *C. vulgaris* (no effect observed). As daphnids are a source of food for organisms of higher trophic levels, the impairment on its population is prone to have consequences in the entire ecosystem. The biomarkers activities measured in daphnids and fish seemed to be useful tools in the assessment of trichlorfon effects, especially ChE activity which was the most sensitive biomarker tested for all organisms. Trichlorfon showed to be teratogenic for zebrafish embryos leading to anomalies in the absorption of the yolk sac, spine bending and pericardial oedemas. The present research suggests that further work is urgently needed in order to monitor environmental concentrations of trichlorfon and test the long term effects of environmental realistic doses of this compound.

Keywords: Trichlorfon, biomarkers, sublethal effects, *Danio rerio*, *Daphnia magna, algae*

1. Introduction

In the last three decades, aquaculture has shown a worldwide expansion, playing an important role in the economy of many countries, especially the less developed ones. However, many issues, particularly in the context of health protection and environmental impact have to be dealt with (Focardi et al., 2005). In commercial aquaculture, the use of chemicals to treat diseases, to prevent infections and promote growth (such as food supplements, antimicrobial agents and antiparasitic drugs) is essential for a successful production (Subasinghe et al., 1996) and became part of management strategies (Tonguthai, 1996). However the overuse of these substances and inappropriate treatment of wastewaters pose risks for human and environmental health.

Trichlorfon (TCF) is a selective organophosphate insecticide used as a treatment for various parasitic infestations acting against arthropods as an acetylcholinesterase inhibitor (EPA, 1997). The widespread use of TCF in aquaculture for a long time is generating concerns about the impact on public (Tonguthai, 1996) and environmental health (Graslund and Bengtssona, 2001). In Brazil TCF is used by fish farmers to control two common ectoparasites (*Lernae* and *Argulus*) but is very often used indiscriminately, repeatedly, at high concentrations and without specialized technical orientation (Guimarães and Calil, 2008). In many south-east Asian countries TCF is used in shrimp farming to kill disease vectors such as crabs and small shrimps but there is an almost complete lack of information about the quantities of chemicals used (Graslund and Bengtssona, 2001). The absence of information about the use and fate of TCF makes very difficult to assess its impact in the environment.

As effects of environmental concentrations very rarely cause lethality, the risk assessment of TCF and other long term pollutants in aquatic ecosystems is better achieved if a battery of sublethal endpoints is used in ecotoxicity testing. In this context, biomarkers are useful tools that have been used as early indicators of environmental pollution due to their ability to detect effects at very low concentrations. Moreover, due to the specific modes of action of chemicals, organisms are affected differently according to their trophic levels thus; it is

important to include in the study organisms from different levels to allow a better understanding of effects in the entire ecosystem.

This work aims at 1) evaluating the acute toxicity of TCF using organisms of different trophic levels: the algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*, the crustacean *Daphnia magna* and young and adult stages of the fish *Danio rerio* and 2) assess the usefulness of using biomarkers in the detection of sub-lethal effects of TCF in daphnids and fish (young and adult stages). The biomarkers selected for this study include cholinesterase (EC 3.1.1.8, ChE), an important enzyme in the maintenance of normal nerve function (Olsen et al., 2001); glutathione S-transferase (EC 2.5.1.18, GST), a family of enzymes with a key role in the general biotransformation of xenobiotics and endogenous substances (Hyne and Maher, 2003), lactate dehydrogenase (EC 1.1.1.27, LDH) which is involved in the carbohydrate metabolism (Diamantino et al., 2001) and catalase (EC 1.11.1.6, CAT) which is an antioxidant enzyme protecting organisms against hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) generated in many situations of stress (Oruc and Uner, 2000). Zebrafish, *D. rerio*, is a small freshwater fish belonging to the Cyprinidae family and has been selected for this study because it is an important model vertebrate in a diversity of disciplines (Hill et al., 2005) and has been used in studies of developmental biology, molecular genetics, physiology and toxicology (Oliveira et al., 2009). *D. magna* was also selected for this study because it is very sensitive to changes in the chemical composition of aquatic environments and plays an important role in aquatic food webs placed between primary producers and fish. The green algae *P. subcapitata* and *C. vulgaris* are important species in aquatic trophic chains as primary producers and also as food for invertebrates and fish. Changes in the abundance and functions of any of these species can cause community or ecosystem- level responses (Barbosa et al., 2008, Perez et al., 2010).

This work is an attempt to elucidate how TCF toxicity varies across trophic levels and consequently which level will be more prone to be in risk due to long term TCF usage. Moreover, we will evaluate TCF effects at sub lethal level in daphnids and fish by analysing different biochemical markers. A selection of the most sensitive species and the most sensitive biochemical marker will be done for further use in studies of assessment of long term effects.

2. Material and Methods

2.1 Test Chemical

TCF ($C_4H_8Cl_3O_4P$), purity 97 % (m/v), (PESTANAL, Sigma-Aldrich, Taufkirchen, Germany) was used in all the performed assays.

2.2 Zebrafish assays

The zebrafish (*D. rerio*) facility established at the Department of Biology, University of Aveiro (Portugal) provided all organisms (zebrafish eggs and adults) used in the present study. In the zebrafish facility, organisms are maintained in carbon-filtered water complemented with salt “Instant Ocean Synthetic Sea Salt” (Spectrum Brands, USA), at 27.0 ± 1 °C and under a 16 :8 h light: dark photoperiod cycle (conductivity: 750 ± 50 µS, pH: 7.5 ± 0.5 and dissolved oxygen > 95 % saturation). This water was used as dilution water in the preparation of test solutions in all assays performed with fish. Temperature and photoperiod conditions mentioned above were used in all assays. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular, ZMsystems, Hampshire, UK) and brine shrimp.

2.2.1 Early life-stages assay:

The assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) Test (OECD, 2006a) and is described in detail in Domingues et al (2010). The test started with newly fertilized eggs exposed to the nominal concentrations of 0, 2.5, 5.0, 10, 20, 40, 80, 160 mg/L of TCF and run for 5 days. Forty eight eggs per treatment (6 replicates) were selected and distributed in 24-wells microplates. Embryos and larvae were daily observed under a stereomicroscope (Stereoscopic ZoomMicroscope-SMZ 1500, Nikon Corporation, Japan) (magnification used for observations was 70 x for eggs and 40 x for larvae). In the embryo phase the following parameters were evaluated: egg coagulation, eye and body pigmentation, somite formation, tail circulation,

detachment of the tail-bud from the yolk sac, absorption of the yolk sac and hatching. After hatching, oedemas, tail malformation and mortality were observed and reported. A second test was performed for collection of larvae for biomarkers analyses. Concentrations used were the same except for the last 3 concentrations that were skipped due to high mortality rates previously observed. Test ended at day 4 and 10 clusters of eight larvae per treatment were snap-frozen in microtubes (Eppendorf, Hamburg, Germany) for biomarkers analyses.

2.2.2 Adult fish assay

The assay using adult fish followed the OECD guideline 203 (OECD, 1992) in semi static test conditions. Adult zebrafish of similar length and age (2 ± 1 cm, 6 months old) were selected for the test. In each treatment 12 fish were equally distributed in 4 aquaria (each one containing 1 L of test solution). The nominal concentrations of TCF used were: 0, 2.5, 5, 10, 20, 40, 60 and 80 mg/L and test solution was replaced each second day. Test run for 96 hours. Fish were not fed during the test period and their mortality and behavioural changes were recorded daily. A second test using a similar design was run to allow the use of organs for biomarker analysis (12 fish per treatment were used). Concentrations used were the same except for the last 3 concentrations that were skipped due to high mortality rates previously observed. At the end of the test the living fish were sacrificed on ice by decapitation. Heads, muscles, liver and gills were isolated and snap-frozen in microtubes.

2.3 Daphnids assays

D. magna culture (clone A, *sensu* (Baird et al. 1989a)) is established in the lab under a photoperiod of 16 h light: 8 h dark, light intensity of 100 – 1000 Lux and at 23 ± 1 °C in ASTM (1980) hardwater enriched with an organic extract (Marinure, Glenside Group, UK). The culture media has a total hardness of 175.41 ± 5.53 mg/l CaCO3, pH of 8.15 ± 0.27 and a conductivity of 577.63 ± 9.01 µS/cm. Medium is renewed three times per week and Daphnids are fed daily with *C. vulgaris* algae at a concentration of 3.0 x 10^5 cells/ml. Newly released *D. magna*

neonates (< 24 h old) from the third to fifth broods were used in the assay. Experimental conditions were similar to culture conditions.

2.3.1 Acute Immobilization Assay

The immobilization test was performed in accordance with the OECD guideline 202 (OECD, 2004). Neonates were exposed to control medium and TCF at the nominal concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, 1 and 2 µg/L. In each treatment 15 juveniles of *D. magna* were equally distributed in 3 glass beakers (each one containing 100 ml of test solution). No food was provided during the assay. The number of immobilized daphnids was recorded at 24 and 48 hours of exposure (immobilization was defined as the inability to swim or move until 15 second of gentle agitation, and was taken to indicate lethality).

2.3.2 Assay for sampling for Biomarkers analysis

A second assay with a similar design was performed for collection of daphnids for biomarkers analysis. However, larger beakers containing 1 L of test solution and 50 daphnids in triplicate were used. Nominal concentrations tested were 0, 0.01, 0.025, 0.05, 0.1 and 0.25 µg/L of TCF. At the end of the test daphnids were rinsed with phosphate buffer, 0.1 M, pH 7.4 and frozen in pools of seven in microtubes.

2.4 Algae assay

The microalgae *P. subcapitata* and *C. vulgaris* which have been currently recommended as standard species for algal toxicity tests (OECD, 2006b) were obtained from nonaxenic batch cultures with Woods Hole MBL medium at 20 ± 2 °C and with a 16 :8 h light/dark photoperiod. For the maintenance of the laboratory cultures and the start of new cultures, algae were harvested while still in the exponential growth phase (5 – 7 days old) and inoculated in fresh media.

2.4.1 Algae inhibition test

The algae growth inhibition test was used to evaluate the toxicity of TCF and was based on the OECD guideline 201 (OECD, 2006b). *P. subcapitata* and *C. vulgaris* were exposed in two independent experiments to the nominal concentrations of 0, 1, 3.2, 10, 32, 100 and 300 mg/L. Exposure conditions and analysis of endpoints were as described in Perez et al (2010).

2.5 Biomarkers determinations

Assays were performed to analyse ChE, GST, CAT and LDH activities on larvae and adults of *D. rerio* and *D. magna*. All collected samples were immediately frozen at - 80 ºC in adequate buffer (phosphate buffer, 0.1 M, pH= 7.2 for ChE and CAT; phosphate buffer, 0.1 M, pH= 6.5 for GST and Tris-NaCl buffer, 0.1 M, pH= 7.2 for LDH) until analysis. On the day of enzymatic analysis, samples were defrosted on ice and homogenised (Ystral GmbH D-7801, Dottingen, Germany).

ChE was analysed in the heads and muscle of adult zebrafish, in clusters of 8 larvae or 7 daphnids. LDH was analysed in the muscle of adult zebrafish, in clusters of 8 larvae or 7 daphnids. GST was analysed in the heads, liver, gills and muscle of adult zebrafish, in clusters of 8 larvae or 7 daphnids. The procedure for ChE, LDH and GST determinations is described in Domingues et al (2010). Briefly, ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm the conjugation product between thiocoline (a product of the degradation of acetylthiocoline) and 5.5-dithiobis-2-nitrobenzoic acid (absorbance increase) according to the method of Ellman (1961). GST assay was based on the measurement of the conjugation product between the 1-chloro-2, 4-dinitrobenzene (substrate) and glutathione at 340 nm (absorbance increase) according to the method of Habig and Jakoby (1981). Determination of LDH was based in the decrease of absorvance due to the oxidation of NADH measured at 340 nm according to the method of Vassault (1983).

CAT was analyzed in the liver and muscle of adult zebrafish, in clusters of 8 larvae or 7 daphnids using the homogenates prepared for ChE analysis. CAT

activity was determined based on the method described by Clairborne (1985). Fifty µl of homogenate were mixed with 500 µL H_2O_2 0.030 M, and 950 µL K-Phosphate 0.05 M (pH 7.0) in spectrophotometer quartz cell and decomposition of the substrate (H_2O_2) measured at 240 nm.

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration in samples was determined in quadruplicate by the Bradford method (Bradford, 1976), at 595 nm, using γ-globulin as standard. A Labsystem Multiskan EX microplate (Labsystems Inc, Franklin MA, USA) reader was used for all biochemical determinations except CAT for which a Jenway 6505 uv/vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK) was used.

2.6 Statistical analysis

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS, 2004). A One-way ANOVA was used to detect the significant differences between the groups for normally distributed data sets. When data did not pass the Kolmogorov Smirnov normality test and the homogeneity of variance test, a Kruskal-Wallis test was used. If significant results were found, the Dunett or Dunn's test (depending on the nature of the test, parametric or non-parametric respectively) was used to verify differences between the tested concentrations and control. EC_{50} for binary responses (zebrafish and daphnia survival) were calculated with the PriProbit software package (Sakuma, 1998) while EC_{50} for continuous responses (algae growth) were calculated with Sigma Stat software package using a non-linear allosteric decay function (SPSS, 2004). All statistical analyses based on 0.05 significance level.

3. Results

3.1 Zebrafish early life-stages assay

Organisms from the control group presented a normal embryo development as described by Kimmel et al. (1995). TCF proved to be embryotoxic with LC_{50} values of 25.4 (18.2-32.6) and 45.13 (27.5 -62.7) mg/L for 96 and 72

hours respectively (95% confidence interval (CI) in between brackets) (Fig 1 a). In the first day of exposure, effects were observed in the eye and body pigmentation and in the detachment of the tail at the highest concentration tested (160 mg/L) (Fig 2, 3 a and 3 b) causing the dead of 89.6 % of these organisms by the second day.

On day 2, frequency of embryos with weak body pigmentation, delayed yolk sac absorption and pericardial oedemas was higher at 80 mg/L (Fig 2, 3 c and 3 d). Embryos exposed to 0, 2.5 and 5 mg/L of TCF started to hatch.

On day 3 all alive embryos had hatched except at the concentration of 40 mg/L where a significant delay was observed with only 23.75 % of hatching (Kruskal–Wallis H= 28.029 p< 0.001). Incidence of oedemas and delayed absorption of the yolk sac was verified in larvae exposed at 20 and 40 mg/L (Fig 2, Fig 3 e and 3f).

On day 4, 97.9 % of the embryos exposed at 40 mg/L died while embryos exposed at 20 mg/L still presented a high incidence of oedemas and abnormal yolk sac absorption. (Fig 2, 3 g and 3 h). EC and LOEC (lowest observed effect concentration) values of developmental parameters are presented in Table 1. Biomarkers were also analysed at day 4 and were all responsive (Fig 4 e-h): GST activity increased at 20 mg/L, ChE showed to be dose dependent inhibited (IC_{50} = 2.47 ± 0.46 mg/L); LDH activity was inhibited at 20 mg/L and CAT activity inhibited at the two highest concentrations tested.

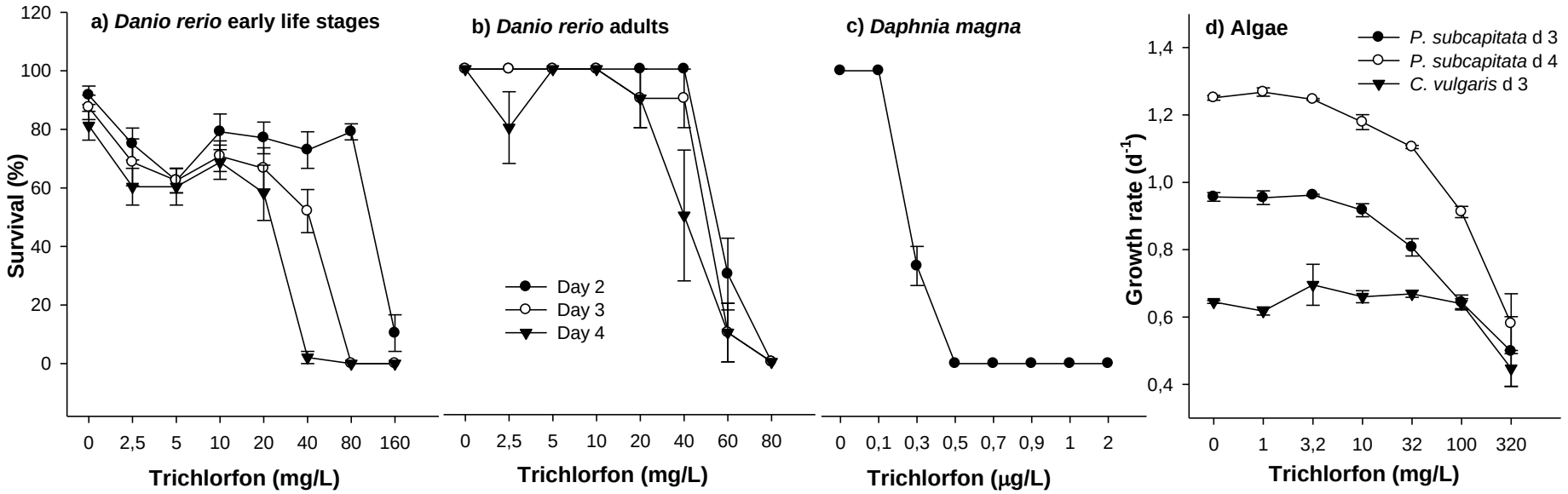


Fig 1 Effects of trichlorfon in the tested species. a), b) and c) show percentage of survival (mean ± standard error) for *D. rerio* early life stages, adults and *D. magna* respectively. d) shows algae growth rate (mean ± standard error). “d” stands for “day”.

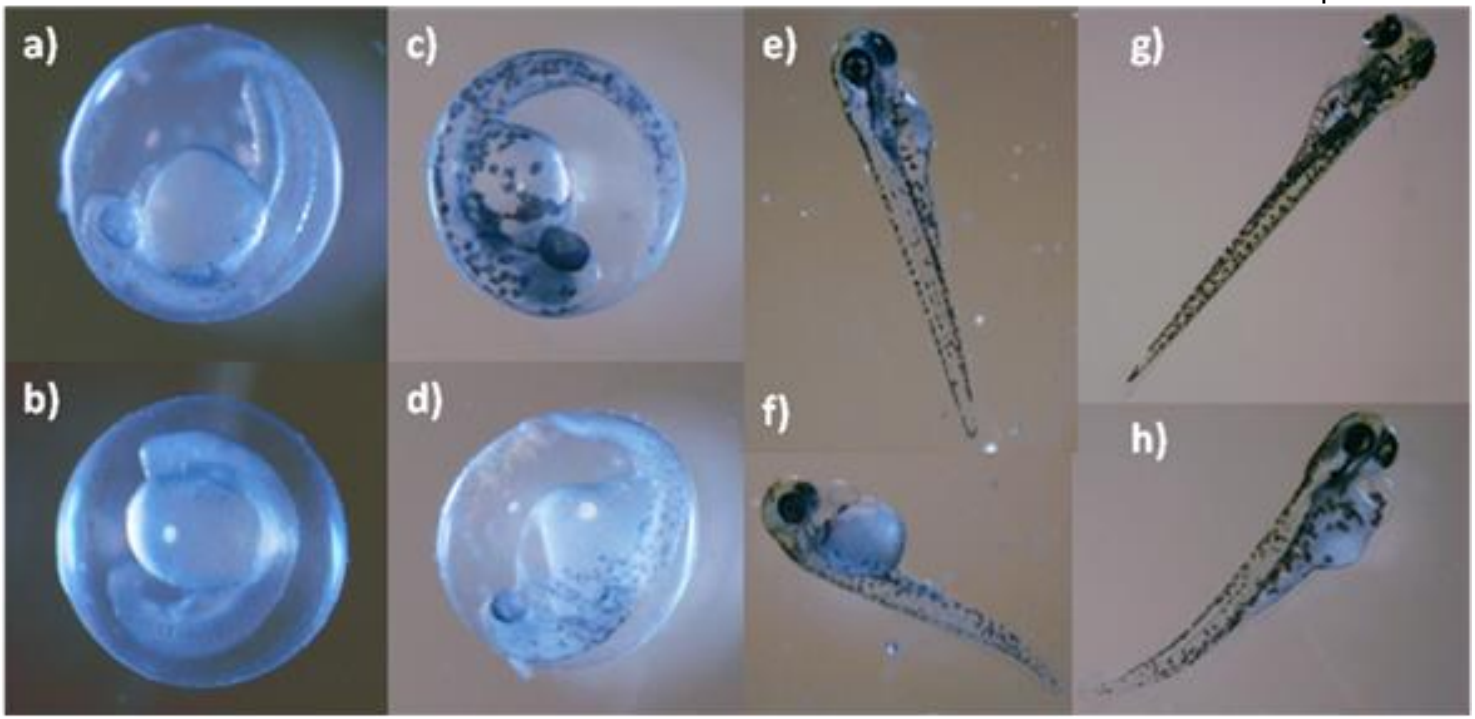


Fig 2 Embryo and larval development anomalies on zebrafish exposed to trichlorfon. **a)**, **c)**, **e)** and **g)** show control organisms with normal development after 1, 2, 3 and 4 days respectively; **b)** shows a 1 day-old embryo exposed to 160 mg/L of trichlorfon with weak eye and body pigmentation; **d)** shows a 2 days-old embryo exposed to 80 mg/L with weak body pigmentation, pericardial oedema and delay in the absorption of the yolk sac; **f)** shows a 3 days-old larvae exposed to 40 mg/L with pericardial oedema, spine bending and delay in the absorption of the yolk sac; **h)** shows a 4 days-old larvae exposed to 20 mg/L with spine bending, delayed yolk sac absorption and pericardial oedema.

3.2 Adult zebrafish assay

The following LC_{50} values were calculated for 48, 72 and 96 hours respectively: 58.4 (56.0 -60.9), 43.8 (34.5 -53.9) and 28.8 (14.2 -74.0) (95 % CI between brackets) (Fig 1 b). Behavioural alterations of fish were observed during the assay including erratic swimming, slow movements and long periods standing in the bottom of the flask, especially at the highest concentration, however no detailed record of this information is available.

GST activity was analysed in four types of zebrafish tissue: liver, head, gills and muscle (Fig 4 i). GST activity measured in the liver and head was a very sensitive parameter, responding with an increment at the lowest concentration tested (2.5 mg/L); however, while GST in the liver maintained increased activities at 5 and 10 mg/L, GST in the head was inhibited at 5, 10 and 20 mg/L. GST measured in the muscle and in the gills was not affected by TCF. ChE activity was dose dependent inhibited in the head and in the muscles (IC_{50} = 2.05 ± 0.23 mg/L). LDH activity measured in the muscle was inhibited at 10 and 20 mg/L. CAT measured in the muscle was not affected by TCF while CAT measured in the liver showed a steady inhibition at the last three concentration (5, 10 and 20 mg/L) (Fig 4 i-l).

3.3 D. magna assay

D. magna proved to be very sensitive to TCF with a 48 h-LC_{50} of 0.29 (95 % CI: 0.27 -0.31) µg/L (Fig 1 c). GST activity increased only at concentration of 0.05 µg/L, while ChE showed a dose dependent inhibition (IC_{50} = 0.052 ± 0.01 µg/L). LDH was not affected by TCF exposure and CAT was induced at 0.05 and 0.1 µg/L (Fig 4 a-d).

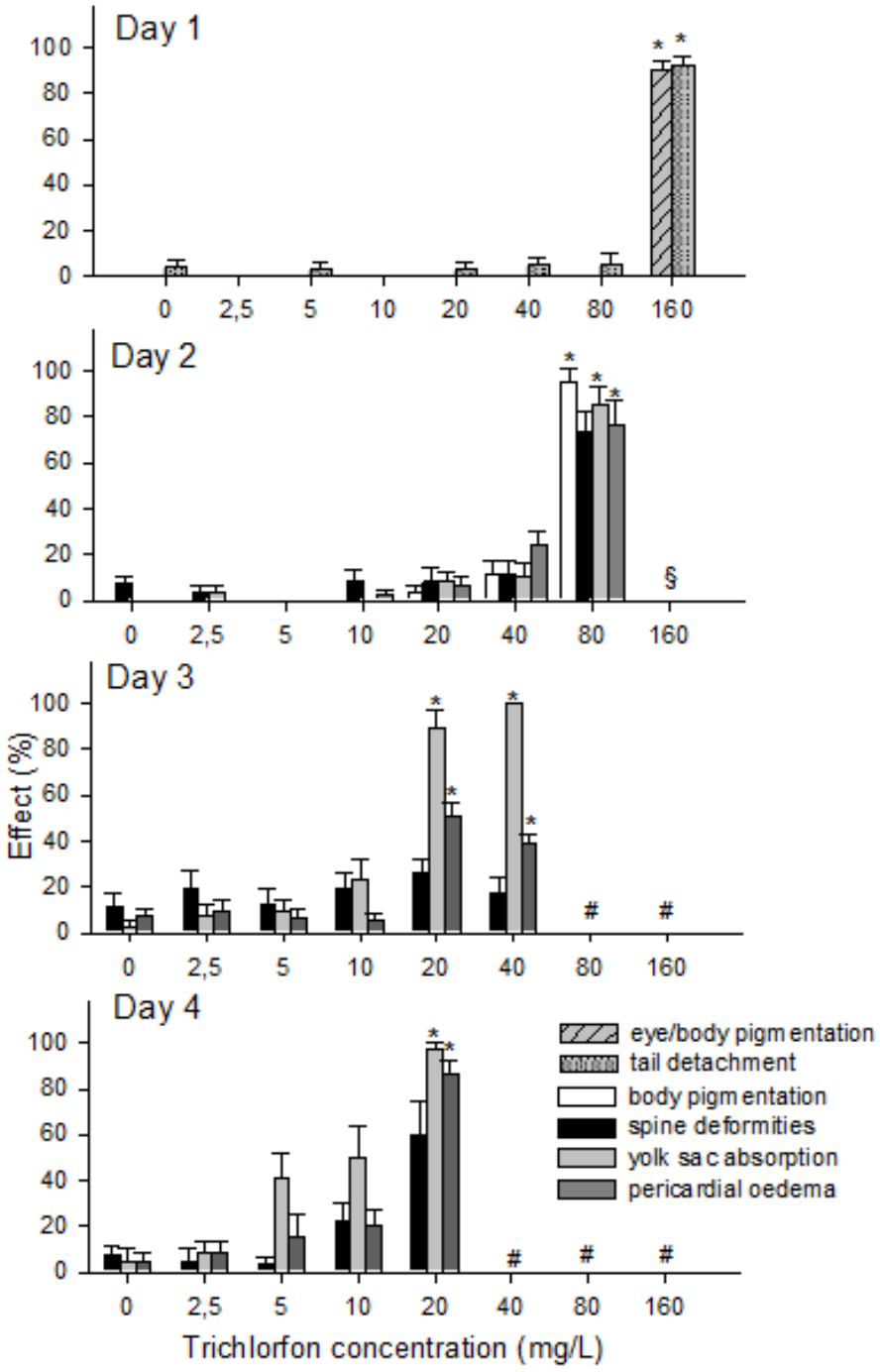


Fig 3 Incidence of anomalies (mean ± standard error) during four days of development of embryos exposed to trichlorfon. "*" above the bars shows a result statistically different from the respective control (Dunn's method, $p<0.05$ after Kruskal-Wallis). "§" indicates that number of surviving organisms was too low to evaluate developmental endpoints. "#" indicates no survival.

3.4 P. subcapitata and C. vulgaris growth inhibition test

P. subcapitata proved to be very resistant to TCF exposure with a 96 h-EC_{50} (for growth rate) of 274.5 (95 % CI: 216.6 -332.4) mg/L (Fig 1 d). *C. vulgaris* growth rate was not affected by exposure to TCF (Kruskal-Wallis; H= 10.11, P= 0.072; Fig 1 d).

Table 1- Effects of different concentrations of trichlorfon on developmental parameters of zebrafish embryos and larvae.

Endpoint	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Eye pigmentation	160	n.e.	_	_
Body pigmentation	160	**56.6** (3.4)	_	_
Somite/ Otolith formation	n.e.	_	_	_
Tail detachment	160	_	_	_
Pericardial oedema	160	**42.2** (4.3)	**12.3** (29.0)	**11.9** (2.2)
Yolk sac absorption	_	**61.8** (3.1)	**13.7** (1.1)	**7.4** (1.7)
Spine deformities	_	**65.8** (3,9)	n.e.	**10.6** (1.9)
Tail blood circulation	_	n.e.	n.e.	n.e.

Values are LOECs (lowest observed effect concentration). Bold values are EC_{50} (in mg/L) of dose responsive endpoints followed by the Standard Error in between brackets. “n.e.” means no effect on the endpoint analysed.

4. Discussion

4.1 Acute toxicity

Based on the LC_{50} values calculated, the crustacean *D. magna* (48 h-LC_{50} = 0.29 µg/L) is the most sensitive organism tested, followed by zebrafish early life stages and adults (with similar 96 h-LC_{50} of 25.4 and 28.8 mg/L respectively) and finally by the algae *P. subcapitata* (96 h-LC_{50} = 274.5 mg/L) and *C. vulgaris*. Fig 5 clearly shows the substantial difference in the sensitivities of the different organisms studied. The higher sensitivity of *D. magna* was the expected result

given that TCF targets species of arthropods parasites. LC_{50} value agrees with values found in literature (0.26 µg/L in the work of Yoshimura and Endoh (2005), and 0.21 µg/l in the work of Ren (2007)) for this crustacean. The relative insensitivity of algae compared to other trophic levels is already reported in literature (eg. Papst and Boyer, 1980); moreover some works also suggest algae ability for organophosphorous (OP) pesticides degradation (Caceres et al., 2008).

4.2 Biomarkers sensitivity

Biomarkers levels were measured after exposure of zebrafish early life stages and adults and *D. magna* to TCF.

Induction of GST activity has been used as a biomarker of exposure to xenobiotics with electrophilic centers. In this work, a transient induction of GST was observed in *D. magna* (only verified at 0.05 µg/L). Few works are available in literature dealing with effects of OP pesticides in daphnids GST, however absence of response to fenitrothion was observed in the work of Damasio (2007) suggesting that GST is not a very important biomarker in the assessment of OP effects in daphnids. GST measured in adult zebrafish varied according with the different organs. While no response was observed in the muscle and gills, and an irregular response was observed in the head (only verified at 2.5 mg/L and inhibited in the following concentrations) a steady induction (except for the last concentration) was observed in the liver, indicating that this organ is the most suitable to evaluate TCF effects. In fact, the liver has an important role in degradation and bioactivation of pesticides (Oruc and Uner, 2000); induction of hepatic GST was also observed in *Oreochromis niloticus* exposed to diazinon (Uner et al., 2007) and in *O. mossambicus* exposed to monocrotophos (Rao, 2006a) indicating ongoing detoxification mechanisms. This tissue-dependent response of GST in zebrafish adults was also observed in works such as Kavitha and Rao (2009) with *O. mossambicus* exposed to profenofos and it is possibly the result of different isoforms of the enzyme present in the tissues. In zebrafish early life stages GST was not a very sensitive endpoint as a response (induction) was only observed in the last concentration (20 mg/L) which is close to the LC_{50} value.

ChEs are responsible for the degradation of the neurotransmitter acetylcholine in the cholinergic synapses. A dose dependent inhibition of ChE was the expected response for all organisms tested given that TCF, as all OPs have an anticholinergic action. ChE inhibition under OP exposure is already described in literature for *D. magna* (Barata et al., 2004; Printes and Callaghan, 2004), zebrafish early life stages (Kuster and Altenburger, 2006), and adults (Frasco and Guilhermino, 2002; Rao, 2006b; Kavitha and Rao, 2007). In this work, ChE activity proved to be, for all organisms tested, the most sensitive endpoint tested, with IC_{50} values for ChE inhibition 19.7 (head of adult fish), 7.4 (fish early life stages) and 5.4 (*D. magna*) times lower than the respective LC_{50} values. ChE activity in *D. magna* was affected at concentrations as low as 0.025 µg/L.

LDH is a key enzyme in the anaerobic pathway of energy production, responsible for the catalysis of the interconversion of pyruvate to lactate in glycolysis and has been used as general biomarkers of stress in fish (Almeida et al., 2001; Osman et al., 2007; Vieira et al., 2008). In this work LDH did not prove to be a useful biomarker for TCF exposure as it did not response (*D. magna*) or responded only at very high concentrations (zebrafish adults and early life stages).

Several works in which fish was exposed to OP pesticides found an induction of LDH (indicating the use of the anaerobic pathway of energy production based on pyruvate) as it is the case of *P. reticulate* exposed to dimethoate (Frasco and Guilhermino, 2002) and *O. mossambicus* (gill and brain) exposed to monocrotophos (Rao, 2006a). However, in the same work of Rao (2006a), LDH activity decreased in liver and muscle similarly to what happened in this work, indicating, in these cases, possible tissue damage and muscular harm.

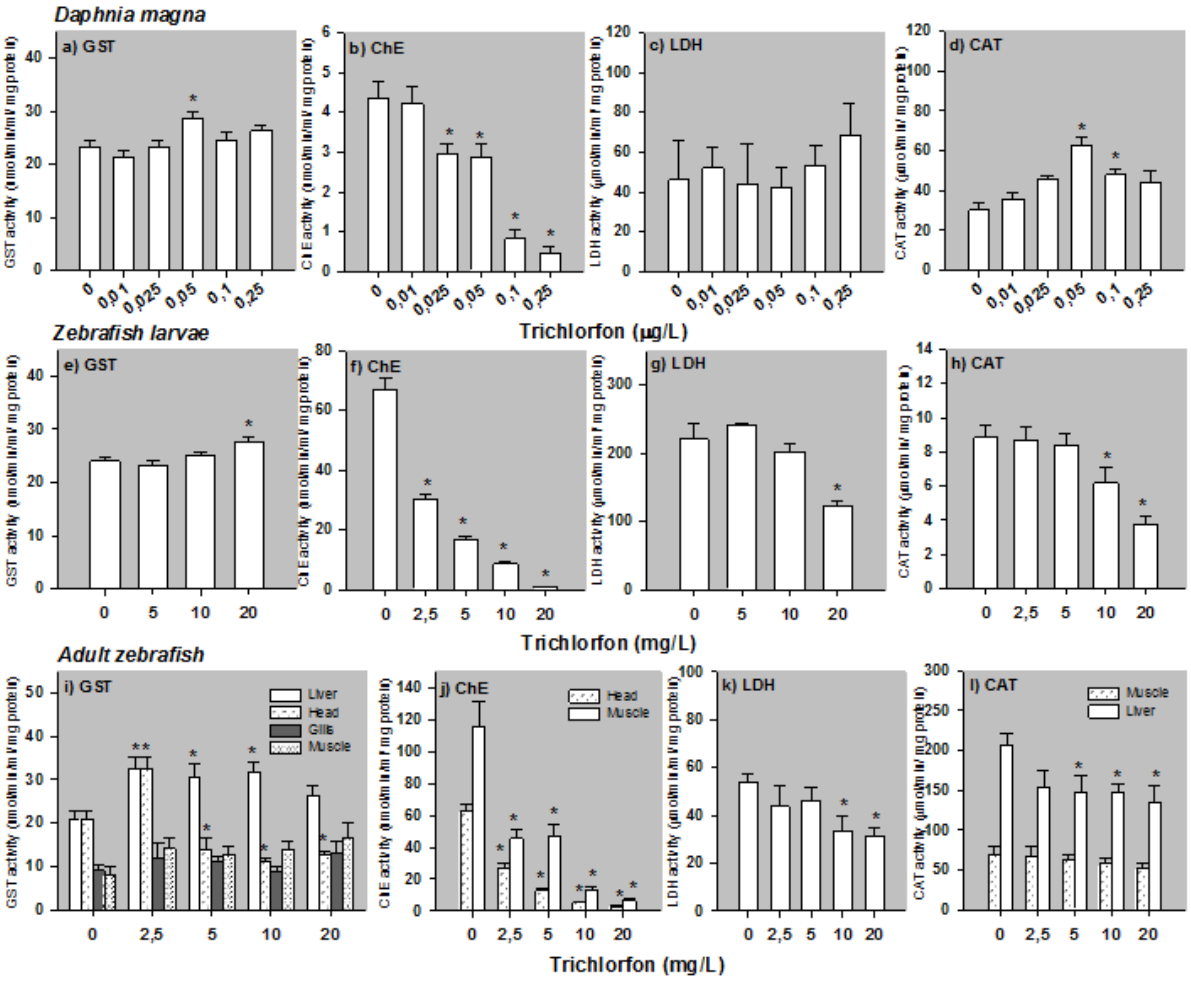


Fig 4 Variation of biomarkers activities (mean value ± standard error) on *Daphnia magna* and zebrafish larvae and adults after exposure to trichlorfon. Asterisks mean significantly different from the respective control treatment (Dunett test $P<0.05$ after 1-way ANOVA).

Several studies suggest that non-specific toxicity of OPs can be caused by the production of ROS and consequent oxidative stress (Oruc and Uner, 2000; Pena-Llopis et al., 2003). CAT belongs to the first line of defense against oxidative stress and occurs in peroxisomes, detoxifying H_2O_2 to O_2 and H_2O. In this work the response of CAT varied between daphnids and fish: while in *D. magna* a transient induction was observed at 0.05 and 0.1 µg/L suggesting the activation of the antioxidant defense mechanism; in zebrafish early life stages and adults (liver) an

inhibition was observed at the highest concentrations. TCF has been reported to induce oxidative stress in fish (Demael et al., 1990; Hai et al., 1997; Pena-Llopis et al., 2003; Thomaz et al., 2009). Although an inhibition (or no effect when measured in the muscle) of CAT in zebrafish was found in this work, oxidative damage is not excluded as suggested by works such as Feng et al. (2008) in which *T. nilotica* CAT was not affected by TCF but oxidative stress was proved to occur (by the depletion of GSH which indicates that ROS could be involved in the toxic effects). Other works with fish species suggest that besides their anticholinergic action, OP pesticides may also lead to oxidative stress, such is the case of *O. mossambicus* exposed to profenofos, *G. affinis* exposed to chlorpyrifos and monocrotophos, *Sparus aurata* exposed to malathion and *O. niloticus* exposed to azinphosmethyl (Kavitha and Rao, 2009, 2008 and 2007, Rosety et al., 2005 and Oruc and Uner, 2000. However, in these works CAT is not always induced, meaning that this enzyme may or not have an active role in the antioxidant defense mechanism and may be even inhibited due to the excessive generation of ROS as it happened in this present work. In future works, the activity of other enzymes of oxidative stress and indicators of oxidative damage (such as lipid peroxidation) should be included in order to elucidate this mechanism.

4.3 Zebrafish early life stages assay

Fish embryo testing has been suggested as an alternative to fish testing (because fish embryos are not subject to the Directive 86/609/EEC, which regulates the use of animals in scientific experiments) (Braunbeck and Lammer, 2006). Fish embryos are also excellent models for the understanding of toxic mechanisms and long-term effects of pollutants (Scholz et al., 2008). In the case of zebrafish embryo assay a strong correlation in acute toxicity between embryonic stages and adults has already been verified (Nagel, 2002; Lammer et al., 2009). Moreover, it has been observed that very often biochemical parameters (biomarkers) are more sensitive in early life stages (Oliveira et al., 2009; Domingues et al., 2010) and that important information is provided by effects at embryo development level, which has no equivalent in the adult fish test. In this work TCF proved to be embryotoxic: the most important endpoints of embryo

development at 24 h were the anomalies in the pigmentation of eye and body and the lack of tail detachment which proved to be lethal as embryos died the next day. Between 48 and 96 h, hatching delay, pericardial oedema, delay in the absorption of the yolk sac and spine bending were the most important effects observed. These effects in the embryo development are likely to be general responses of zebrafish embryos to organic pollutants and were also observed in *O. niloticus* embryos exposed to TCF (Guimarães et al., 2007) and zebrafish embryos exposed to other OP pesticides such as diazinon (Osterauer and Kohler, 2008).

96 h-EC_{50} values calculated for the dose responsive endpoints analyzed in this test, indicate that the most sensitive endpoint was ChE activity (2.47 mg/L), followed by the absorption of the yolk sac (7.4 mg/L), spine bending (10.6 mg/L) and pericardial oedema (11.9 mg/L) proving that these are useful endpoints in the detection of sublethal effects of TCF (Fig 5). A further link between these effects and future fitness impairment of zebrafish should be investigated to evaluate their ecological relevance.

Comparing biomarkers responses in the early life stages assay with the adult assay, except for ChE activity that was equally sensitive, all biomarkers were more sensitive in the adult assay. This was not the expected since in previous works with different types of toxicants, the sensitivity of biomarkers in early life stages seemed to be higher than in adults (Oliveira et al., 2009).

4.4 Environmental relevance

Based on the results obtained for *D. magna* and as previous described on literature, the main concern for TCF is its effects on non-target crustaceans (Graslund and Bengtssona, 2001). The IC_{50} value of 0.052 µg/L calculated for ChE inhibition is significantly below values measured in the environment such as 10 µg/L found in several wells in Georgia (EPA, 1997) and has unknown consequences at population level. In the environment, an eventual reduction on the daphnids population under exposure to TCF would have direct effects on both upper and lower levels of the trophic chain. Organisms such as fish that feed on these crustaceans would face feeding constrains while algae due to the lack of predation and due to the natural lower sensitivity to TCF would have their

populations augmented (Daam et al., 2008) with pernicious consequences to the ecosystem such as water eutrofication.

Acute toxicity data for fish species ranges from highly toxic to practically non-toxic depending on the species (EPA, 1997). Most of the sub-lethal endpoints analyzed in zebrafish (enzymes and embryo development parameters) were affected at doses between 2 and 10 mg/L. The lack of information about aquaculture usage (frequency and amounts), the efficacy of waste water treatments and the concentrations of effluents actually reaching aquatic ecosystems is of special concern because of two factors that can potentiate TCF effects on the environment. The first is the hydrolysis of TCF into the more toxic compound dichlorvos (Guimarães et al., 2007). This hydrolysis highly depends on pH and temperature, making necessary a careful planning of treatments taking into account these factors (Roth et al., 1993). The risk of unexpected fish kills is highly increased when doses are not respected. Second, the potential for combined toxicity of the several types of chemicals used simultaneously in aquacultures (fertilizers, pesticides, disinfectants, antibiotics, probiotics, vaccines) (Graslund and Bengtssona, 2001). The possibility of interactions between different types of chemicals resulting in synergistic effects highly increases ecological risk and was already verified, for instance, between TCF and the detergent sodium dodecyl sulfate (Feng et al., 2008).

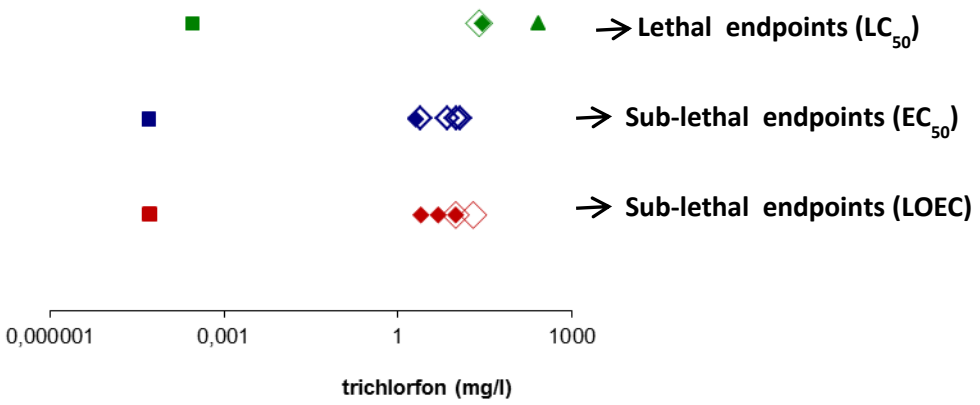


Fig 5 Comparative sensitivity of the endpoints tested in the different organisms. ■ Daphnids data; ♦ zebrafish adults; ◇ zebrafish embryos; ▲ algae (*P. subcapitata*)

5. Conclusions

Organisms from the different trophic levels tested had varying levels of sensitivity towards TCF exposure. *D. magna* (48 h-LC_{50} = 0.29 µg/L) showed highest sensitivity among organisms tested, followed by zebrafish early life stages (96 h-LC_{50} = 25.4 mg/L), zebrafish adults (96 h-LC_{50}= 28.8 mg/L) and finally by the algae *P. subcapitata* (96 h-LC_{50} = 274.5 mg/L) and *C. vulgaris*. The impairment on daphnids population is prone to have consequences in the entire ecosystem given that it would affect organisms of higher levels (such as fish) that feed on them and it would increase populations of organisms of lower levels (algae) on which daphnids feed, disturbing the equilibrium within the ecosystem. The biomarkers activities measured in daphnids and fish seemed to be useful tools in the assessment of TCF effects, especially ChE activity which was the most sensitive biomarker tested for all organisms, showing to be a suitable tool for assessment of OP exposure in fish and daphnids. Moreover, other biomarkers tested proved to be very sensitive; namely, CAT in *D. magna* and CAT and GST in the liver of adult zebrafish. TCF showed to be teratogenic for zebrafish embryos leading to anomalies in the absorption of the yolk sac, spine bending and pericardial oedemas. Given the potential environmental risk posed by TCF use and based on data from the present work, further work should be planned in order to monitor environmental concentrations of TCF and its degradation product, dichlorvos and test long term effects of normally used doses of these compounds.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCTPortugal) by the financial support through the grants SFRH/BPD/31752/2006 (Inês Domingues) and SFRH/BD/62605/2009 (Rhaul Oliveira) and University of Aveiro.

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

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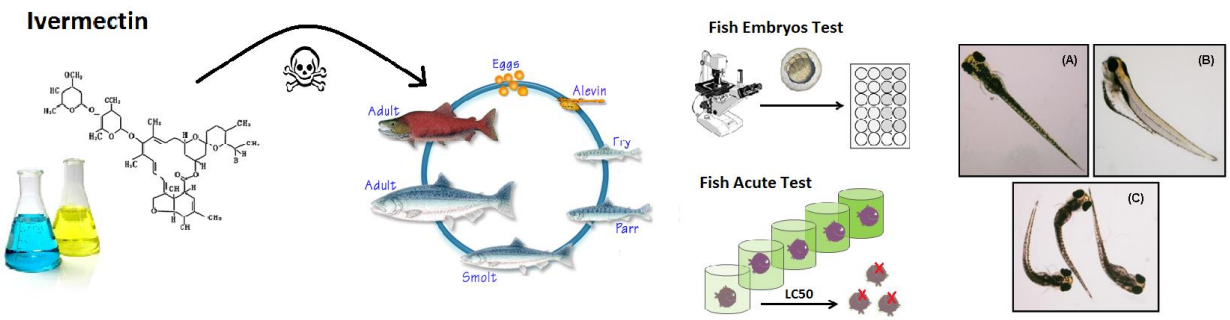
Chapter 6

Effects of ivermectin on zebrafish early life-stages and adults



Effects of ivermectin on zebrafish early life-stages and adults

Rhaul Oliveira; Cesar K. Grisolia; António J.A Nogueira; Amadeu M.V.M. Soares & Inês Domingues

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

Environmental toxicology and pharmacology

Abstract

Several studies have shown high toxicity of ivermectin for aquatic invertebrates, however, few data is found for fish species. The present study evaluated the acute toxicity of ivermectin to embryos, juveniles, and adults of zebrafish. Toxicity tests were performed based on OECD protocols and mortality and behavioural changes were assed for all stages. Biochemical responses were assessed in adults and embryos and included cholinesterases, (ChE), catalase (CAT) (only in embryos), glutathione-S-Transferase (GST), lactate dehydrogenase (LDH) and vitellogenin (VTG) like proteins (only in embryos). Genotoxicity was evaluated in adults. Results showed a higher sensitivity of juvenile and adults of zebrafish (96 h-LC50 values of 18.5 and 76.7 µg/L, respectively). For embryos a 96 h-LC50 of 3391.9 µg/L was calculated, moreover developmental anomalies and hatching inhibition were observed only at high concentrations (> 400 µg/L), whereas biochemical and behavioural responses occurred at lower concentrations (< 60 µg/L). Behavioural responses (lethargy) occurred in all life stages and at least in embryos can be linked to the inhibition of ChE, indicative of a potential disruption of nervous system. Behavioural endpoints are particularly important from an ecological perspective as they can be directly linked to important functions such as feeding, reproduction or predators' avoidance which are vital to the species survival. Other biochemical responses observed included the inhibition of GST in adults and changes in CAT, LDH activities and VTG levels in embryos. Ivermectin did not show to be genotoxic for adult fish. The species sensitivity distribution analysis, based on fish and invertebrate species, indicated a HC5 value of 0.057 µg/L; suggesting high sensitivity of both groups to ivermectin and a high risk of this compound to aquatic ecosystems.

Keywords: *Danio rerio*, ivermectin, developmental abnormalities, biomarkers, genotoxicity and behaviour

1. Introduction

At the end of the 1970s, Burg et al., (1979) isolated avermectins from the actinomycete *Streptomyces avermitilis* and noted their potent anthelmintic activity. Since the 1980s, within the group of avermectins many macrocyclic lactones were identified and characterized including ivermectin, doramectin, abamectin, emamectin and eprinomectin. Among these different macrocyclic lactones ivermectin is one the most used worldwide in the treatment of arthropod and nematode infestations in domestic animals and agricultural activities (e.g. aquaculture) and antifilarial chemotherapy in humans (Lumeret, 2012).

Ivermectin is composed by at least 80% of 22,23-dihydroavermectin-B1a and not more than 20% of 22,23-dihydroavermectin-B1b. It acts by interfering with the parasites nervous system affecting gamma-aminobutyric acid (GABA)-related chloride channels present in nerves and muscle cells, causing cell hyperpolarization and subsequent death (Brownlee et al., 1997). Additionally, in nematodes, ivermectin block nerve signals by interfering with the glutamate-gated ion channels receptors (GluCl). The mode of action of ivermectin in vertebrates is unclear, especially for fish. Ivermectin weak interaction with the GABA-gated chloride channel receptors in vertebrates (mammals) might be explained by the large size of ivermectin molecule. Thus, the organisms are protected from its effects by the blood-brain barrier (Lumeret, 2012).

In livestock systems (e.g. cattle, pigs, and sheep) ivermectin can be administered in oral, injectable or topical formulations. A high percentage (35 ±10 %) of ivermectin administrated to cattle is not metabolized and passes into the terrestrial environment through faeces (Fernandez et al., 2009). The erosion of ivermectin-containing particulate matter, and direct excretion of treated animals into surface water bodies are relevant entry pathways to freshwater ecosystems (Boxall et al., 2004).

In the past decades ivermectin was also widely used in salmon aquacultures. (Athanassopoulou et al., 2001) reported its use in the Mediterranean to control the

infestations by the ectoparasites *Lernathropus kroyeri* (copepod) and *Ceratothoa oestroides* (isopod). Burridge et al., (2010) and Sanderson et al., (2007) claim the decline of ivermectin use in salmonids farming in many countries and its replacement by emamectin. However, in freshwater aquacultures ivermectin is still used and poses a high risk for the environment as reported by Rico and Van den Brink, (2014) in a case study performed in South-East Asia. The direct application of ivermectin in aquatic ecosystems can be, thus, considered another important pathway for ivermectin entrance to aquatic environments. Liebig et al. (2010) indicated risk of ivermectin for several compartments in freshwater ecosystems. Indeed, cladocerans are extremely sensitive to ivermectin exposure with a 21 d-NOEC for reproduction of 0.3 pg/L (*Daphnia magna*) and 1 pg/l (*Ceriodaphnia dubia*) (Boonstra et al., 2011; Garric et al., 2007; Lopes et al., 2009; Schweitzer et al., 2010). Most of the studies found in current literature about ivermectin effects in aquatic organisms are focused in invertebrates (**Table 1, Table S1**); little information is found for fish species, particularly at sub lethal level, chronic effects and on early life-stages. Toxicological studies where ivermectin is used as veterinary drug in the treatment of parasites reported clinical signs in fish; exposure to ivermectin via food, injection or dissolved in water lead to a range of effects on swimming, feeding activity, colour darkening and biomolecular alterations (summarized in **Table S1**). The side effects after ivermectin administration might give interesting clues on its effects on fish species.

Table 1 - Review of ivermectin short-term toxicity tests using fish and aquatic invertebrates with duration between 1 and 6 days (values in µg/L).

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effect measured	Values (µg/L)	References
Anguilla anguilla	Saltwater	01	LC50	Mortality	0.2	(Geets et *al.,* 1992)
Danio rerio	Freshwater	02	EC50	Movement	171	Weil et *al.,* (2009)
Danio rerio	Freshwater	01	EC50	Movement	440	Carlsson et *al.,* (2013)
Lepomis macrochirus	Freshwater	04	LC50	Mortality	4.8	(Halley et *al.,* 1989)
Salmo gardneri	Freshwater	04	LC50	Mortality	3	Halley et *al.,*(1989)
Salmo salar	Saltwater	04	LC50	Mortality	17	(Kilmartin et *al.,* 1996)
Biomphlaria glabrata	Freshwater	01	LC50	Mortality	30	(Matha and Weiser, 1988)
Carcinus maenas	Saltwater	04	LC50	Mortality	957	(Grant and Briggs, 1998)
Crassostrea giga	Saltwater	04	LC50	Mortality	460	Kilmartin et *al.,* (1997) in (Davies, 1997)
Daphnia magna	Freshwater	02	EC50	Immobilisation	0.0057	Garric et *al.,* (2007)
Daphnia magna	Freshwater	02	LC50	Mortality	0.025	Halley et *al.,* (1989)
Gammarus fossarum	Freshwater	04	LC50	Mortality	3	(Alonso et *al.,* 2010)
Gammarus fossarum	Freshwater	04	LC50	Mortality	3.5	Alonso et *al.,* (2010)
Gammarus pulex	Freshwater	04	LC50	Mortality	5	Alonso et *al.,* (2010)
Gammarus pulex	Freshwater	04	LC50	Mortality	4.5	Alonso et *al.,* (2010)
Gammarus sp.	Freshwater	04	LC50	Mortality	0.033	Grant and Briggs (1998)
Littorina littorea	Saltwater	04	LC50	Mortality	580	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)

Table 1 - Review of ivermectin short-term toxicity tests using fish and aquatic invertebrates with duration between 1 and 6 days (values in µg/L).

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effect measured	Values (µg/L)	References
Lumbriculus variegates	Saltwater	03	LC50	Mortality	490	(Ding et *al.,* 2001)
Monodonta lineata	Saltwater	04	LC50	Mortality	780	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Mytilus edulis	Saltwater	04	LC50	Mortality	400	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Neomysis integer	Saltwater	04	LC50	Mortality	0.07	Davies et *al.,* (1998)
Neomysis integer	Saltwater	02	LC50	Mortality	0.026	Grant and Briggs (1998)
Nereis diversicolor	Saltwater	04	LC50	Mortality	7.5	Grant and Briggs (1998)
Nucella lapillus	Saltwater	04	LC50	Mortality	390	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Palaemonetes varians	Saltwater	04	LC50	Mortality	54	Grant and Briggs (1998)
Patella vulgata	Saltwater	04	LC50	Mortality	600	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Pecten maximus	Saltwater	04	LC50	Mortality	300	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Sphaeroma rugicauda	Saltwater	04	LC50	Mortality	348	Grant and Briggs (1998)
Tapes semidecussatus	Saltwater	04	LC50	Mortality	380	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Tapes semidecussatus	Saltwater	04	LC50	Mortality	600	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)

Table 1 - Review of ivermectin short-term toxicity tests using fish and aquatic invertebrates with duration between 1 and 6 days (values in µg/L).

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effect measured	Values (µg/L)	References
Tubifex tubifex	Freshwater	01	EC50	Movement	2000	Gerhardt (2009)
Tubifex tubifex	Freshwater	01	LC50	Mortality	1820	Gerhardt (2009)

With this study we aim at acquiring a deeper knowledge on ivermectin effects in different life stages of fish at lethal and sub lethal levels, using zebrafish (*Danio rerio)* as model organism. The zebrafish was chosen because it is widely used as a model organism for developmental biology, physiology, molecular genetics, and toxicology (Briggs, 2009; Scholz et al., 2008). Thus, the main objectives of this study were (a) to compare the survival and behavioural response to ivermectin among the zebrafish different life stages (embryos, juveniles and adults) and with other fish species and with invertebrates though a Species Sensitivity Distribution analysis (SSD); (b) to evaluate ivermectin effects on embryo development; (c) to evaluate biochemical responses to ivermectin in embryos and adults by looking at biomarkers of neurological stress (e.g. cholinesterase (ChE), oxidative stress (catalase (CAT) and glutathione-S-transferase (GST)), energetic metabolism (lactate dehydrogenase (LDH)); and estrogenicity (Vitellogenin like proteins (VTG)) and (d) to evaluate the genotoxicity of ivermectin (looking at micronuclei (MN) and nuclear abnormalities (NA)) in adults.

2. Materials and methods

2.1 Chemicals

Ivermectin (CAS Number: 70288-86-7, Empirical Formula: $C_{15}H_{16}Cl_3N_3O_2$, 97 % purity; primarily B1 A), acetone (CAS Number: 67-64-1, ≥99.5 %), cyclophosphamide (CAS Number: 6055-19-2, 97.0-103.0 %) and all chemicals for biomarker analysis were purchased from Sigma-Aldrich.

2.2 Test organisms maintenance

All the organisms used in this work were provided by the zebrafish facilities of the Department of Biology, University of Aveiro. Only organisms of the ABwt lineage were used. The fishes were maintained in a ZebTEC (Tecniplast) recirculating system

using water obtained by reverse osmosis. Water passes through several levels of filtration (activated carbon filters and biological filters), is disinfected by UV light and automatically adjusted for pH and conductivity. The temperature is maintained at 26.0 ± 1 ºC, conductivity at 750 ± 50 µS, pH at 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. Nitrate, nitrite and ammonia are regularly monitored. This water was used as dilution water in the preparation of test solutions in all assays performed. A 16:8 h (light: dark) photoperiod cycle and continuous and uniform cool-white light was used (2000 lux) in all assays. The adult fish are fed twice a day with commercially available artificial diet (ZM 400 Granular) and brine shrimp nauplii.

2.3 Zebrafish embryos assays

2.3.1 Embryos extended toxicity test

On the day before spawning, tanks with adult fish (3.2 L) were filled with a layer of marbles. The following morning, during mating, the eggs released fell amongst the marbles, preventing most eggs from being eaten by the adults. Marbles were gently removed from the aquarium and eggs collected with the help of a Pasteur pipette and a strainer. Eggs were then rinsed with water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation) to exclude non-fertilized or non-viable eggs.

The extended assay with embryos (Fish Embryo Extended Test- FEET) was adapted from the OECD protocol Fish Embryo Toxicity Test (OECD, 2013). Zebrafish embryos were exposed in 24-well microplates to seven treatments: control, solvent control (100 µL/L of acetone), and nominal concentrations of 100, 200, 400, 600 and 800 µg/L of ivermectin. The stock solution was prepared by dissolving ivermectin in acetone. Test solutions were prepared by successive dilution of the stock solution in water. Seventy two eggs per treatment were placed individually in the wells with 2 ml of the test solution. Organisms were observed daily with the help of a stereomicroscope. The test was initiated with eggs at the end of the blastula stage (30% epiboly) and was continued for 144 h. Developmental parameters were daily

evaluated over the test period using a magnification of × 70 for eggs and × 40 for post hatched embryos. Before hatching, the following parameters were evaluated: egg coagulation, otolith formation, eye and body pigmentation, somites formation, presence of heartbeat (only at 48h), blood circulation in the tail, detachment of the tail-bud from the yolk sac, and hatching. After hatching, the following parameters were evaluated: oedemas, spine deformities, abnormal position (side-laying), and mortality.

2.3.2 Embryos behavioural test

For the evaluation of behaviour effects on embryos, the test was repeated using lower concentrations of ivermectin (10, 20, 40, 80, 100, 200 and 400 µg/L) plus a control and a solvent control (100 µL/L acetone). Petri dishes were used, with 10 ml of ivermectin solution and 10 organisms each. Thirty embryos were used per treatment (divided in 3 replicates). Observations of spontaneous movement of the embryos (before hatching) and posture (post-hatching) were taken daily as an endpoint. Each Petri dish was placed under the stereomicroscope and the number of embryos not presenting any movement or with an abnormal posture (laying in their sides) during 5 min was recorded.

2.3.3 Embryos biomarkers assay

A third test was performed to collect embryos for biomarkers analysis using concentrations of 10, 20, 40, 60 and 80 µg/L plus a control and a solvent control (100 µg/L acetone). In this test, 6-well polystyrene microplates were utilized, with 20 ml of treatment solution and 15 organisms per well (and a total of 20 wells per treatment). The test ended after 96 h when a minimum of five groups of 7 embryos were snap-frozen in eppendorfs with adequate buffer (described below). All collected samples were stored at - 80 ºC until the enzymatic analysis of AChE, BChE, PChE, CAT, GST, LDH and quantification of VTG like proteins.

2.4 Zebrafish juveniles assay

To assess ivermectin toxicity for juvenile zebrafish, a static acute toxicity test was performed based on the OECD Guideline TG 203 (OECD, 1992). All test solutions were prepared by successive dilution of stock solutions in water. The temperature was set to 26 ± 1 ºC to provide a better comparison with FEET assay. Zebrafish juveniles (40-days old) with similar length (0.8 ± 0.2 cm) were selected according to the classification indicated by the Zebrafish Model Organism Database (ZDB-STAGE-010723-51) (ZFIN, 1994). Eight treatments were used: control, acetone control (100 µl/L), and 10, 20, 40, 60, 80, and 100 µg/L of ivermectin. Twelve juveniles (divided in 3 replicates) were exposed per treatment in glass vessels. The fish were not fed during the test period. Mortality and behavioural alterations were recorded at 24, 48, 72, and 96 h of exposure. Organisms were considered to have behavioural alterations if any of the following behaviour was observed in a 5 min period: erratic swimming, inhibition of spontaneous movement, abnormal posture or abnormal operculum movement.

2.5 Zebrafish adults assay

The assay with adult fish was adapted from the OECD Guideline TG 203 (OECD, 1992) in static test conditions. Zebrafish adult of similar length and age (2 ± 0.5 cm, 1 year old) were selected for the test. All test solutions were prepared by successive dilution of the stock solution in water. The temperature was set to 26 ± 1 ºC to provide a better comparison with FEET assay. A total of eight treatments were used: control, acetone control (100 µl/L) and ivermectin treatments at nominal concentrations of 10, 20, 40, 60, 80, 100 and 200 µg/L. An extra treatment using 50 mg/L of cyclophosphamide was included as a positive control for the genotoxic evaluation. Twelve adults (divided in 3 replicates) were exposed per treatment in glass vessels with 4 L of test solution. The fish were not fed during the test period. Mortality and behaviour alterations were recorded at 24, 48, 72, and 96 h of exposure. Organisms were considered to have behavioural alterations in any of the following behaviour was observed in a 5 min period: erratic swimming, inhibition of spontaneous movement, abnormal posture or abnormal operculum movement. At the

end of the each test surviving fish were sacrificed and organs dissected, immediately frozen by liquid nitrogen and stored at - 80 ºC until enzymatic analysis. Heads were used for AChE analysis, muscles for LDH analysis, liver for CAT and GST analyses and gills for GST analysis. In addition, the organisms from a low and an intermediary concentrations of 20 and 80 µg ivermectin /L and 50 mg cyclophosphamide/ L had 50 µL of blood sampled for micronucleus and nuclear abnormalities assay.

2.6 Biochemical analyses

Enzymatic determinations were performed spectrophotometrically (Thermo Scientific Multiskan® Spectrum) in quadruplicate at 25 °C using 96 wells microplates. The levels of each biomarker were normalized by the amount of the protein in each sample. The protein concentration in all the samples (0.9 ± 0.2 mg/ml) was determined in quadruplicate by the Bradford method (Bradford, 1976), at 595 nm, using γ-globulin as the standard.

On the day of enzymatic analysis, samples were defrosted on ice, homogenised using a sonicator (KIKA Labortechnik U2005 Control^{TM}) and centrifuged during 20 minutes at 1,0000 *g* in order to isolate the post-mitochondrial supernatant (PMS) (Jesus et al., 2013).

ChEactivity was evaluated using acetylthiocholine iodide (AsCh). In addition, in the 96 h - embryos, ChE activity was also assayed using butyrylthiocholine iodide (BsCh) and propionylthiocholine iodide (PsCh) as substrates, since the main ChE form present in this early life stage is not fully known. The activities were determined by monitoring (every 1 minute, during 5 minutes) at 414 nm the conjugation product between substrates and 5,5-dithiobis-2nitrobenzoic acid (DTNB) (absorbance increase) based on the Ellman's method (Ellman et al., 1961), adapted for microplate analysis (Guilhermino et al., 1996). For each reaction 50 µL of PMS were mixed with 250 µL of reaction solution (acetylthiocholine iodide or butyrylthiocholine iodide or propionylthiocholine iodide (75 mM) and DTNB (10 mM)). All solutions were prepared using K-phosphate buffer (0.1 M, pH 7.2).

CAT activity was measured at 240 nm by monitoring (every 10s, during 2 minutes) the decrease of absorbance due to degradation of H_2O_2, as described by Claiborne, (1985). All solutions were prepared using K-phosphate buffer (0.05 M, pH 7.0). Fifteen µL of PMS were mixed with 135 µL of reaction solution (H_2O_2, 30 mM), and 150 µL of K-phosphate buffer.

GST activity was determined according to Habig et al., (1974), using 1-chloro-2, 4-dinitrobenzene as substrate and monitoring the increase in the absorbance at 340 nm (every 20s, during 5 minutes). All solutions were prepared in in K-phosphate buffer (0.05 M, pH 6.5). Activity determinations were made using 100 µL of PMS of the sample and 200 µL of reaction mixture (10 mM reduced glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene).

LDH activity was determined at 340 nm by continuously monitoring (every 20 s, during 5 min) the decrease in absorbance due to the oxidation of NADH, following the methodology described by Vassault, (1983), with the modifications introduced by Jesus et al., (2013). Activity determinations were made using 40 ml of PMS of the sample, 250 ml of NADH (0.24 mM) and 40 ml of piruvate (10 mM) in Tris–NaCl buffer (0.1 M,pH 7.2). All solutions for LDH analyses were performed in Tris–NaCl buffer (0.1 M, pH 7.2).

2.7 Vitellogenin like-proteins quantification

Samples were homogenized through sonication, in homogenization buffer (125 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA and 1 mM dithiothreitol at pH 8; ratio 1 ml per 200 g of tissue) and then centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was then used to quantify total protein content and VTG-like proteins levels. VTG was then determined by the indirect alkali-labile phosphate method following the protocol presented in Gagné and Blaise, (2000) with some alterations (Hallgren et al., 2009). Briefly, 100 µL of the supernatant were mixed with 54 µL of acetone (35 % of final volume) during 5-10 min at room temperature and then mixed with a vortex at least three times and then centrifuged at 10,000 *g* for 5 min. After

acetone removal, 50-100 µL (depending of pellet size) of 1M NaOH were added to samples that were then maintained for 90 min at 70 °C (Hallgren et al., 2009), to allow hydrolysis of bound phosphate (Gagné and Blaise, 2000). The levels of free phosphates were determined in the aqueous phase according to the phosphomolybdenium method (Stanton, 1968). Results are expressed as mg PO_4/mg protein.

2.8 Micronuclei and nuclear abnormalities assay

The blood samples were obtained immediately after decapitation using a micropipette with heparinised tips. Fifty µl of blood was smeared on a microscope slide. After fixation in methanol for 15 min, slides were left to air-dry and then were stained with Giemsa 5 %. The stained slides were viewed under a microscope at a magnification of 1,000 x, and 3,000 erythrocyte cells per fish were scored for the presence of MN. The criteria for the identification of fish micronucleated erythrocytes were as follows: (a) MN should be smaller than one-third of the main nuclei, (b) MN should be circular or with ovoid chromatin bodies and show the same staining pattern as the main nuclei (c); MN should not touch the main nuclei (Al-Sabti and Metcalfe, 1995). NAs were classified as: (i) binuclear (for cells with two nuclei); (ii) blebbed nuclei (for cells that present a small invagination of the nuclear membrane, which contains euchromatin); (iii) lobed nuclei (for cells with invagination larger than the blebbed nuclei which could have several lobes and) and (iv) notched nuclei (for cells showing vacuoles and appreciable depth into a nucleus that does not contain nuclear material) (Carrasco et al., 1990).

2.8 SSD analysis

In order to perform a SSD analyses EC_{50} values for mortality, movement, and immobilization were summarized including data from the present study and values from the current literature (**Table 1** and **Table 2**). All concentration values refer to the active ingredient concentration of ivermectin. Short-term toxicity data from tests with the duration between one and six days were used. When more than one value was

found for the same species the LC_{50} from the longest study was chosen; for studies with the same duration the toxicity data were summarized as geometric means. A logistic curve (log) was fitted to the data using nonlinear regression. The predicted toxicities for the 5% and 50% most sensitive organisms were estimated (Hazardous Concentration for 5% (HC_5) or 50% (HC_{50}) of the population). The SSD plot was generated as described by Melo et al., (2015) using the U.S. Environmental Protection Agency spreadsheet built over excel (USEPA, 2005).

2.9 Statistical analysis

Differences between the control and control solvent were assessed by t-student test. In the case no differences were found data from the two controls were pooled together. In the case of difference, control solvent was taken for further analysis and comparison with treatments.

For genotoxicity and biochemical data an ANOVA (one-way analysis of variance) with appropriate post hoc test (Dunnett's or Dunn's test) was conducted to assess differences towards control. 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 (SPSS, 2004) and a significance level of 0.05.

Lethal concentration (LC_{10} and LC_{50}) and effect concentration values (EC_{10} and EC_{50}) were calculated for each 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 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 2**.

3. Results

3.1 Acute toxicity of ivermectin

Three static toxicity tests were performed to assess the acute toxicity of ivermectin on different life stages in zebrafish**.** At 96 h, the most sensitive stage was the juvenile (LC_{50} = 17.21 ± 1.97 µg/L) followed by adults (LC_{50} = 74.88 ± 1.99 µg/L) and embryos (LC_{50} > 800 µg/L). At the end of the extended embryo test (144 h) an LC_{50} of 518.5 ± 28.07 µg/L was calculated (**Figure 1**, **Table 2, Figure S1).**

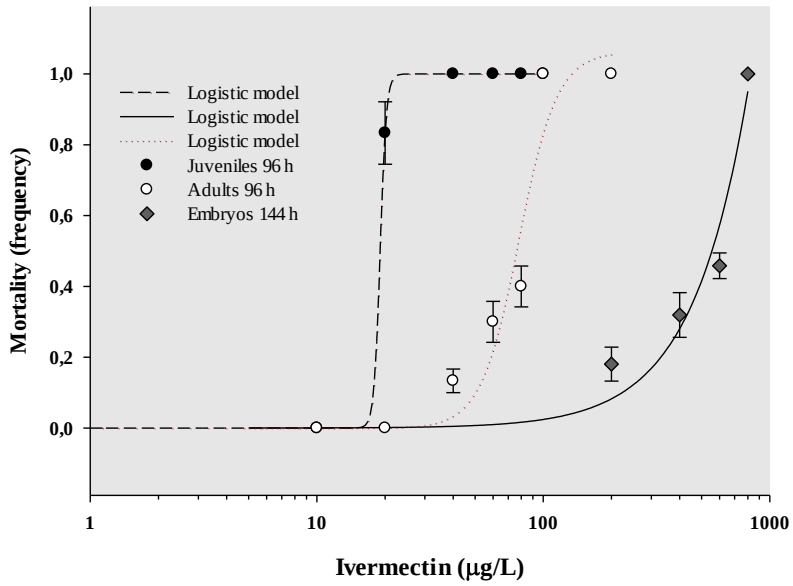


Figure 1 – Mortality of zebrafish embryos (grey diamonds), juveniles (black circles) and adults (white circles) after exposure to ivermectin (mean value ± standard error).

In the embryo test the mortality in the control group was 8.3 % in the first 96 h of experiment. In **Figure 2** an overview of the effects of ivermectin on zebrafish embryos can be seen. Embryos mortality was always below 50 % until the 144 h of exposure where a high proportion of non-hatched embryos died at highest concentrations (see below results for hatching). At 72 h of exposure only 19.4 % of the eggs exposed to the highest concentration (800 µg/L) had died while at 144 h this proportion was 100 %.

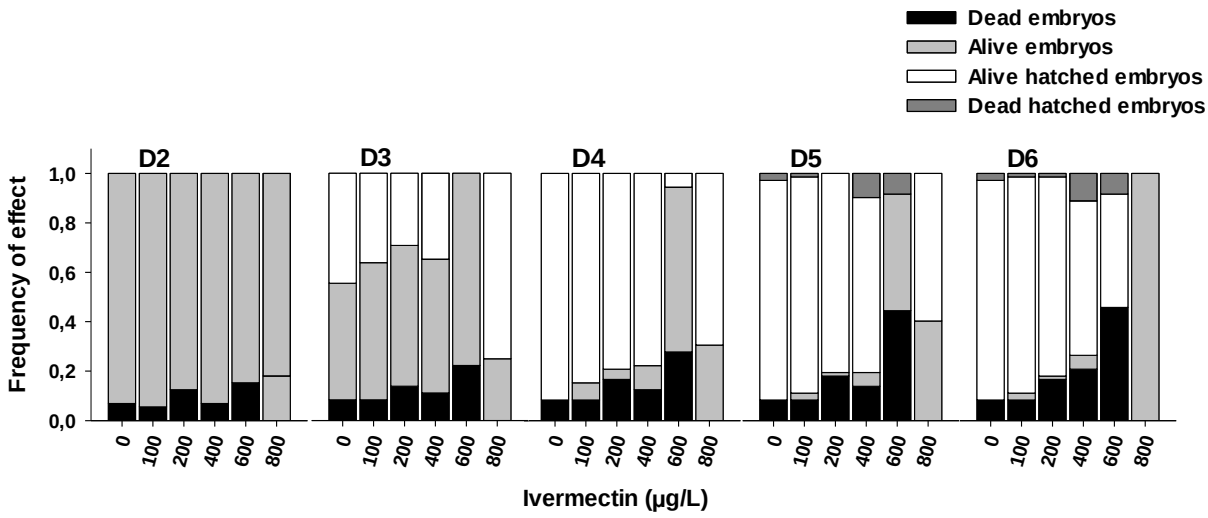


Figure 2 – Overview of effects of ivermectin on zebrafish early-life stages. The proportion of dead, alive and hatched organisms is represented by different colors.

3.2 Developmental alterations

The effects of ivermectin on the embryos development were observed during 144 h. Embryos development in the control treatment was normal, as described by Kimmel et al., (1995): embryos presented a well-developed head, body, and tail throughout the entire test period.

Ivermectin inhibited hatching at concentrations above 600 µg/L (see **Table 2** for EC_{50} values). At 72 h of exposure , the embryos from the control, 200 and 400 µg/L treatments showed more than 30% of hatching while at 600 and 800 µg/L no hatching was observed **(Figure S2)**. From 96 h to 144 of exposure, hatching was still 100 % inhibited at 600 and 800 µg/L treatments resulting in mortality of all organisms at the end of experiment.

Several developmental anomalies were observed in embryos exposed to ivermectin (see **Table 2** for EC_{50} values). At 96 h spine deformities and lateral position (side-laying) of the larvae could be seen (**Figure 3** and **Figure S3 A**). At 120 h these effects persisted (**Figure S3 B** and **C**). Embryos exposed to concentrations

from 600 µg/L were not able to hatch; moreover these embryos showed an opaque chorion, preventing an accurate visual analysis of the embryos morphological features.

Figure 3 – Zebrafish embryos malformations after 96 hours of exposure to ivermectin: (A) normal embryo with normal body structure and position, (B) embryos exposed to 200 µg/L with spine curvature and (C) embryo exposed to 400 µg/L with abnormal position (side laying) in the plate, oedemas (oe), and spine curvature (sc).

Other developmental endpoints such as otolith formation, eye and body pigmentation, somite formation, heartbeat, tail blood circulation and detachment of the tail-bud from the yolk sac were not affected by ivermectin exposure.

3.3 Behavioural alterations

Alterations in the behaviour of adults, juveniles and embryos exposed to ivermectin were observed (see **Table 2** for EC_{50} values).

Embryos behaviour was affected by ivermectin exposure in a dose-dependent pattern. During the first 48 h behaviour of organisms exposed to the highest concentrations (200 and 400 µg/L) of ivermectin was characterized by an inhibition of spontaneous movement compared to the control group. At 72 h and 96 h the hatched embryos exposed to concentrations above 80 µg/L showed abnormal posture (appears to be laid down in the bottom of the microplate well with a differential competence to respond a mechanical stimulus).

Juvenile and adult zebrafish exposed to ivermectin also showed behavioural alterations including slow movement and erratic swimming with periods of paralysis (however organisms still reacted in response to a mechanical stimulus). Increased operculum movement was also observed in adults.

3.4 Biomarkers levels

A battery of biochemical analysis was performed to assess possible alterations at biochemical level on embryos and adults exposed to ivermectin.

In the case of embryos, enzymatic assays were performed with pools of whole body homogenates of embryos and results are depicted in **Figure 4** and **5**. The ChE activity was inhibited at the highest concentration tested (80 µg/L), independently of the substrate used in the enzymatic assay (AsCh:, $F_{5, 69} = 10.35$; $p = 0.001$; PsCh: $F_{5, 69} = 10.35$; $p = 0.001$; BsCh: $F_{5, 69} = 10.35$; $p = 0.001$; **Figure 4** A, B and C).

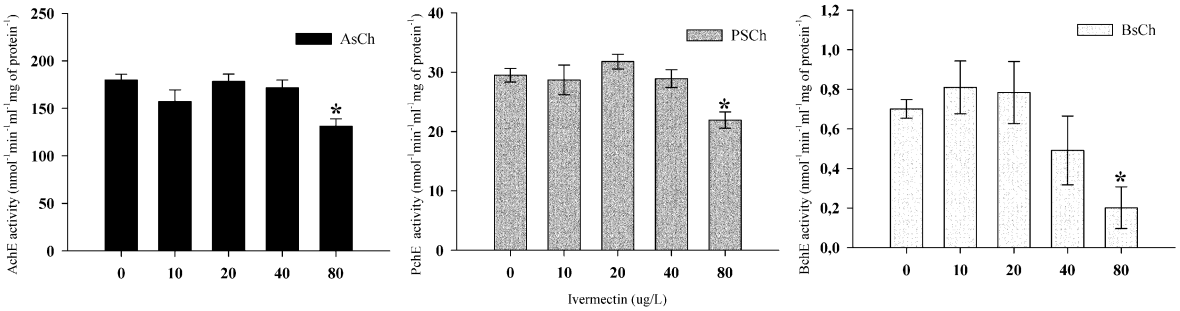


Figure 4 – ChE activities (mean ± standard error) in zebrafish embryos after 96 h exposure to ivermectin. ChE activity assayed with the following substrates (A) acetylthiocholine (AsCh), (B) propionythiocholine (PSCh) and (C) butyrylthiocholine (BsCh). "*" means significantly different from control treatment (Dunnet test $p<0.05$).

For oxidative stress related enzymes, an inhibition of CAT was observed at 80 µg/L ($F_{5, 65} = 10.40$; $p = 0.001$ **Figure 5** A) whereas no alterations were found on the activity of GST ($F_{5, 69} = 1.50$; $p = 0.203$ **Figure 5** B).

LDH presented a bell shape pattern of response ($F_{5, 65}$ = 10.40; p = 0.001 **Figure 5** C) being induced from 10 to 40 µg/L and was not different from the control at 80 µg/L.

Vitellogenin like proteins were induced with a peak at 40 µg/L returning to the basal levels at 80 µg/L ($F_{5, 65}$ = 10.396; p = 0.001 **Figure 5** D).

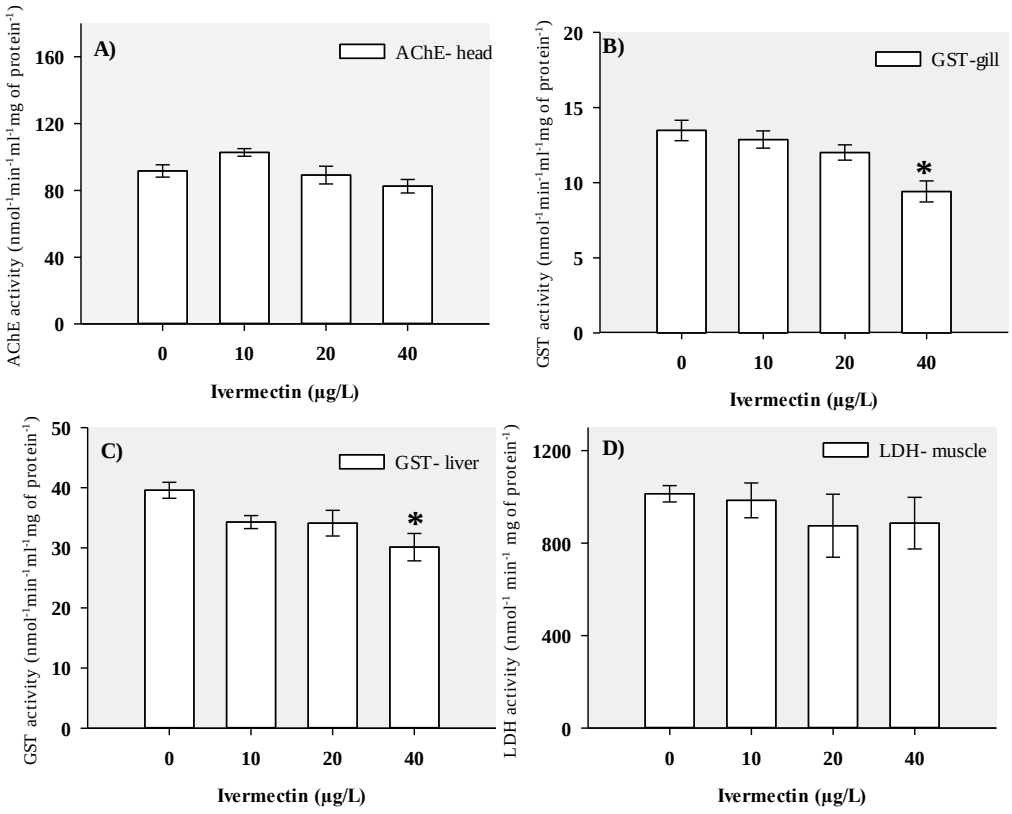


Figure 6 – Zebrafish adults biochemical markers activities (mean ± standard error) after 96 h exposure to ivermectin: (A) AChE measured in the head, (B) GST activity measured in the gills, (C) GST activity measured in the liver and (D) LDH activity measured in the muscle.“*” means significantly different from control treatment (Dunnet test p<0.05).

In the case of adult fish, biochemical markers were analysed in specific tissues or organs (**Figure 6**). The activity of AChE measured in the head was not changed after exposure to ivermectin when compared to the control, although the statistical analysis revealed an overall effect of the treatments (AChE: $F_{3, 49} = 3.36$; $p = 0.027$, **Figure 6** A). In the case of GST measured either in the gills or in the liver a clear dose-dependent inhibition was observed (GST_{gill}: $F_{3, 49} = 6.51$; $p < 0.001$, GST_{liver}: $F_{3, 39} = 5.43$; $p = 0.003$; **Figure 6** B and C). The activity of LDH measured in the muscles was not changed after ivermectin exposure ($H = 1.22$; $p = 0.75$; **Figure 6** D).

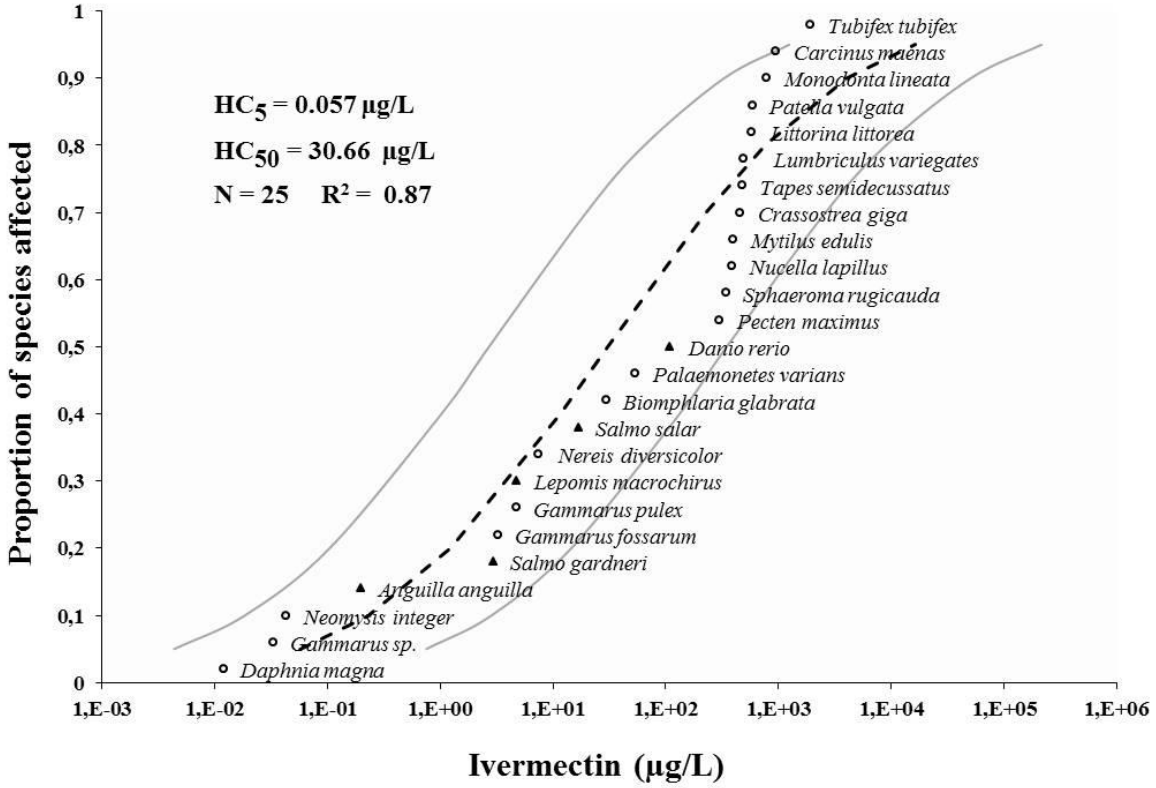


Figure 7 – Species sensitivity distribution showing the affected fraction of species versus ivermectin concentration (µg/L). The black triangles (▲) represent the different fish species and the white circles (○) invertebrates' species.

3.6 Genotoxic responses

For the assessment of ivermectin genotoxicity, the MN and NA assay were performed using adults' blood. The results showed differences only between the control and positive control group for MN parameters ($F_{4, 41}$ = 7.58, p<0.001) and no effect for NA ($F_{4, 27}$ = 2.79, p = 0.05) (**Table 3**).

3.6 SDD analysis

The SSD results are depicted in **Figure 7** with effective concentrations values from different species regarding mortality and behavioral parameters. The maximum average LC_{50} value reported among all the studies was 1907 µg/L for the worm *Tubifex tubifex* whereas the minimum values were found for crustaceans, namely *Daphnia magna* with an average EC_{50} = 0.012 µg/L; *Gammarus sp.* EC_{50} = 0.03 µg/L; *Neomysis integer* average EC_{50} = 0.043 µg/L **(Table 1)**. The predicted toxicities for the 5% and 50% most sensitive species are, respectively, 0.057 µg/L (upper limit = 0.754 and lower limit = 0.004 µg/L) and 30.66 µg/L (upper limit = 347.6 and lower limit = 2.7 µg/L) of ivermectin.

4. Discussion

Acute toxicity of ivermectin seemed to be higher in juvenile and adults when compared to embryonic stages. The 96 h-LC_{10} values calculated for the different life stages were: 14.0 µg/L for juveniles, 55.4 µg/L for adults and 147.1 µg/L for embryos. No LC_{50} values were found in literature to zebrafish although information is available for other fish species (**Table 1**). For instance, juveniles of *Salmo salar* (96 h-LC_{50} = 17 µg/L; see Kilmartin et al., (1996)) showed a very similar sensitivity to ivermectin when compared to zebrafish juveniles (96 h-LC_{50} = 14 µg/L), the most sensitive life stage tested in our study. Regarding zebrafish embryos and according to Weil et al., (2009), an exposure of 48 h at concentrations of ivermectin up to 400 µg/L did not result in increased mortality, agreeing with our results where no significant mortality was found at 48 h for even higher concentrations (800 µg/L). The toxicity test with zebrafish

embryos has been recommended to replace acute toxicity tests with adult fish (Lammer et al., 2009). The higher sensitivity of juveniles and adults suggest that using only embryos in the effect assessment of ivermectin might underestimate the risk of this compound for fish. In Europe, an approach including different life stages of invertebrates is already adopted for terrestrial risk assessment of avermectins in dung fauna. An approach with different fish life stages, as complementary tests, might be appropriate to estimate the ivermectin effects on wild fish populations.

Ivermectin showed to act on embryo development leading to morphological abnormalities and hatching delay at high concentrations. The increase in spine deformities (120 h-EC_{10} = 311.3 µg/L) and oedemas were the most pronouced morphologicals effects. Carlsson et al., (2013) reported edemas in zebrafish exposed to ivermectin in concentration between 1000 – 10,000 µg/L agreeing with our results. Concerning the hatching, embryos from the control and treatments ≤ 400 µg/L started to hatch at 48 h reaching about 80% of hatching after 96 h. On the contrary, ivermectin completely inhibited hatching in embryos exposed to concentrations ≥ 600 µg/L (Figure 2). All unhatched embryos at 800 µg/L eventually died at the end of the test. Carlsson et al., (2013) performed tests with ivermectin and doramectin (another commonly used avermectin) and for both chemicals effects on hatching were observed, namely in the time to hatch and hatching inhibition. Effects were observed at concentration ranging between 1,000 and 10,000 µg/L in accordance to the effective concentration of 120 h-EC_{50} = 506.5 µg/L obtained in our study for hatching rate. No explanation is found in the literature to explain how ivermectin and others avermectins inhibit the hatching in fish embryos. Though, since the embryo movement is essential for breaking the chorion, one could speculate that the observed effects on hatching at high doses of avermectins are a consequence of inhibition of the embryos movement in the eggs.

Effects on movement, swimming, equilibrium, coordination and activity, are well documented for different avermecitins (e.g. abamaecitn, doramectin, amamectin, ivermectin) (Carlsson et al., 2013; Raftery et al., 2013; Weil et al., 2009). Our results

showed behavioural alterations at all zebrafish life stages. The 72 h-LC_{10} values calculated for the different stages were: 16.1 µg/L for juveniles, 22.5 µg/L for adults and 35.9 µg/L for embryos indicating higher sensitivity of juveniles followed by adults and embryos. Our behavioural data is supported by previous literature reporting behavioural alterations due to ivermectin exposure (**Table S1**). Bard and Gadbois, (2007) and Mladineo et al., (2006) performed short term assessments of the behavioural effects of ivermectin in *Fundulus heteroclitus* and *Sparus aurata*, respectively. In both studies exposed fish showed alterations in behaviour including lethargy, postural changes and loss of activity. Those effects are very similar to the ones observed in zebrafish adults and juveniles in our study. Fish behaviour responses are very important from an ecological perspective as they can be directly linked to important functions of organisms such as predation avoidance, reproduction or feeding; upon which their survival depends. Indeed, effects on feeding rate, total time of feeding, loss of appetite or complete feeding inhibition are reported in several studies with *Salmo salar*, *Sparus aurata*, *Oncorhynchus kjsutch* and *Oncorhynchus tshawytscha* (**see Table S1**).

For embryos, the use of behavioural parameters greatly improved the sensitivity of the fish embryo toxicity test suggesting behaviour alterations (e.g. side-laying) as a valuable endpoint. For concentrations between 80 and 400 µg/L of ivermectin, embryos exposed for 96 h showed alterations in movement rates agreeing with the studies by Weil et al., (2009) and Carlsson et al., (2013) where the same effect was found for embryos exposed to ivermectin. In (Weil et al., 2009), embryos showed a complete lack of movement after 48 h of exposure at concentrations above 250 µg/L and a 48 h-EC_{50} of 171 µg/L was calculated. This value is somewhat higher than the 72 h- EC_{50} of 93.2 µg/L in the present study. Effects on zebrafish embryos activity were also observed by Raftery et al., (2013) for two other avermectins (abamectin e amamectin) were a complete elimination of spontaneous activity in embryos was observed at 436 µg/L of abamectin.

Neurological synaptic transmissions are the basis of all behavioural actions. Our results for ChE activity suggest that the behavioural changes may be related to a disruption of the cholinergic system. ChE was included in this study based on evidences of cholinergic action of ivermectin (Krause et al., 1998; Ucán-marín et al., 2012). Moreover, behavioural symptoms obtained for the different life stages of fish in this study are consistent with ChE depression. ChE are a group of enzymes belonging to the family of esterase that promote hydrolyses of carboxylic esters playing a key role in the maintenance of normal nerve function. In zebrafish embryos acetylcholinesterase is necessary for normal neural and muscular development (Behra et al., 2002). In 48 h zebrafish embryos the main ChE form present in whole embryo tissues is mainly AChE (Kuster et al. 2005). In our study the rate of hydrolysis decreases in the order: AsCh > PrSCh > BsCh. This suggests that the main ChE form present in 96 h zebrafish embryos is still AChE. However, this should be confirmed with further studies with specific inhibitors of the different ChEs forms. In embryos, the ChE activity assayed with the different substrates (AsCh, PrSCh, BsCh) was inhibited at the highest concentration tested (80 µg/L). An inhibition of cholinesterase was also observed in freshwater snails (*Physa acuta*) after 24 and 48 h of exposure to 19.2 and 27.4 µg/L of the similar compound abamectin (Ma et al., 2014). For zebrafish adults no effect could be observed in the AChE activity measured in the heads. In a study with *Salmo salar* the brain AChE activity is induced after 30 days treatment with ivermectin via food at either 0.05 mg/Kg b.w. or 0.25 mg/Kg b.w. (Ucán-marín et al., 2012).

The low activity and erratic movements observed for the different life stages of zebrafish are probably linked to the effect of ivermectin on central nervous system. The action of ivermectin in gamma-aminobutyric acid (GABA) receptors its well described even for mammals, in which ones high doses of exposure can provoke severe acute neurological damage (Dorman, 2000). Several studies with fish have demonstrated that ivermectin can pass through the blood–brain barrier of fish. Additionally, the P-glycoproteins efficacy in the fish blood–brain barrier is

considerably lower than in mammals (Lumeret, 2012). Thus, neurotoxic effects and consequent behavioural alterations on fish can be expected not only due to ivermectin mode of action but also due to the intrinsic low efficacy of fish blood-brain barrier to block ivermectin intake into the fish central nervous system.

The GST, CAT, LDH and VTG are biomarkers widely used as tools for chemical toxicity assessment in laboratory and field conditions (Domingues et al., 2010). However, the effects of ivermectin exposure on these markers were not studied before in aquatic organisms. These markers were chosen because they represent a variety of important biochemical pathways in fish (biotransformation, oxidative stress, energetic metabolism, and vitellogenesis).

The GST family of enzymes plays a role in the phase II of the detoxification pathway via conjugation of xenobiotics and/or endogenic compounds with glutathione (GSH) (Oost et al., 2003). Given the high partition coefficient octanol water of ivermectin (it is a lipophilic substance), it is expected an important role of GST in the detoxification of this compound. In the present work, GST activities measured in liver and gills of adult fish were dose-dependent inhibited after exposure to ivermectin. Although an effect in GST activity was previewed, an increase and not a decrease in the activity was expected. This may be related to the bell-shape curve of response of these kind of enzymes (maybe to high concentrations were used) or to the response time of the enzymes (physiological response of the organism to the compound is always difficult to predict, GST “induction window” may have occurred days earlier or be still to occur). Alternatively, as there are some evidences for oxidative stress caused by ivermectin (see discussion for CAT), GST activity may be reduced due to depletion of GSH, which is an antioxidant involved in fighting of reactive oxygen species, being in the process converted into oxidized glutathione (GSSG) and thus less available for conjugation with GST. Chronic effects of ivermectin to adult zebrafish studied within our group also reported the inhibition of GST after the 21 days exposure (unpublished data). For embryos, no effects were observed at the tested concentrations, corroborating the lower toxicity of ivermectin to embryos

compared to adults, also at biochemical level. Despite an expected increase in the detoxification process (and, consequently, in the GST activity in embryos and adults); either no response or an inhibition was exhibited. The inhibition of GST may compromise the capacity of fish to metabolize the toxicants and response such as oxidative stress can be triggered. This inhibition pattern of activity has already been described for fish exposed to different types of contaminants including pesticides and metals (Al-Ghais and Ali, 1999; Domingues et al., 2010; Wang et al., 2012).

LDH is an important enzyme in muscular physiology. In case of chemical stress, metabolic hypoxia is a common response, which can increase the anaerobic pathway of energy production and consequently increase LDH activity (Oliveira et al., 2009). In our study, this non-specific biomarker was the most sensitive endpoint for embryos, with a bell-shaped induction (lowest observed effect concentration (LOEC) = 10 µg/L); however, no differences were found in adults (muscle). Some of the points discussed earlier for explaining lack of GST response (bell shape responses and time for physiological response) also apply for LDH. Moreover, the higher responsiveness of LDH at embryos can be related to the different physiological role of this enzyme during the life cycle of fish species. Further studies linking changes in LDH activity at different life stages and the ecological consequences for fish populations should be carried out.

CAT is an antioxidant enzyme involved in the response to reactive oxygen species (ROS), more specifically fighting hydrogen peroxide (H_2O_2). An inhibition of CAT reflects a disturbance in the antioxidant balance of the organism, potentially compromising the capacity to cope with oxidative stress. Other works also report an effect of ivermectin or related compounds in the antioxidant system: Sakin et al., (2012) also observed an inhibition of CAT in rainbow trout exposed by intraperitoneal injection to 0.01 and 0.02 mg/kg of ivermectin and Varó et al., (2010) studied the liver protein profile of gilthead sea bream after oral administration of 0.2 mg/Kg IVM for 10 days and observed changes in the expression of proteins involved in oxidative stress response.

VTG is a precursor of the major yolk protein vitellin and it is used as a biomarker of reproductive endocrine disruption as it is frequently affected by exposure to chemicals with oestrogenic modes of action (by increasing levels in juvenile and male fish) (Henry et al., 2009; Pait and Nelson, 2003). In our work, the induction of VTG observed in zebrafish larvae corroborates some findings accounting for the reproductive and developmental toxicity of avermectins which has been verified mainly in insects. Some examples are the inhibition of ovary development and vitellogenesis in the tick *Amblyomma hebraeum* after injection of 100 µg/L of avermectin (Lunke and Kaufman, 1992); the developmental abnormalities in *Scathophaga stercoraria* after exposure to 0.5 µg/L of ivermectin (West and Tracy, 2009), the effects in the development of *Musca nevilli* eggs born in the dung of cattle injected with 0.2 mg/ Kg body mass and the fertility reduction of individuals that successfully emerged (Krüger and Scholtz, 1998). In a study recently published by Ucán-Marín et al. (2012) were *Salmo salar* were exposed to ivermectin in the food during 30 days the concentrations of VTG in the plasma were lower in female fish when compared to control group. Moreover, the VTG plasma levels continued depleted even after the depuration period. Future work should address the consequences of VTG alterations in the reproductive fitness of fish, especially in a chronic exposure scenario.

Cytogenetic assays performed with fish blood cells have previously been used to assess the damage of chemicals on DNA (Oost et al., 2003). The MN assay is a widely used test to reveal the consequences of spindle anomalies and chromosomal breakage, and this technique has been applied to feral fish (Oost et al., 2003). In addition, NAs, other than MN, are also considered to be indicators of genotoxic damage complementing MN scoring (Cavaş and Ergene-Gözükara, 2005). No effects were found in adults exposed to ivermectin **(**see **Table 3)**. For a more precise genotoxicity evaluation of ivermectin other tools of DNA damage assessment such as the comet assay can be included in further studies, a technique is available to perform the comet assay in fish embryos (Kosmehl et al., 2006).

The different life stages of zebrafish tested showed to be as sensitive to ivermectin exposure as several invertebrates (see **Table 1**; **Figure 7**). Together, mortality and behavioural parameters were essential in the ivermectin acute toxicity assessment. Addition of sub lethal endpoints in the zebrafish tests has proved to be useful, increasing the sensitivity of toxicity tests and providing additional information on the mode of action of ivermectin. Despite the very high sensitivity of crustaceans to ivermectin exposure our data highlight fish as a taxonomic group highly sensitive to ivermectin exposure. Indeed, among the ten most sensitive species to ivermectin exposure three are fish (**Figure 7**). Overall, the SDD analysis indicate that concentrations as low as 0.057 µg/L of ivermectin might affect a fraction of 5 % of species (HC5) in contaminated waters and concentrations of 30.66 µg/L might affect 50 % of organisms (HC50). These results emphasize the potential of ivermectin to affect aquatic communities even at nano – micrograms levels. Thus, our findings corroborate others studies where it is demonstrated the risk of ivermectin contamination in different environmental compartments such as surface water and sediments (Liebig et al., 2010; Rico and Van den Brink, 2014).

5. Conclusions

The results from this study indicated higher toxicity of ivermectin to juvenile and adults than to embryos. Biochemical responses in embryos suggested an involvement of ivermectin in the oxidative defence mechanism (inhibition of CAT), an activation of the anaerobic pathway of energy production (changes in LDH activity), some estrogenic action (increase in VTG) and a potential disruption of the cholinergic system (inhibition of ChE). The effects observed in LDH and ChE were not seen in adults but a dose dependent inhibition of GST was observed suggesting some interference in the detoxification mechanism possibly linked to activation of the oxidative defence system. Moreover, genotoxicity was not detected for adult zebrafish. The inhibition of ChE levels may be linked to the behavioural alterations observed in embryos. Although ChE was not affected in adults, behavioural alterations (lethargy and paralysis) were also observed and showed to be a very

sensitive endpoint. Behavioural endpoints are particularly important from an ecological perspective as they can be directly linked to important functions such as feeding, reproduction or predators' avoidance which are vital to the species survival.

In embryos, developmental anomalies and hatching inhibition were observed but only at high concentrations (> 4000 µg/L), whereas the biochemical and behavioural responses mentioned occurred at much lower concentrations (< 60 µg/L).The SDD analysis showed that Zebrafish is as sensitive to ivermectin as various species of invertebrates and other fish species. Overall, a HC5 value of 0.057 µg/L was obtained suggesting that a high risk is expected for aquatic ecosystems.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCT – Portugal) by the financial support through the grants SFRH/ BPD/31752/2006, SFRH/BD/62605/2009 and SFRH/BPD/100448/2014, and University of Aveiro. The authors also acknowledge the Ministry of Education, and Ministry of Science and Technology of Brazil for the scholarship provided to Cesar K. Grisolia (*Bolsa de Produtividade 1C* – CNPq) and Rhaul Oliveira through the program Science without Borders (*Bolsa Jovem Talento A* – CNPq).

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

6. References

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Multilevel assessment of ivermectin effects using different zebrafish life stages

Rhaul Oliveira[1,2*], Cesar K. Grisolia[1], Marta S. Monteiro[2], Amadeu M.V.M. Soares[2] and Inês Domingues[2]

[1]Departamento de Genética e Morfologia, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, Distrito Federal, Brazil

[2]Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[*]Corresponding author

E-mail addresses: rhaul.oliveira@gmail.com (R. Oliveira*), gisolia@unb.br (C.K. Grisolia), mmonteiro@ua.pt (M.S. Monteiro), asoares@ua.pt (A.M.V.M. Soares), inesd@ua.pt (I. Domingues)

Tel.: +55 61 31073084; Fax: +55 61 32734942

Short-title: Lethal and sub lethal effects of ivermectin to zebrafish life-stages

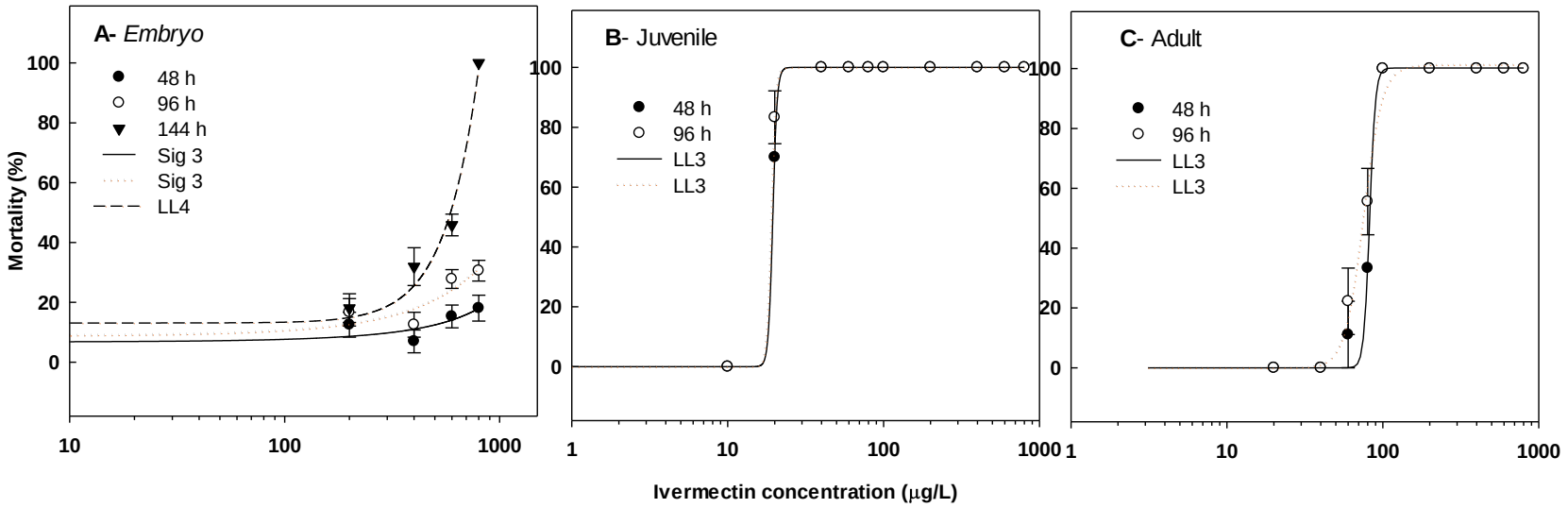

Figure S1 – Mortality of zebrafish embryos (A), juveniles (B) and adults (C) after exposure to ivermectin (mean value ± standard error).

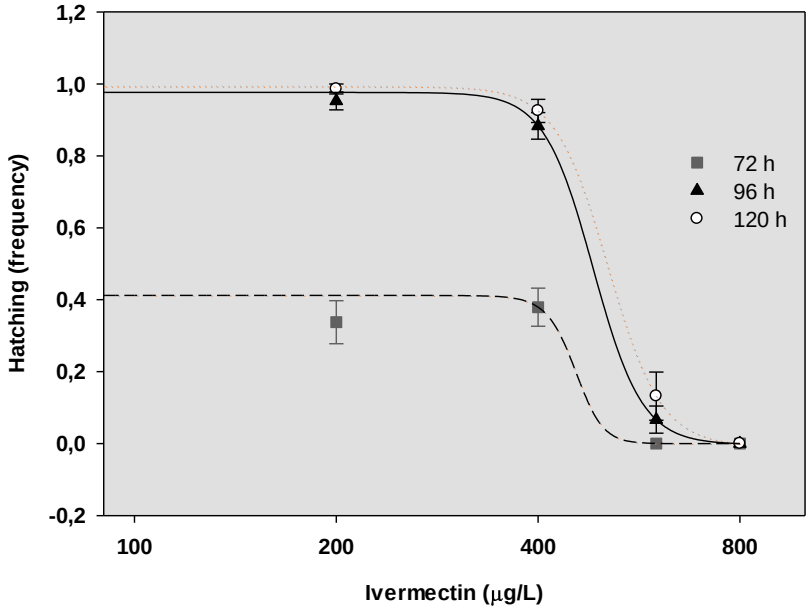

Figure S2 – Hatching of zebrafish embryos (mean value ± standard error) from 72 to 120 h.

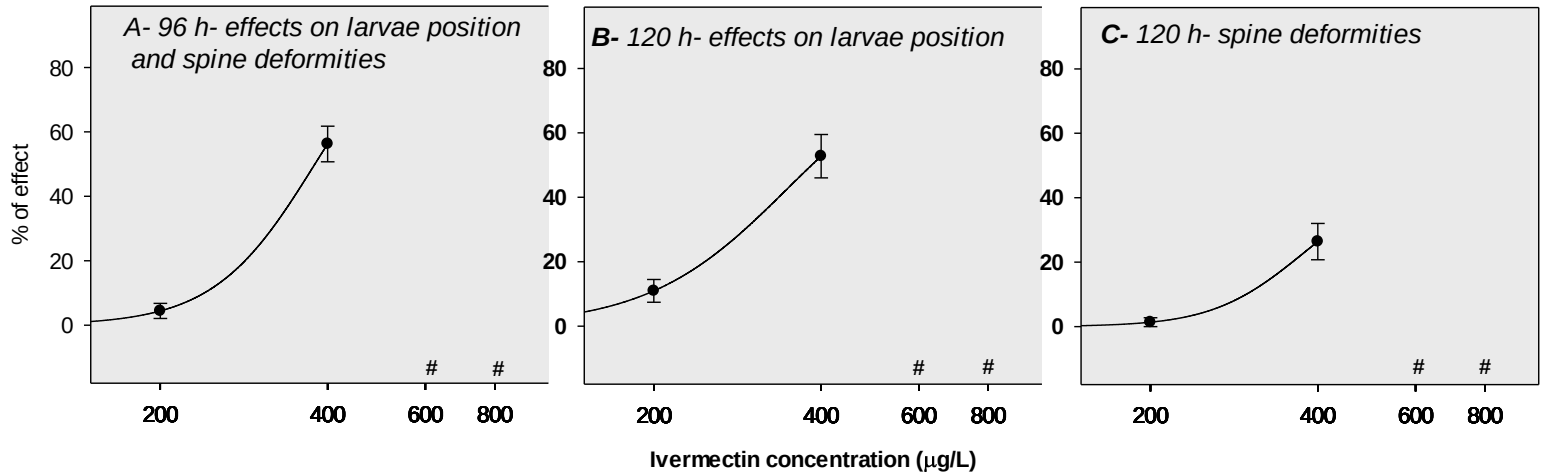

Figure S3 – Embryo development anomalies (mean value ± standard error) at 96 and 120 hours of exposure. # indicates concentrations in which a high percentage of exposed embryos did not hatch and presented an opaque chorion, preventing a proper assessment of deformities.

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Fish						
Anguilla anguilla[a]	Saltwater	01	LC50	Mortality	0.2 µg/L	Geets et *al*., (1992)
Lepomis macrochirus	Freshwater	04	LC50	Mortality	4.8 µg/L	Halley et *al*., (1989)
Salmo gardneri	Freshwater	04	LC50	Mortality	3 µg/L	Halley et *al.,*(1989)
Salmo gardneri	Freshwater	04	NOEC	Mortality	0.9 µg/L	Halley et *al.,*(1989)
Salmo trutta	Saltwater	04	LD50	Mortality	300 µg/Kg b.w. oral intubation	Kilmartin et *al.*, (1996)
Salmo salar	Saltwater	04	LD50	Mortality	500 µg/Kg b.w. oral intubation	Kilmartin et *al.,*(1996)
Salmo salar	Saltwater	04	LC50	Mortality	17 µg/L	Kilmartin et *al.,*(1996)
Sparus aurata	Saltwater	10	Adverse effects	Hepatic protein expression	0.2 µg/Kg b.w. Day	Varó et *al.*, (2010)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Sparus aurata	Saltwater	35	Adverse effects	Loss of appetite, lethargy and darkening	800 µg/Kg injection	Katharios and Spiropoulos, (2002)
Danio rerio	Freshwater	02	EC10	Movements	167 µg/L	Weil *et al*., (2009)
Danio rerio	Freshwater	02	EC50	Movements	171 µg/L	Weil et *al.,* (2009)
Danio rerio	Freshwater	02	LC10	Mortality	> 4000 µg/L	Weil et *al.,* (2009)
Danio rerio	Freshwater	02	LC50	Mortality	> 4000 µg/L	Weil et *al.,* (2009)
Danio rerio	Freshwater	02	LOEC	Gene expression *hmox 1*	4000 µg/L	Weil et *al.,* (2009)
Danio rerio	Freshwater	02	LOEC	Heart rate	4600 µg/L	Carlsson et *al*., (2013)
Danio rerio	Freshwater	01	EC50	Movements	440 µg/L	Carlsson et *al.,* (2013)
Danio rerio	Freshwater	01	LOEC	Movements	1000 µg/L	Carlsson et *al.,* (2013)
Danio rerio	Freshwater	02	NOEC	Heart rate	2200 µg/L	Carlsson et *al.,* (2013)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Danio rerio	Freshwater	01	NOEC	Organ formation	1000 - 10000 µg/L	Carlsson et *al.,* (2013)
Danio rerio	Freshwater	02	NOEC	Organ formation	1000 - 10000 µg/L	Carlsson et *al.,* (2013)
Danio rerio	Freshwater	01	NOEC	Movements	460 µg/L	Carlsson et *al.,* (2013)
Danio rerio	Freshwater	06	NOEC	Hatch	1000 - 10000 µg/L	Carlsson et *al.,* (2013)
Labeo rohita[b]	Freshwater	28	Adverse effects	Lethargy and darkened body color	200 µg/Kg b.w. day	Hemaprasanth et *al.*, (2012)
Danio rerio[c]	Freshwater	28	Adverse effects	Mortality, darkened body color, reduced movement, erratic swimming.	≥ 0.05 µg/Kg b.w. day	Collymore et *al*., (2014)
Oncorhynchus mykiss	Saltwater	50	NOEC	Mortality	≥ 50 µg/Kg d.w. food	Johnson et *al.*, (1993)
Oncorhynchus mykiss	Saltwater	50	Adverse effects	Histopatology intestine and spleens	≥ 50 µg/Kg d.w. food	Johnson et *al.,* (1993)
Oncorhynchus kjsutch	Saltwater	50	Adverse effects	Feeding activity	≥ 50 µg/Kg d.w. food	Johnson et *al.,* (1993)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Oncorhynchus kjsutch	Saltwater	50	Adverse effects	Darkened colouration	100 µg/Kg d.w. food	Johnson et *al.,* (1993)
Oncorhynchus tshawytscha	Saltwater	50	Adverse effects	Feeding activity	≥ 50 µg/Kg d.w. food	Johnson et *al.,* (1993)
Oncorhynchus tshawytscha	Saltwater	50	Adverse effects	Slow movement	100 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	NOEC	Mortality	100 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	LOEC	Mortality	200 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	NOEC	Feeding activity	50 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	LOEC	Feeding activity	100 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	NOEC	Loss of equilibrium and darkened coloration	200 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	27	LOEC	Loss of equilibrium and darkened coloration	500 µg/Kg d.w. food	Johnson et *al.,* (1993)
Salmo salar	Saltwater	30	Adverse	Loss of equilibrium, darkened	50 µg/Kg b.w.	Ucán-marín et *al.*,

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
			effects	coloration, reduced feeding behavior, Lethargy.		(2012)
Salmo salar	Saltwater	30	Adverse effects	Acetilcholinesterase induction in males	50 µg/Kg b.w.	Ucán-Marín et *al.,* (2012)
Salmo salar	Saltwater	30	Adverse effects	Vitellogenin inhibition in females	250 µg/Kg b.w.	Ucán-Marín et *al.,* (2012)
Salmo salar	Saltwater	30	Adverse effects	Wet weight	250 µg/Kg b.w.	Ucán-Marín et *al.,* (2012)
Salmo salar	Saltwater	30	Adverse effects	Liver somatic index	250 µg/Kg b.w.	Ucán-Marín et *al.,* (2012)
Invertebrates						
Arenicola marina	Saltwater	10	LC50	Mortality	17.9 µg/Kg w.w. sediment	Allen et *al.*, (2007)
Arenicola marina	Saltwater	10	LC50	Mortality	14.8 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	LOEC	Inhibition in casting rate	5 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	LOEC	Inhibition in casting rate	4 µg/Kg w.w. sediment	Allen et *al.,* (2007)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Arenicola marina	Saltwater	10	NOEC	Inhibition in casting rate	3 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	NOEC	Inhibition in casting rate	2 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	EC50	Inhibition in casting rate	6.5 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	EC50	Inhibition in casting rate	5.5 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	100	LC50	Mortality	6.8 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	100	LOEC	Inhibition in casting rate	0.5 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	100	NOEC	Inhibition in casting rate	< 0.5 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	100	EC50	Inhibition in casting rate	7.4 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Arenicola marina	Saltwater	10	LC50	Mortality	23 µg/Kg d.w. sediment	Thain, (1997)
Arenicola marina	Saltwater	10	LOEC	Mortality	24 µg/Kg d.w.	Thain, (1997)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
					sediment	
Arenicola marina	Saltwater	10	NOEC	Mortality	15 µg/Kg d.w. sediment	Thain, (1997)
Arenicola marina	Saltwater	10	Adverse effects	Feeding	< 5 µg/Kg d.w. sediment	Thain, (1997)
Arenicola marina	Saltwater	10	Adverse effects	Burrowing	> 8 µg/Kg d.w. sediment	Thain, (1997)
Artemia salina	Saltwater	01	LC50	Mortality	> 300 µg/L	Grant and Briggs, (1998)
Asterias rubens	Saltwater	10	LC50	Mortality	23600 µg/Kg d.w. sediment	Davies, (1998)
Biomphlaria glabrata	Freshwater	01	LC50	Mortality	30 µg/L	Matha and Weiser, (1988)
Caenorhabditis elegans	Freshwater	04	NOEC	Reproduction	≤ 1.0 µg/L	Liebig et *al*., (2010)
Caenorhabditis elegans	Freshwater	04	NOEC	Reproduction	100 µg/Kg d.w. sediment	Liebig et *al.,* (2010)
Capitella sp.	Saltwater	21	Adverse effects	Mortality	81 - 810 µg/m^2	Black, (1997)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Carcinus maenas	Saltwater	04	LC50	Mortality	957 µg/L	Grant and Briggs (1998)
Chironomus riparius	Freshwater	51	NOEC	Emergence	263 µg/Kg d.w. dung	Schweitzer et *al.*, (2010)
Chironomus riparius	Freshwater	10	NOEC	Larval survival	263 µg/Kg d.w. dung	Schweitzer et *al.,* (2010)
Chironomus riparius	Freshwater	10	NOEC	Larval length	263 µg/Kg d.w. dung	Schweitzer et *al.,* (2010)
Chironomus riparius	Freshwater	10	NOEC	Laval dry weight	263 µg/Kg d.w. dung	Schweitzer et *al.,* (2010)
Chironomus riparius	Freshwater	10	LOEC	Number of surviving larvae	50 µg/Kg d.w. sediment	Egeler et *al.*, (2010)
Chironomus riparius	Freshwater	10	NOEC	Number of surviving larvae	25 µg/Kg d.w. d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	10	EC10	Number of surviving larvae	34 µg/Kg d.w.	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	10	EC50	Number of surviving larvae	64 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus	Freshwater	10	NOEC	Length	12.5 µg/Kg d.w.	Egeler et *al.,* (2010)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
riparius					sediment	
Chironomus riparius	Freshwater	10	LOEC	Length	25 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	10	NOEC	Individual dry weight	3.1 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	10	LOEC	Individual dry weight	6.3 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	LOEC	Emergence ratio for females	12.5 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	NOEC	Emergence ratio for females	6.3 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	EC10	Emergence ratio for females	3.2 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	EC50	Emergence ratio for females	9 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	NOEC	Development rate (females)	6.3 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Chironomus riparius	Freshwater	28	LOEC	Development rate (females)	12.5 µg/Kg d.w. sediment	Egeler et *al.,* (2010)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Corophium volutator	Saltwater	10	LC50	Mortality	22 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Corophium volutator	Saltwater	10	LC50	Mortality	21.9 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Corophium volutator	Saltwater	28	EC50	Growth	> 29 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Corophium volutator	Saltwater	28	EC50	Inhibition in casting rate	16.7 µg/Kg w.w. sediment	Allen et *al.,* (2007)
Corophium volutator	Saltwater	10	LC50	Mortality	18000 µg/Kg d.w. sediment	Davies, (1998)
Crangon septemspinosa	Saltwater	04	LC50	Mortality	11.5 µg/g d.w. food	Burridge and Haya, (1993)
Crangon septemspinosa	Saltwater	04	LC50	Mortality	7.23 µg/g d.w. food	Burridge and Haya (1993)
Crangon septemspinosa	Saltwater	04	LC50	Mortality	7.76 µg/g d.w. food	Burridge and Haya (1993)
Crangon septemspinosa	Saltwater	04	NOEC	Mortality	2.6 µg/g d.w. food	Burridge and Haya (1993)
Crassostrea	Saltwater	04	LC50	Mortality	80-100 µg/L	Kilmartin et

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
gigas(larva)						*al.,* (1997) in Davies et *al.*, (1997)
Crassostrea gigas(spat)	Saltwater	04	LC50	Mortality	460 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Daphnia magna	Freshwater	02	EC50	Immobilisation	0.0057 µg/L	(Garric et al., 2007)
Daphnia magna	Freshwater	21	LOEC	Growth rate	0.000001 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	21	NOEC	Growth rate	0.0000003 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	21	LOEC	Reproduction	0.000001 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	21	NOEC	Reproduction	0.0000003 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	21	LOEC	Sex ratio	0.000001 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	21	NOEC	Sex ratio	0.0000003 µg/L	Garric et *al.,* (2007)
Daphnia magna	Freshwater	02	LC50	Mortality	0.025 µg/L	Halley et *al.,* (1989)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Daphnia magna	Freshwater	02	NOEC	Mortality	0.01 µg/L	Halley et *al.,* (1989)
Daphnia magna	Freshwater	02	LC50	Mortality	39 µg/Kg d.w. soil	Halley et *al.,* (1993)
Daphnia magna	Freshwater	51	NOEC	Abundance and biomass	53 µg/Kg d.w. dung	Schweitzer et *al.*, (2010)
Gammarus fossarum (adult)	Freshwater	04	LC50	Mortality	~ 3 µg/L	Alonso et *al.*, (2010)
Gammarus fossarum (juvenile)	Freshwater	04	LC50	Mortality	~ 3.5 µg/L	Alonso et *al.,* (2010)
Gammarus pulex (adult)	Freshwater	04	LC50	Mortality	~ 5 µg/L	Alonso et *al.,* (2010)
Gammarus pulex (juvenile)	Freshwater	04	LC50	Mortality	~ 4.5 µg/L	Alonso et *al.,* (2010)
Gammarus sp.[c]	Freshwater	04	LC50	Mortality	0.033 µg/L	Grant and Briggs (1998)
Hydrobia ulvae	Saltwater	04	LC50	Mortality	> 10000 µg/L	Grant and Briggs (1998)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Littorina littorea	Saltwater	04	0 % effect	Mortality	> 1000 µg/g d.w. sediment	Grant and Briggs (1998)
Littorina littorea	Saltwater	04	100 % effect	Mortality	> 10000 µg/g d.w. sediment	Grant and Briggs (1998)
Littorina littorea	Saltwater	04	LC50	Mortality	580 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Lumbriculus variegates	Saltwater	03	LC50	Mortality	490 µg/L	Ding et *al.*, (2001)
Lumbriculus variegates	Freshwater	28	LOEC	Total dry weight per replicate	500 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus variegates	Freshwater	28	NOEC	Total dry weight per replicate	160 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus variegates	Freshwater	28	EC10	Total dry weight per replicate	650 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus variegates	Freshwater	28	EC50	Total dry weight per replicate	2980 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus	Freshwater	28	LOEC	Total number of worms	500 µg/Kg d.w.	Egeler et *al.,* (2010)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
variegates					sediment	
Lumbriculus variegates	Freshwater	28	NOEC	Total number of worms	160 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus variegates	Freshwater	28	EC10	Total number of worms	450 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Lumbriculus variegates	Freshwater	28	EC50	Total number of worms	6440 µg/Kg d.w. sediment	Egeler et *al.,* (2010)
Monodonta lineata	Saltwater	04	LC50	Mortality	780 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Mytilus edulis	Saltwater	04	LC50	Mortality	400 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Neomysis integer	Saltwater	04	LC50	Mortality	0.07 µg/L	Davies et *al.,* (1998)
Neomysis integer	Saltwater	02	LC50	Mortality	0.026 µg/L	Grant and Briggs (1998)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Nereis diversicolor	Saltwater	04	LC50	Mortality	7.5 µg/L	Grant and Briggs (1998)
Nucella lapillus	Saltwater	04	LC50	Mortality	390 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Palaemonetes varians	Saltwater	04	LC50	Mortality	54 µg/L	Grant and Briggs (1998)
Patella vulgata	Saltwater	04	LC50	Mortality	600 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Pecten maximus	Saltwater	04	LC50	Mortality	300 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Potamopyrgus jenkinsi	Saltwater	04	LC50	Mortality	< 9000 µg/L	Grant and Briggs (1998)
Potamopyrgus jenkinsi	Saltwater	04	LC10	Mortality	~ 1800 µg/L	Grant and Briggs (1998)
Sphaeroma rugicauda	Saltwater	04	LC50	Mortality	348 µg/L	Grant and Briggs (1998)

Table S1 - Review of lethal and sub lethal effects of acute and chronic exposures to ivermectin on several aquatic organisms.

Species scientific name	Media type	Time of exposure (days)	Endpoint	Effects measured	Values	References
Tapes semidecussatus (larvae)	Saltwater	04	LC50	Mortality	380 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Tapes semidecussatus (spat)	Saltwater	04	LC50	Mortality	600 µg/L	Kilmartin et *al.,* (1997) in Davies et *al.,* (1997)
Tubifex tubifex	Freshwater	01	EC50	Movements	2000 µg/L	Gerhardt, (2009)
Tubifex tubifex	Freshwater	01	LC50	Mortality	1820 µg/L	Gerhardt, (2009)

[a] Organisms infected with *Anguillicola crassus;*[b]Organisms infected with *Argulus siamensis*; [c]organisms infected with *Pseudocapillaria tomentosa*; [d]A mixture of two species *G. dueheni* and *G. zaddachi* in a ratio of approximately 1 to 4.
d.w. means dry weight
w.w. means wet weight
b.w. meas fresh body weight

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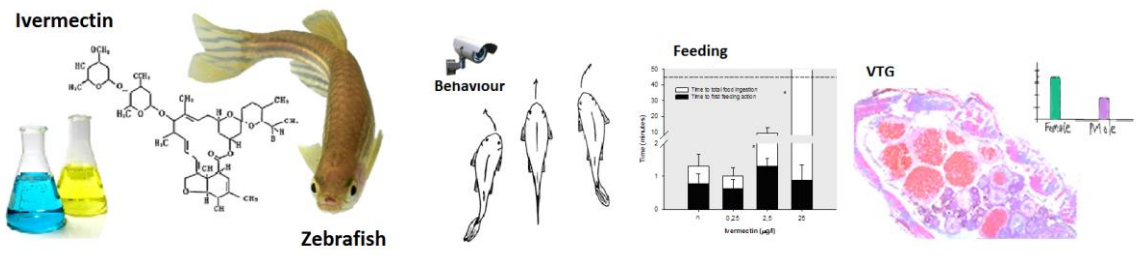
Chapter 7

Ivermectin exposure to *Danio rerio* affects its growth rate, behavior and vitellogenin levels



Ivermectin exposure to *Danio rerio* affects its growth rate, behavior and vitellogenin levels

Oliveira, R.[*†]; Domingues, I.*[†]; Leal, P.; Soares A.M.V.M. & Amorim M.J.B.

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

Aquatic toxicology

Abstract

Ivermectin (IVM) is an active ingredient used in various veterinary pharmaceuticals. It has been shown that IVM enters the aquatic compartment and adversely affects organisms including fish. Hence, in this study, a 21-days test was performed using Danio rerio as a model organism. IVM was tested in a range of concentrations (0.25-25 µg/l) following standard procedures. At test end, a range of endpoints were measured: weight, behaviour (swimming and feeding) and cellular markers including endocrine-disruption (vitellogenin-VTG), oxidative stress (catalase-CAT and glutathione-S-transferase-GST) and neurotransmitter (cholinesterase-ChE). Results showed that IVM affected weight of fish when exposed to 2.5 and 25 µg IVM/l for male and female respectively. Further, VTG levels were reduced in females at 25 µg IVM/l and the oxidative stress mechanism was activated. Changes in behaviour were observed, including decrease in swimming performance (0.25 µg IVM/l) with fish spending longer periods at the lower levels of the aquarium and having a decreased ability to feed (2.5 µg IVM/l). This poses an important ecological impact, e.g. in predator-prey interactions where their competitive advantage can be decreased. The current study based on a one experiment multiple endpoint (anchored) allowed results to be integrated and linked towards a mechanistic understanding. **Keywords***: Ivermectin, vitellogenin, feeding behavior, biomarkers, growth rate, Danio rerio*

† The two authors contributed equally to the study

1. Introduction

Veterinary pharmaceuticals (VPs) have an important role in livestock production, treating and preventing diseases. In 2003, in the United States of America the VPs market accounted for U$3.3 billion per year (Boxall et al. 2003). In the European Union, an amount of 6051 tons of VPs active substances are used per year (Kools et al. 2008).

The avermectins are naturally occurring compounds with insecticidal and anthelmintic properties, being included in the composition of various VPs. Ivermectin (IVM) is a semisynthetic avermectin which is composed of at least 80% of the 22,23-dihydroavermectin B1a and not more than 20% 22,23-dihydroavermectin B1b. It was the first avermectin to be commercialized and is the most used parasiticide in livestock, humans and pets (Wolstenholme and Rogers 2005).

First studies on IVM mode of action showed IVM interference with the nervous system, targeting at gamma-aminobutyric acid (GABA) gated chloride ion channels (Omura 2002), although later Duce and Scott (1985) found additional IVM targets. In nematodes, IVM acts at the GABA receptor channels located in the peripheral nervous system and also by opening glutamate gated chloride channels located in nerve and muscle cells, causing increased chloride conductance and paralysis of movement (Schaeffer 1989). Chronic effects were also observed including reduction in the production of new larvae (Wolstenholme and Rogers 2005). In vertebrates, no glutamate gated chloride channels can be found and IVM acts by opening the GABA receptor chloride channels, increasing chloride permeability, hyperpolarizing the membrane potential and reducing nerve transmission (Stevens and Breckenridge 2001). As GABA receptors channels are mostly found in the central nervous system, the blood-brain barrier prevents the entrance of IVM (because it is a large molecule) protecting organisms from effects at therapeutic dosages; however, some studies indicate that blood-brain barrier may not be sufficient to prevent entrance of IVM, especially in fish (Hoy et al. 1992).

Soil and water compartments are exposed to IVM residues due to its large scale use. IVM is excreted by treated cattle relatively unaltered, reaching soils through manure and then reaching waters by run-off (Metcalfe et al 2008). Pond aquacultures are a source of IVM especially in Asian countries such as China and Vietnam (Rico and Van der Brink 2014). Open water aquacultures in the marine environment (especially salmon farming) were also a source of IVM residues both in the water column and sediment (Cannavam et al 2000; Davies and Rodger, 2000). Although IVM has never been licensed for fish, it was used in marine aquaculture in Ireland, Canada and Chile until 2000 (Horsberg 2012). Most of the studies assessing environmental

concentrations of IVM focus on sediments. For instance Cannavam et al. (2000) measured 5 ng IVM/ g (wet weight) in the 3 cm top layer of sediments at an Atlantic salmon aquaculture. For freshwater assessments, Liebig et al (2010) calculated a Predicted Environmental Concentrations (PEC) of 0.2 to 2.5 ng/L for surface water nearby pasture animal (Phase II, Tier A PECS, calculated according to EMEA (2008)) and Metcalfe et al (2008) indicated a PEC of 13 ng/L for surface water (Phase I, calculated following CVMP guidelines (2005) and assuming surface water PEC is 1/10 of the groundwater PEC). A recent probabilistic risk assessment of veterinary medicines used in Asian aquaculture indicated a high ecological risk (median probability of Risk Quocient > 100) for IVM used in the production of tilapia (China), shrimp (China) and pangasius (Vietnam) (Rico and Van der Brink 2014). These authors followed procedures adopted for developed countries (VICH 2000, 2004).

Even low concentrations (ng/L) of IVM can pose a threat to non-target organisms and consequent equilibrium of the ecosystems. For instance, Garric et al (2007) derived a lowest observed effect concentration (LOEC) as low as 0.001 ng/L for the growth rate, reproduction and sex ratio of *Daphnia magna* in a 21 days semi-static reproduction test. Indoor microcosm studies have also revealed high toxicity of IVM to invertebrates either in the water column (Boonstra et al. 2011) or in the sediment (Brinke et al. 2010). A recent semi-field study aiming at assessing the dynamic of IVM in runoff and drainage waters from dung treated soils showed that concentrations measured in water under different scenarios are high enough to harm aquatic and benthic communities. Total IVM concentrations (dissolved and particle-bound) of 19 ng/L (drainage water, pasture scenario), 88 ng/L (runoff water, arable land scenario) and 118 ng/L (runoff water, spray application scenario) were measured, which indicates a risk to aquatic systems adjacent to pasture areas of treated animals or soil fertilized with their manure (Fernandez et al. 2011).

Due to its particular mode of action and multiple sources of entrance into the environment, IVM has received increasing attention by regulatory authorities of VPs. Recently Lumaret et al. (2012) reviewed the literature regarding ecotoxicity of macrocyclic lactones, including IVM, showing that it also causes a range of effects in non-target insects such as delayed reproductive development, reduced fecundity, disruption of water balance, interference with moulting and emergence, and developmental abnormalities, suggesting a potential effect in the endocrine system rather than simply an indirect effect of reduced feeding activity. Interference with development and reproductive processes was observed, for instance,in the tick *Amblyomma hebraeum* where inhibition of the process of vitellogenesis is observed (Lunke and Kaufman 1992).

Few data can be found in literature concerning long term effects of IVM to fish. Values for acute toxicity (96h LC_{50}) range from 3 □g/L for rainbow trout (*Oncorhynchus mykiss*) and 4.8 □g/L for bluegill sunfish (*Lepomis macrochirus*) according to Halley et al. (1989), to 17 □g/L for Atlantic salmon (*Salmo salar*) according to Kilmartin et al. (1996). Chronic effects of IVM were studied, for instance, by Ucan-Marin et al. (2012) in Atlantic salmon exposed through food (evaluating growth, behaviour and physiologic parameters) and by Varó et al. (2010) in sea bream (*Sparus aurata*) exposed orally (evaluating hepatotoxicity using a proteomic approach). Chronic effects have to be further studied in fish as evidences suggest that IVM can pose an environmental risk to aquatic environments

Hence, in this study, physiological and biochemical endpoints were assessed in zebrafish after 21 days of exposure to IVM. Weight, feeding and swimming behaviour were measured as they may be impaired by neurotoxic compounds. Biochemical endpoints included vitellogenin (VTG), cholinesterase (ChE), catalase (CAT) and glutathione-S-transferases (GSTs). VTG is a precursor of the major yolk protein vitellin and it is used as a biomarker of reproductive endocrine disruption as it is frequently affected by exposure to chemicals with oestrogenic modes of action (by increasing levels in juvenile and male fish) (Henry et al. 2009; Jin et al. 2009; Pait and Nelson 2009). ChE was selected to assess if the neurotransmission was impaired, while CAT will inform about the oxidative stress status and GST will give a measure of ongoing detoxification processes in the organism.

2. Material and Methods

2.1 Test Chemicals

Ivermectin (IVM) ($C_{15}H_{16}Cl_3N_3O_2$, CAS no 70288-86-7, 97 % purity; (~95% B1A and ~5% B1B) was purchased from Sigma-Aldrich (product reference: I8898 Sigma).

2.2 Test organisms

Danio rerio was used as test species. Organisms were kept in culture under controlled conditions, in a ZebTEC (tecniplast) recirculating system. Culture water is reverse osmosis and activated carbon filtered tap water, complemented with 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, salinity 0.35 ppt and dissolved oxygen equal or above 95 % saturation. A 16:8 h (light:dark) photoperiod cycle was maintained. Nitrogen compounds were regularly monitored and levels kept always below 0.1 mg/L (nitrate) and 0.01 mg/L (nitrite and

ammonia). The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd) and brine shrimp nauplii. Sexually mature male and female of the same size (0.29 g ± 0.02 for females and 0.20 g ± 0.01 for males) and age (between 16 and 18 weeks) were selected for the tests.

2.3 Tests procedure

Acute test

A 96 hours acute assay with adult fish, based on the OECD Guideline TG 203 (OECD 1992) in semi-static test conditions was performed to assess survival of adult zebrafish exposed to IVM. Temperature and photoperiod conditions were similar to the culture conditions. Uniform cool-white light was used (2000 lux). All test solutions were prepared by successive dilution of a stock solution in culture water. IVM treatments range was 10, 20, 40, 60, 80, 100 µg/L plus a control and an acetone control (100 µl/l) treatments. As IVM degrades quickly in aquatic environment in the presence of light (half-life of 12 to 39 hours according to Bloom and Matheson (1993)), renewal of test solutions was performed daily to ensure exposure concentrations. Nine *D. rerio* (divided in 3 replicates) were exposed per treatment in glass vessels with 4 L of test solution. The fish were not fed during the test period. Mortality was recorded at 96 hours of exposure.

For both tests water physico-chemical properties (temperature, conductivity, pH and dissolved oxygen) were daily monitored in each exposure vessel after medium renewal and were in the range of values established for culture water.

Chronic test

Test procedure was based on the OECD guideline 204 (1984). Temperature and photoperiod conditions were similar to the culture conditions. Uniform cool-white light was used (2000 lux). Adult fish previously weighted were exposed for 21 days to 0.25, 2.5 and 25 µg/l of IVM plus an acetone control. Control contained the same concentration of acetone (100 □l/l) to resemble the amount used in the highest IVM treatment. IVM concentrations were selected based on the results of the acute test; the highest concentration represents half of the 96h LC_{10} (56.4 µg/l, 36.4<CI<64.8). Test solutions were obtained by successive dilution of a stock of IVM in culture water. Test solutions were daily renewed. Ten fish (5 male and 5 female) were used per test container (5 L aquarium with 4 L test solution). Two replicates per treatment were used except in the last treatment (25 µg/l) where 3 replicates were used. Fish were fed twice daily (in the morning and after medium renewal) with ZM-300 fish food (Zebrafish Management Ltd) and brine shrimp nauplii *ad libitum*.

During the test, any fish mortality or abnormal behaviour was recorded. At day 21 feeding and swimming behaviour were evaluated as follows. Feeding behaviour was evaluated by transferring 5 fish of each treatment to an aquarium with the same IVM concentration and by adding 10 particles of ZM300. Time until first biting/feeding action and time required for total food ingestion (until a maximum of 45 minutes) were recorded. Swimming behaviour was analysed by transferring each of the 10 fish from each treatment to an aquarium individually with the same IVM concentration, marked at 3 different heights (5, 10 and 15 cm) and video recorded over 10 min. Video records were analysed for swimming behaviour by measuring the time spent by each fish in each of the layers of the aquarium (bottom: 0 to 5 cm, middle: 6 to 10 cm and upper: 11 to 15 cm). Data was then used to calculate a swimming frequency per altitude. All fish were then weighted, placed in ice to reduce metabolism, euthanized by decapitation and sexed (through dissection and direct observation of the gonads). Tail, trunk and head of each fish were placed separately in cryotubes, snap frozen in liquid nitrogen and stored at -80ºC for further analysis of biochemical parameters, i.e., vitellogenin, catalase, glutathione-S-transferase and cholinesterase. Vitellogenin and weight variation were evaluated for male and females separately.

2.4 Biochemical analysis

Sample processing

VTG levels were determined in fish tails. Samples were defrosted on ice, homogenized in dilution buffer (included in the Zebrafish Vitellogenin Elisa Kit; Biosense Laboratories) (1:20 weight: volume) and centrifuged (20 min, 4ºC, 10000 *g*).

CAT, GST and ChE activities were determined in head and body of zebrafish adults. Samples were defrosted on ice, homogenised in K-phosphate buffer (0.05 M, pH 7.4) and centrifuged (20 min, 4ºC, 10000 *g*) to separate the post-mitochondrial supernatant (PMS) (Howcroft et al. 2011).

All sample homogenizations were done in a Ystral X10/20 homogenizer.

VTG analysis

Vitellogenin (VTG) was analysed using a Zebrafish Vitellogenin Elisa Kit (Biosense Laboratories). The technique is based in the specific binding between antibodies and VTG and the detailed procedure is included in the commercial Kit. Overall, the process includes the following stepwise: i) preparing dilutions of standard and samples, ii) incubation with standard and diluted samples, iii) incubation with detecting antibody, iv) incubation with secondary antibody and v) calculations of results.

CAT analysis

CAT activity was measured at 240 nm by monitoring (every 10 s, during 2 min) the decrease of absorbance due to degradation of H_2O_2, as described by Clairborne (1985). Fifteen µl of PMS were 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).

GST analysis

GST activity was determined at 340 nm by monitoring the increase in absorbance every 20 s, during 5 min, following the general methodology described by Habig and Jakoby (1981) with modifications as introduced by Frasco and Guilhermino (2002). Activity determinations were made using 100 µl of PMS and 200 µl of reaction mixture (10 mM reduced glutathione (GSH)) and 60 mM 1-chloro- 2.4-dinitrobenzene in K-phosphate buffer (0.05 M, pH 6.5).

ChE analysis

ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm, every 20 s, during 5 min the conjugation product between thiocoline (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase) in K-phosphate buffer (0.1 M, pH 7.2), 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 K-phosphate buffer (0.1 M, pH 7.2)).

CAT, ChE and GST enzymatic activities were determined spectrophotometrically in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration in samples was determined in quadruplicate by the Bradford Method (Bradford 1976), at 595 nm, using γ-globulin as a standard.

2.5 Data analysis

Effects on weight were calculated as the specific growth rate (r) according to the following equation (OECD 2000):

$$r = \frac{\ln W_2 - \overline{\ln W_1}}{t_2 - t_1} \times 100$$

Where:

$\overline{\ln W_1}$ = average of the logarithms of the fish weights at the start of the experiment

ln W_2 =logarithm of the weight of a particular fish at the end of the experiment

t_1, t_2 = time (days) at start and end of study period

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS, 2004). A One-way ANOVA was used to detect differences between the treatments for normally distributed data sets. If significant differences were found, the post-hoc Dunnetts' test (two-sided) was used to discriminate differences towards control. When data did not pass the Kolmogorov–Smirnov normality test and the Levene's homogeneity of variance test, a Kruskal–Wallis test was used (as it was the case of VTG data for females and GST activity in head homogenates). In the case of significant differences, a Dunn's test was run to discriminate differences towards control. All statistical analyses were based on 0.05 significance level. Effect Concentrations (EC) calculations were performed modelling data to logistic or threshold sigmoid 2 parameters regression models, using the Toxicity Relationship Analysis Program (TRAP) software. EC values for swimming behaviour were calculated using the values corresponding to the top swim. For the feeding, time for total food ingestion was used.

3. Results

Water physico-chemical parameters were always within the culture range. Differences between the replicates (tanks) were not significant for any measured variable.

In the acute adult test a dose response curve was obtained for mortality and LC values calculated (Table 1, Fig. 1).

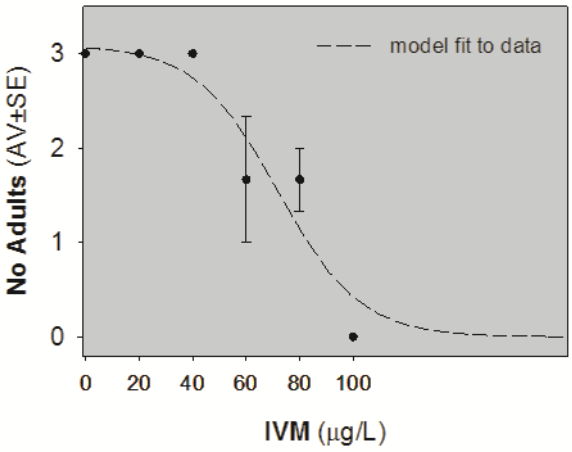


Fig. 1 Results from the acute exposure of *Danio rerio* to Ivermectin for 96 hours. Data expressed as average ± standard error (AV±SE).

In the chronic test, for the highest concentration (25 µg IVM /l), fish were lethargic and inactive and presented morphologic alterations, namely darker coloration and mild curvature of the spine.

Table 1: Summary of Effect Concentrations (ECx), No/Lowest Observed Effect Concentration (NOEC/LOEC) and respective model for calculation, for results of exposure of adult *Danio rerio*

to Ivermectin. Endpoints include 96 h survival, feeding behaviour based on the time for total food ingestion and swimming behaviour based on the time spent at the top layers.

Endpoint / Effect level	**EC_{10}** (µg / L)	**EC_{50}** (µg / L)	**EC_{80}** (µg / L)	**NOEC / LOEC** (µg / L)	**model**
Survival (96h)	41.6 (21.2<CI<62.0)	73.3 (64.1<CI<82.4)	93.2 (78.1<CI<108.3)	40 / 60	Logistic, 2 param
Feeding (21 days)	1.9 (1.4<CI<2.7)	5.1 (2.3<CI<11)	7.9 (1<CI<61)	0.25 / 2.5	Threshold sigmoid, 2 param.
Swimming (21 days)	Not Determined	1.6 (-191<CI<194)	17 (-115<CI<149)	<0.25 / 0.25	Logistic, 2 param.

Effects on weight can be observed in Fig. 2A. The negative growth rates indicate that fish lost weight during the exposure time. IVM caused a weight loss in a concentration related manner. Males were more affected than females, as observed by the significant effects already at 2.5 µg/L (Fig.2).

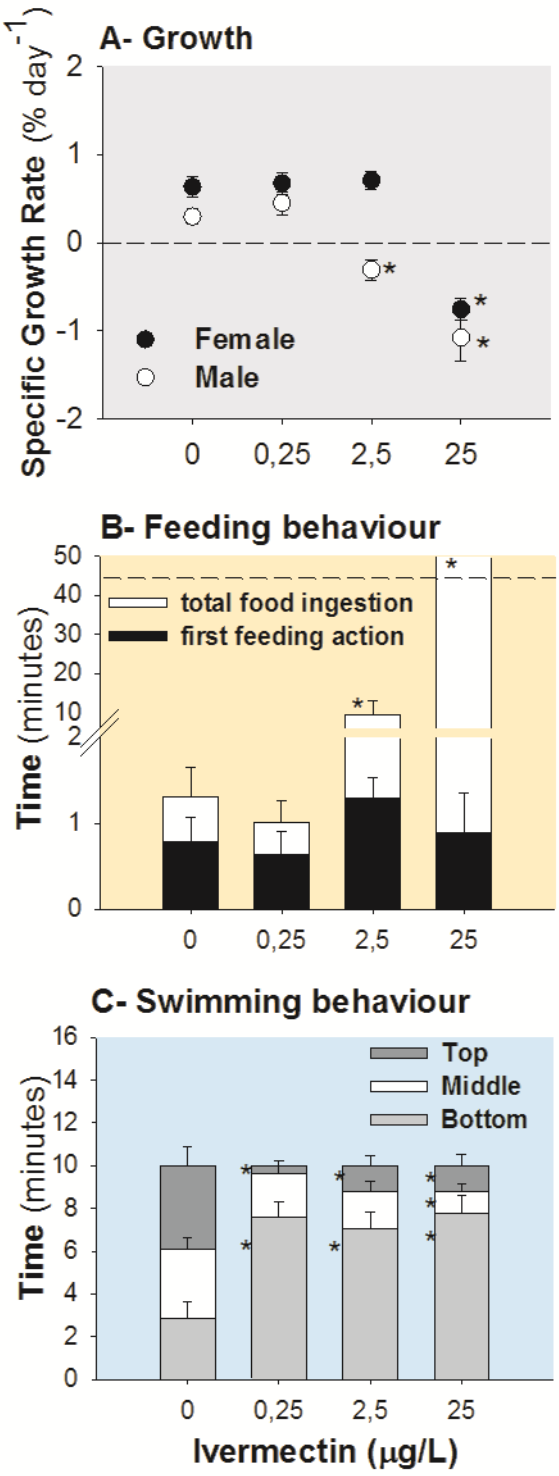


Fig. 2 Results from the exposure of *Danio rerio*, males and females, to Ivermectin for 21 days. All data are expressed as average ± standard error (AV±SE). A- Growth rate as calculated for males and females (n=10); B- Feeding behaviour: recorded as time for first feeding action and time for total food ingestion; at 25 µg/l time total food ingestion exceeds the maximum 45 minutes time pre-established (n=10). C- Swimming behaviour: recorded as the cumulative time swam at different heights, discriminated between top (0-5cm), middle (6-10cm) and bottom (11-15cm) (n=10). (*p<0.05, Dunnetts' Test).

Effects on the feeding behaviour can be observed in Fig. 2B. Time for the first bite/feeding action was not significantly affected by IVM exposure but the time to total food ingestion increased significantly with IVM concentration, exceeding the maximum pre-established time (45 min) at 25 µg IVM /l. From the dose-response curve it can be estimated that 5 µg IVM /l causes 50% effect on the time to total food ingestion45 min as total time).

Effects on the swimming behaviour can be observed in Fig. 2C. The proportion of time spent by fish in each of the layers varies with IVM concentrations. Control organisms swim on all 3 layers of the aquarium for relatively even periods of time, whereas fish exposed to IVM clearly decrease the time spent at more superficial layers (5-10 and 10-15cm), swimming mostly on the bottom. Effects could be observed even at the lowest concentration tested (0.25 µg/l). As estimated, 2 µg/l IVM reduce the ability to swim on top layers by 50% due to the large confidence intervals, this should be used as mere indication).

The results of the biochemical measures can be observed in Fig 3 and 4. Basal levels of the different parameters differ between head and trunk tissues, and the sensitivity of the tissues towards IVM differed. ChE levels (Fig. 3 A and B) did not change significantly, although there was an inhibition trend observed in heads of fish exposed to 25 µg/l. The levels of ChE in trunk were much lower compared to the head. In terms of the oxidative stress biomarkers, CAT activity (Fig. 3 C and D) was inhibited in trunk samples (at 25 µg/L) and GST activity (Fig 3 E and F) was inhibited in head samples at the highest concentrations (2.5 and 25 µg/L).

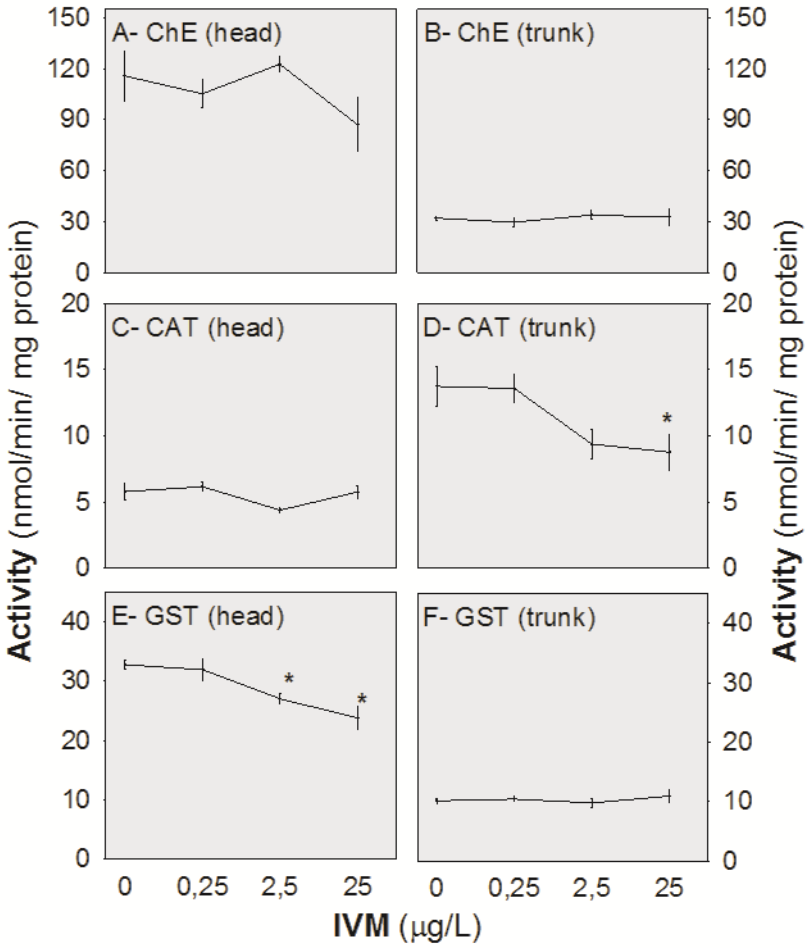


Fig. 3 Results of the quantification of biochemical parameters (expressed as average ± standard error (AV±SE)) in *Danio rerio* after exposure to IVM for 21 days. Marker included cholinesterase (ChE), catalase (CAT) and glutathione-S-transferase (GST) (n=10) (*p<0.05, Dunnetts' Test for D and *p<0.05, Dunns Test for E).

For VTG (Fig. 4), no significant changes occur in males, whereas in females a non-significant inhibition pattern was observed for 25 µg/l.

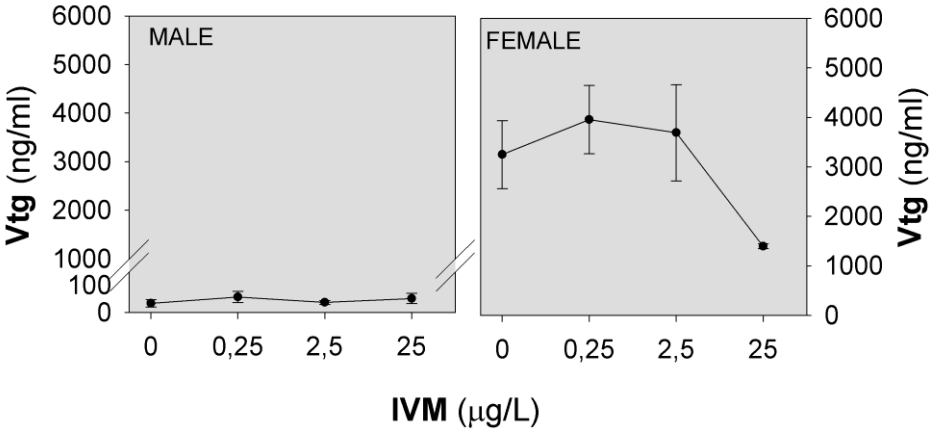


Fig. 4 Results of the quantification of vitellogenin (expressed as average ± standard error (AV±SE)) in *Danio rerio* tails homogenates after exposure to IVM for 21 days (n=10).

4. Discussion

The multiple endpoint approach allowed an integrated interpretation and further understanding of the effects and mechanisms of IVM in fish.

In terms of weight, the observed reduction in males at 2.5 µg IVM /l could be partly related with a decrease in feeding as a consequence of changes in feeding and/or swimming behaviour, e.g., fish exposed for 21 days to 25 µg IVM /l nearly lost the ability to swim in the upper layers, spending most of their time in the bottom (between 0-5cm layer) of the aquariums and hence also not eating or reaching the food as laid on the surface. Effects of IVM on fish swimming performance were reported in two studies where IVM was administrated intraperitoneally to rainbow trout *(Oncorhynchus mykiss)* at sublethal doses: Kennedy et al. (2014) observed changes in critical swimming speed, burst swimming distance and schooling at doses between 0.01 and 0.5 mg/ kg while Sakin et al (2012) observed erratic swimming, loss of reflexes and lethargy at doses of 0.01 and 0.02 mg/kg. Also, administrating IVM via food, Ucan-Marin (2012) observed the same type of behaviour in juvenile Atlantic salmon: reduced weight, reduced mobility and longer time to complete feeding. These effects suggest an effect at the central nervous system (CNS) level and are in agreement with the fact that the brain blood barrier in fish is not fully effective in protecting the brain against IVM (e.g. Katharios et al. 2004). Moreover, although IVM targets the GABA receptors, the behavioural (reduced swimming and feeding) effects observed are similar to those described for anticholinergic agents suggesting that AChE may also be affected. Indeed, in our work, ChE activity measured in heads was reduced for the highest concentration, corroborating the hypothesis that IVM affects the CNS. However, this is not conclusive, given the possible association with lethal effects at this concentration.

The reduction observed for CAT activity suggests that the antioxidant system is also affected. CAT acts against reactive oxygen species by converting hydrogen peroxide in water and oxygen (Jemec et al. 2010). Such inhibition of CAT was also observed in rainbow trout exposed intraperitoneally to 0.01 and 0.02 mg/kg IVM (Sakin et al. 2012). Other evidences of IVM induced oxidative stress include the decreased levels of superoxide dismutase and glutathione peroxidase in pigeon after subchronic exposure to avermectin (20, 40 and 60 mg of avermectin/ kg diet for 90 days) (Li et al. 2013). Moreover, in gilthead sea bream *(Sparus aurata)*, the liver protein profile was studied after oral administration of 0.2 mg/ Kg IVM for 10 days and changes were observed in the expression of proteins involved in oxidative stress response (Varo et al. 2010).

Glutathione- S-transferases belong to a family of enzymes acting in the phase II of the detoxification process, conjugating toxicants with glutathione to facilitate their excretion. The GST inhibition observed may be a consequence of energy depletion, which in turn results from the reduced feeding.

The effects of IVM were also observed in the decrease of VTG in females in the highest concentration, in accordance to the work of Ucan-Marin (2012) with juvenile Atlantic salmon, where VTG levels measured in the plasma of females were also decreased after 30 days of food borne IVM exposure. However, decrease of VTG levels can be a consequence of the general stress condition observed at that concentration and thus cannot be interpreted as an indicator of endocrine disruption (OECD 2012). To test for VTG inhibition in females, and to establish a link to possible endocrine disruption effects, further studies focusing on developmental stage and continuous exposure until maturation should be conducted.

5 Conclusions

IVM affected *D. rerio* at sublethal concentrations. The results indicate the following: IVM seems to primarily act via the central nervous system, which in turn conditions the swimming behaviour to the lower levels of the water column at concentrations as low as 0.25 µg IVM /l. At the same time, this reduction of the swimming performance affects the feeding ability and hence causes the decrease in the weight increment. This decrease is gender differentiated, being more pronounced in males (0.25 µg IVM /l) than in female (25 µg IVM /l). Moreover, a reduction on VTG (only in females), CAT and GST levels was observed in fish exposed to 25 µg IVM /l; although these alterations probably only reflect the general condition of the fish which was significantly compromised at this concentration.

Additionally, the decrease in performance in terms of feeding and swimming behaviour, poses further ecological impacts, e.g. in predator-prey interactions by decreasing their competitive advantage.

The current study was based on a one experiment multiple endpoint (anchored), where results could be integrated. The authors recommend and highlight the benefits of integrating various levels (organism and cellular) towards a knowledge based risk assessment.

Acknowledgements

This study was funded by FEDER through COMPETE e Programa Operacional Factores de Competitividade and by National funding through FCT-Fundação para a Ciência e Tecnologia, within FUBIA FCOMP-01-0124-FEDER-008651 (Ref. PTDC/AAC-CLI/103719/2008) and Climatox FCOMP-01-0124-FEDER-027795 (Ref. PTDC/AAG-GLO/4059/2012), a Post-Doc grant to Inês Domingues (SFRH/BPD//90521/2012) and a PhD grants to Rhaul de Oliveira (SFRH/BD/62605/2009). The authors acknowledge the help provided by Pedro Leal in the laboratorial experiments.

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

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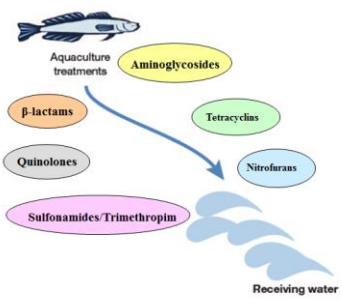
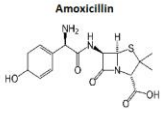
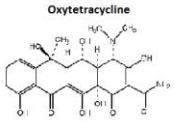
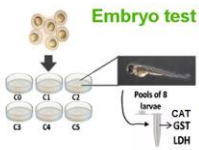
Chapter 8

Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*)









Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (Danio rerio)

Rhaul Oliveira; Sakchai McDonough; Jessica C.L. Ladewig; Amadeu M. V. M. Soares; António J. A. Nogueira and Inês Domingues

This chapter has been published as a original article in:
Enviromental Toxicology and Pharmacology v. 36 pp. 903–912 (2013)

Abstract

Antibiotics have been widely used in human and veterinary medicine to treat or prevent diseases. Residues of antibiotics have been found in aquatic environments, but their effects on fish have been not properly investigated. This work aimed to assess the sub-lethal effects of oxytetracycline and amoxicillin on zebrafish development and biomarkers. Embryos and adults were exposed during 96h to amoxicillin and oxytetracycline following OECD guidelines. Tissues of adults and pools of embryos were used for catalase, glutathione-S-transferases and lactate dehydrogenase determinations. Amoxicillin caused premature hatching (48h-EC50 = 132.4 mg/l) whereas oxytetracycline cause delayed hatching of embryos (72h-EC50 = 127.6 mg/l). Moreover, both antibiotics inhibited catalase and induced glutathione-S-transferases in zebrafish adults. However, only oxytetracycline induced lactate dehydrogenase. Short-term effects of antibiotics were observed at high doses (mg/l) indicating that physiological impairment in fish populations is unlike to occur. However, effects of chronic exposures to low doses of ABs must be investigated.

Keywords: pharmaceuticals; antibiotics; hatching; catalase; glutathione-S-transferases; lactate dehydrogenase

1. Introduction

Antibiotics (ABs) are among the most extensively used pharmaceuticals worldwide. ABs are natural or synthetic drugs with the capacity to kill or inhibit the growth of micro-organisms (Serrano, 2005). ABs are mainly used in hospitals, agricultural fields, and livestock production facilities either to treat or prevent diseases (Bondad-Reantaso et al., 2012; Lipsitch et al., 2002; Nathan, 2004).

Among the different types of livestock systems, aquacultures have especially raised environmental concerns due to the heavy and diverse use of ABs and their potential impacts on downstream aquatic ecosystems (Rico et al., 2012). Amoxicillin (AMX) and oxytetracycline (OTC) are worldwide used in aquacultures. A survey performed to obtain baseline information on the status of chemical use in tilapia aquaculture in the Northeast of Thailand (Moon River, Ubon Ratchathani), in the scope of an international project (Asia-Link, 2010), indicated that ABs are 56.8 % of all chemicals used in cage aquaculture in this region. AMX is considered the most important penicillin-like AB applied in aquaculture (Längin et al., 2009) and it is used to treat systemic bacterial infections diseases such as streptococcosis, furunculosis and pasteurellosis. On the other hand OTC, which belongs to the tetracycline group, is used in the treatment of vibriosis, enteric redmouth and also furunculosis. AMX and OTC are typically administered orally, incorporated into pelleted feed (at doses of 40 - 80 mg kg^{-1} day^{-1} of AMX and 75 mg kg^{-1} $day^{-1}$of OTC) or applied directly in the water of tanks, earth ponds or cages (dose of OTC: 5 - 125 mg/l). Whatever the administration route, these compounds can reach the environment through the uneaten feed or through the faeces of treated organisms (Halling-Sørensen et al., 1998; Lalumera et al., 2004) potentially affecting non-target aquatic organisms (Treves-Brown, 2000).

In recent years, the occurrence of ABs in aquatic ecosystems (derived not only from aquacultures but also from wastewaters effluents, crop fields' runoff and terrestrial livestock manure) and their effects has received increasing attention by

the scientific community (Aarestrup, 2012; Andreozzi et al., 2004; Kümmerer, 2003; Sarmah et al., 2006). For instance, OTC and AMX are reported as pharmaceuticals of high usage and high potential risk for the aquatic environment in England and Korea (Jones et al., 2002; Lee at al. 2008). Since ABs are specifically designed to control micro-organisms they are not acutely toxic to fish (lethal effects would occur only at extremely high doses) (Halling-Sørensen et al., 2000; Park and Choi, 2008). However, effects at developmental and biochemical level can occur, modifying fish health and/or its capacity to respond to changes in the environment. ABs were found to interfere with the normal development of zebrafish causing developmental malformations such as hatching delay, curved body axis, pericardial edema, not inflated swim bladder and yolk sac edema (Anderson and Battle, 1967; Wang et al., 2013).

ABs are known to modulate metabolism via production of reactive oxygen molecules (ROS) eliciting oxidative damage to aquatic organisms including fish (e.g. zebrafish, tilapia, and catfish), invertebrates (e.g. black tiger shrimp) and algae (e.g. *Microcystis aeruginosa* and *Selenastrum capricornutum*) (Tu et al., 2008; Wang et al., 2013; Wang et al., 2009; Madureira et al., 2012; Yang et al., 2013; Zaki et al., 2011). Oxidative stress can be measured using several biomarkers enzymes, such as catalase (CAT) which acts against ROS namely by converting hydrogen peroxide in water and oxygen (Jemec et al., 2010; Li et al., 2010). In addition, by measuring the activity of glutathione S-transferases (GST), the ability of the organisms to biotransform toxicants and mitigate their potential to cause oxidative damage can be assessed. GSTs are an important family of enzymes acting in the phase II of the detoxification process, conjugating toxicants with glutathione to facilitate their excretion. Furthermore, lactate dehydrogenase (LDH), a key enzyme in the anaerobic pathway of energy production, has been suggested as good indicator of stress by antibiotic exposure in fish (Saravanan et al., 2011). Thus, the activity of the above-mentioned enzymes in fish can be a valuable tool for early effect detection of ABs, providing important information about their effects. The main goal of this study was to assess the sub-lethal effects

of AMX and OTC on the biomarkers CAT, LDH and GST using the zebrafish model. The activity of the biomarkers was measured in embryos and in different tissues of zebrafish adults after exposure to the ABs, whereby their responsiveness in different life stages and tissues were assessed. Moreover, effects in the embryonic development were also studied namely malformations, hatching and mortality.

2. Methods

2.1 Test chemicals and preparation of test solutions

Oxytetracycline hydrochloride (CAS: 2058-46-0) and amoxicillin trihydrated (CAS: 61336-70-7) from Sigma Aldrich were used in all the assays. For each assay, stock solutions were carefully prepared by dilution of ABs in water (see below). Stocks were kept refrigerated and protected from light; test solutions were prepared immediately before the beginning of the test by successive dilution of the stock.

2.2 Zebrafish assays

All the zebrafish eggs and adults used in the study were provided by the facilities established at the Department of Biology, University of Aveiro (Portugal). Organisms were maintained in carbon-filtered water, complemented with salt, “Instant Ocean Synthetic Sea Salt”, at 27.0 ± 1 °C and exposed to a photoperiod cycle of 14:10 h light: dark. Conductivity was kept at 750 ± 50 µS/cm, pH at 7.5 ± 0.5 and dissolved oxygen above 95 % saturation. This water was used in the preparation of the test solutions of all assays performed. The above-mentioned temperature and photoperiod conditions were constant in all assays performed. Adult fish were fed twice daily with a commercial artificial diet (ZM 400 Granular) and brine shrimps *nauplii*.

2.2.1 Embryo assay

The Fish Embryo Toxicity test complied with the OECD draft guideline (OECD, 2006). Zebrafish eggs were collected within 30 minutes after natural mating, rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation); unfertilized or injured eggs were discarded.

Test started with newly fertilized eggs, previously selected and exposed to concentrations of 0, 75, 100, 150, 300, 600 and 900 mg/l of OTC and 0, 75, 128, 221, 380, 654 and 1125 mg/l of AMX. Ten eggs per treatment were distributed in 24-wells microplates. Test was performed in triplicate. Tests were carried under low light intensity 32 ± 8.3 lux (mean ± standard deviation) and run for 96 hours. Embryos were daily observed under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation, Japan) using a magnification of 70 x for embryos and 40 x for hatched embryos. In the embryo phase, the following parameters were evaluated: egg coagulation, otolith formation, eye and body pigmentation, somite formation, tail circulation, detachment of the tail-bud from yolk sac, absorption of the yolk sac, alterations of the amniotic fluid and hatching. Subsequent to the hatching were observed and reported, mortality, edema, posture, spine deformities and undersized embryos.

After this assay, sub-lethal doses of both compounds were chosen and the exposure repeated with the aim of collecting material for biomarkers analysis. Concentrations chosen were: 0, 75, 100 and 150 mg/l for OTC and 0, 75, 128 and 221 mg/l for AMX. Six-wells microplates were used for the exposures and teen eggs were placed into each well with 10 ml of the test solution. Teen replicates were used for each AB. At the end of the tests (96 h), a minimum if 7 clusters of eight embryos for each treatment were snap-frozen in microtubes with 0.4 ml of K-phosphate buffer (100 mM, pH 7.4) and kept at - 80 ºC until enzymatic analysis.

2.2.2 Adult fish assay

Considering the low toxicity of the ABs to zebrafish embryos, mortality was not expected to adults at concentrations below 100 mg/L (the maximum exposure concentration recommended by the OECD guideline no 203 for Fish Acute Toxicity testing (OECD, 1992)). Thus, the assay with adults aimed to use sub-lethal doses of OTC and AMX to assess short-term effects on biomarkers activities in different tissues. The following nominal concentrations of AMX and OTC were used: 0, 1, 10, 25, 50 and 100 mg/l. Test was conducted under semi-static test conditions, using the same number of males and females with similar length and age (2 ± 1 cm, one year old). Per treatment, 12 fish were divided into three groups and exposed in aquariums with 2 l of test solution. Test lasted up to 96 h; behavioural changes and mortality were daily reported. The fish were not fed during this period. At the end of the test, the living fish were quickly sacrificed by decapitation. Twelve samples of head, muscle, liver and gills tissues, per treatment, for each antibiotic, were isolated and snap-frozen in microtubes with 1 ml of K-phosphate buffer, 100 mM, pH 7.4. Samples were stored at - 80 ºC until enzymatic analysis.

2.3 Biomarkers determinations

GST, CAT and LDH activities were determined in embryos and in the above-mentioned isolated tissues from adults. On the day of enzymatic analyses, samples were defrosted on ice, homogenised using a sonicator (KIKA Labortechnik U2005 Control^{TM}) and centrifuged for 20 minutes at 10000 g to separate the post-mitochondrial supernatant (PMS) (Howcroft et al., 2011).

CAT activity was measured at 240 nm by monitoring (for 2 minutes) the decrease of absorbance due to degradation of hydrogen peroxide (H_2O_2), as described by Clairborne (1985). Fifteen µl of PMS were diluted with 150 µl of K-phosphate buffer (50 mM, pH 7.0) and mixed with 135 µl of reaction solution (H_2O_2, 30 mM).

GST activity was determined at 340 nm by monitoring the increase in absorbance (for 5 minutes) following the general methodology described by Habig and Jakoby (1981) with modifications introduced by Frasco and Guilhermino (2002). Activity determinations were made using 100 µl of PMS of the sample and 200 µl of reaction mixture (10 mM reduced glutathione and 60 mM 1-chloro- 2.4-dinitrobenzene in K-phosphate buffer (100 mM, pH 6.5)).

LDH activity was measured at 340 nm, by continuously monitoring (for 5 minutes) the decrease in absorbance due to the oxidation of NADH, following the methodology described by Vassault et al. (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 activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration in samples was determined in quadruplicate by the Bradford Method (Bradford, 1976), at 595 nm, using γ-globulin as a standard.

2.4 Statistical analysis

Sigma Stat 3.1 statistical package (SPSS, 2004) was used for statistical analysis. Comparisons between different experimental exposure groups were performed with by a one-way ANOVA followed by the Dunnett post-hoc test. In the case, datasets did not pass the Levene's test and/or the Kolmogorov Smirnov normality test, a Kruskal-Wallis test was performed and the Dunn's post-hoc test was used to verify the differences between each tested concentrations and the control. The effective concentrations (EC_{50}) and lethal concentrations (LC_{50}) were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel. Biomarkers basal activities data sets were analysed by Tukey multiple range tests to assess the degrees of significance between the means of

enzymatic activities of different tissues, including embryos. All statistical analyses based on 0.05 significance level.

3. Results

AMX and OTC were not acutely toxic to zebrafish embryos or adults, with no significant mortality recorded below 100 mg/l. For embryos, test duration and concentrations were extended in order to allow the calculation of LC_{50} values but even though, this was only possible for OTC, for which a 96 h-LC_{50} of 330 ± 7.9 mg/l was calculated (**Fig 1**). For adults, effects on the behaviour were neither observed during the 96 h of exposure to the ABs.

Nonetheless, important developmental effects and biochemical effects on both life stages were found.

3.1 Biochemical effects

Table 2 summarizes enzymatic activities of samples (adult tissues or embryos) from organisms of the control treatment. These activities can be considered as the “basal activities” in the test conditions and allow the detection of differences among tissues sampled and the identification of the tissues with highest and lowest activities for each biomarker. Thus, CAT presented much higher activity in the liver compared to other tissues (Kruskal-Wallis, $H = 25.98$, $p < 0.001$), GST presented much lower activity in the muscle (Kruskal-Wallis, $H = 33.56$, $p < 0.001$) and LDH presented much lower activity in the liver (Kruskal-Wallis, $H = 29.31$, $p < 0.001$).

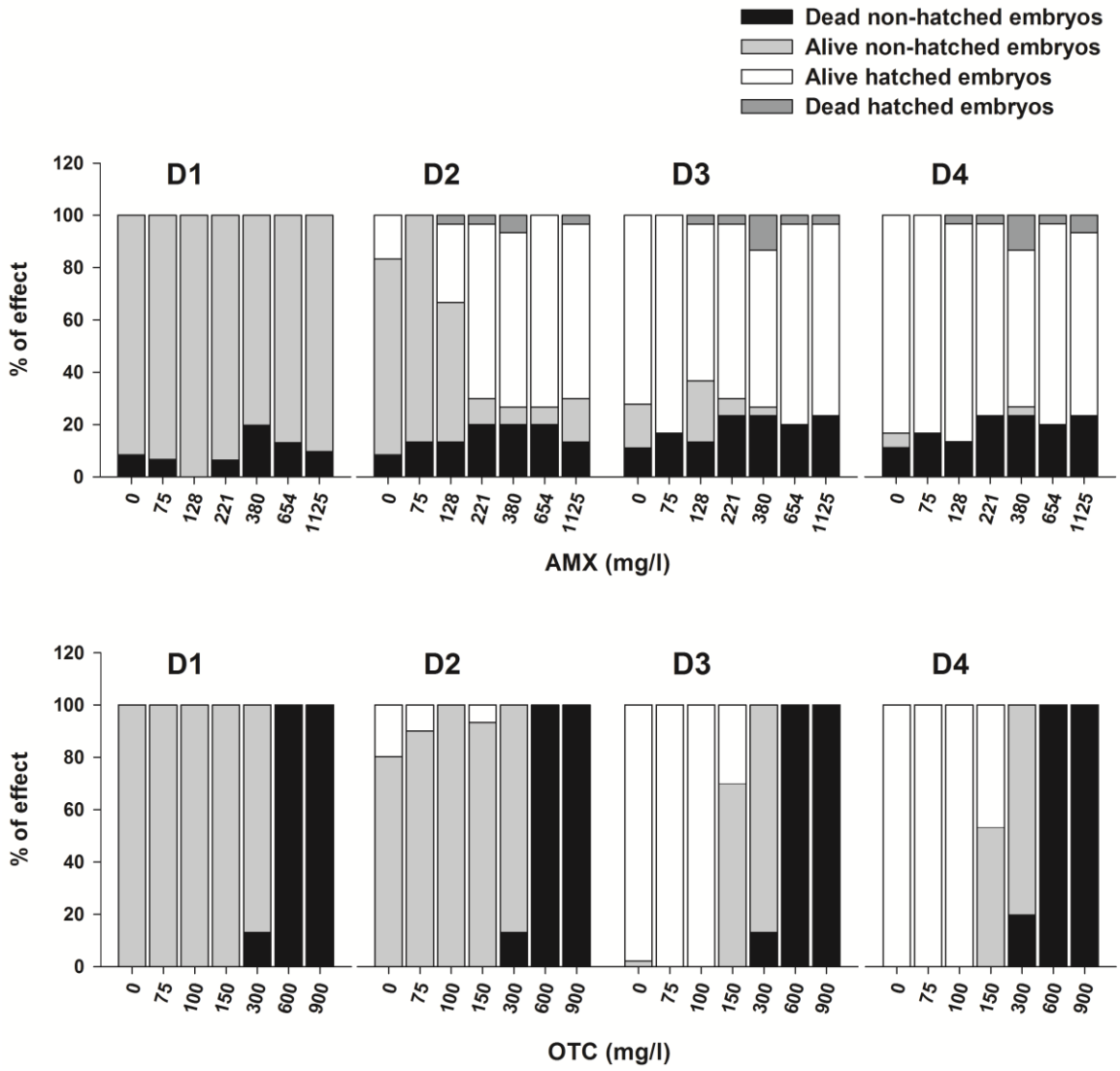


Fig 1 Overview of effects of AMX (above) and OTC (bellow) on zebrafish embryos. In this figure the proportion of eggs and non-hatched embryos that died are represented by black bars; the proportion of embryos that stayed alive but did not hatch are presented as grey bars; those that hatched as white bars and the proportion of embryos that died after hatch as spotted, dark grey bars.

Once the biomarkers activities can differ in more than one order of magnitude between the different tissues (**Table 1**), the biomarkers activities after exposure to ABs are presented as percentages of the control in other to allow a better comparison (**Fig 2 and 3**). Raw data are given in **Online Resource** 1.

Table 1 – Basal activities of biomarkers activity ($nmol^{-1}min^{-1}mg$ of protein) in different zebrafish samples (mean values ± standard deviation)

Samples	CAT	GST	LDH
Head	11.4 ± 8.8^{a}	25.9 ± 4.6^{a}	378.7 ± 78.5^{a}
Muscle	6.0 ± 4.0^{a}	4.1 ± 1.1^{b}	357.6 ± 30.8^{a}
Gill	7.0 ± 2.5^{a}	18.9 ± 4.4^{a}	325.0 ± 88.8^{a}
Liver	200.4 ± 93.2^{b}	23.4 ± 10.2^{a}	128.6 ± 74.0^{b}
Embryos	n.a.	14.7 ± 5.8^{a}	248.7 ± 104.9^{a}

Different letters means statically different groups ($p < 0.05$, Tukey multiple range tests); "n.a." means result not available

3.1.1 AMX exposure

In adults exposed to AMX, CAT activity was inhibited in gills and head samples at the highest AMX concentrations (Gill: Kruskal-Wallis, $H = 18.47$, $p = 0.002$; Head: Kruskal-Wallis, $H = 38.48$, $p < 0.001$) (**Fig 2 A)**. GST activity was induced in muscle samples (One-way ANOVA, $F_{5, 63} = 9.47$, $p < 0.001$) and presented an induction trend in gills as well (Kruskal-Wallis, $H = 15.34$, $p = 0.01$). Moreover, in head samples an inhibition trend was observed (Kruskal-Wallis, $H = 16.54$, $p = 0.01$) (**Fig 2 B)**. No changes in LDH activity were observed in any tissues (**Fig 2 C)**. Concerning embryos, no differences between the treatments were found in GST and LDH activities (**Fig 2 D and E)**.Unfortunately, due to loss of material no results can be presented for CAT activity in embryos.

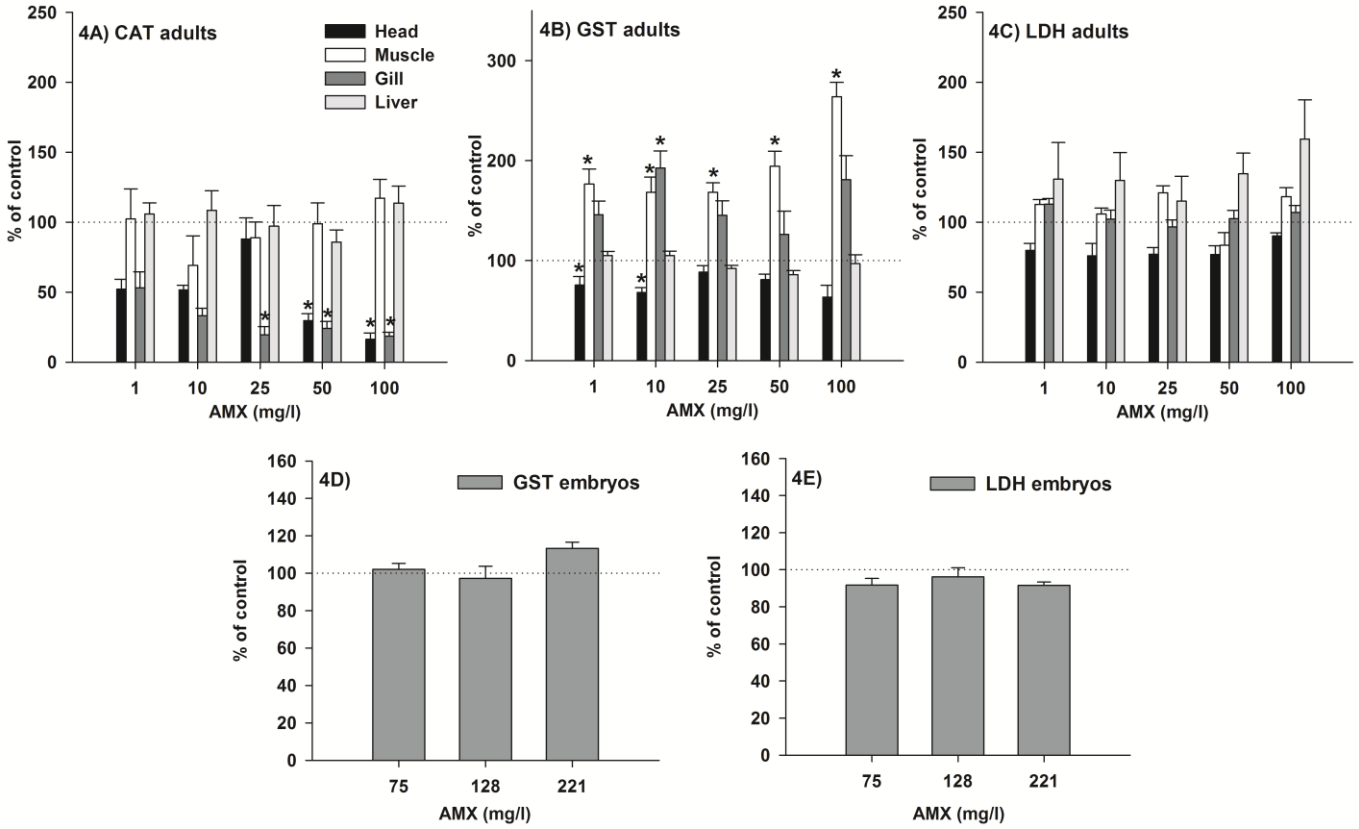


Fig 2 Effects of AMX on zebrafish embryos development after 48 h of exposure. The doted lines (mean values ± standard error) represent cumulative hatching of embryos for each concentration. The grey bars represent embryos that showed malformations, namely edema and tail deformities (mean values ± standard error).

3.1.2 OTC exposure

Zebrafish adults exposed to OTC exhibited a dose-dependent inhibition of CAT activity in head samples (Kruskal-Wallis, $H = 40.47$, $p < 0.001$), whereas no alteration was observed in the other tissues analysed (**Fig 3 A**). GST activities were increased at almost all concentrations above 1 mg/l in muscle and liver samples (Muscle: One-way ANOVA, $F_{5, 71} = 3.82$, $p = 0.004$; Liver: One-way ANOVA, $F_{5, 68} = 3.84$, $p = 0.004$, **Fig 3 B**). LDH activity presented an irregular response in different tissues. LDH activity was induced in muscle at 10 mg/l (One-way ANOVA, $F_{5, 71} = 5.46$, $p < 0.001$), inhibited in head at 50 mg/l (One-way ANOVA, $F_{5, 71} = 5.13$, $p = 0.013$), induced in liver at 10 and 50 mg/l (One-way ANOVA, $F_{5, 71} = 5.18$, $p < 0.001$) whereas no alterations were observed in gills (**Fig 3 C**). Embryos showed changes in enzymatic activities at the highest doses. GST presented an inhibition pattern begging inhibited at the two highest concentrations, 100 and 150 mg/l (One-way ANOVA, $F_{3, 39} = 13.72$, $p < 0.001$, **Fig 3 D**). LDH presented an induction pattern begging induced at 150 mg/l (Kruskal-Wallis, $H = 17.89$, $p < 0.001$, **Fig 3 E**).

3.2 Developmental effects

AMX exposure provoked premature hatching of embryos (48 h-EC_{50} of 132.4 ± 41.6 mg/l, **Fig 4**, **Table 2**). At 48 h only 18.2 % of the embryos hatched in the control group but in the AMX treatments from 221 to 1125 mg/l more than 80 % of the alive embryos had hatched (Kruskal-Wallis: $H = 10.76$, $p = 0.03$). Interestingly, in these same treatments higher occurrences of malformations were observed (**Fig 4**) whereas in the control, embryos presented a normal development as described by Kimmel *et al.* (1995) (**Fig 5 A**). The most frequent malformations due to AMX exposure were edemas and tail deformities (**Fig 5 B and C**).

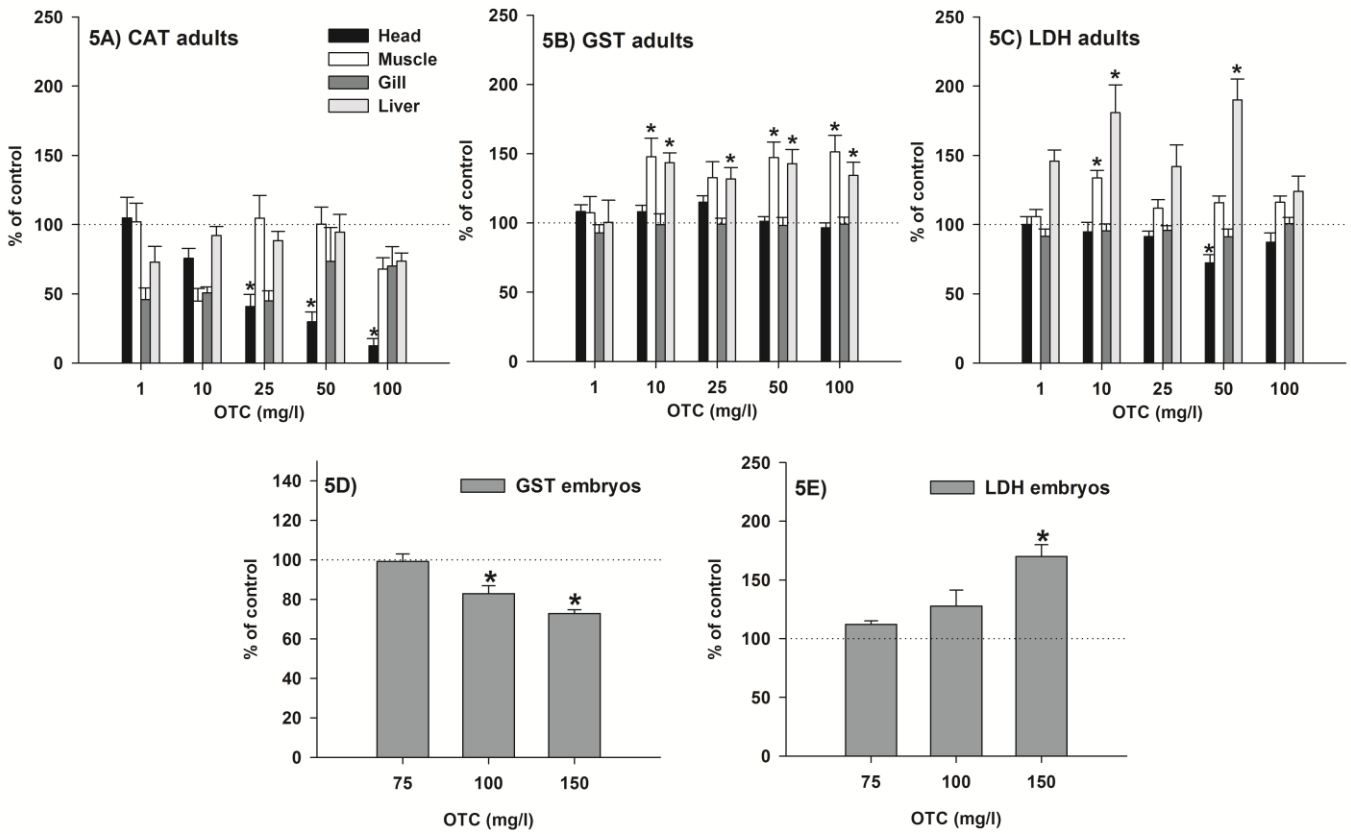


Fig 3 Variation of enzymatic activities after 96 h of exposure to OTC (mean values ± standard error) on embryos and different samples of zebrafish adults "*" means significantly different from the respective control treatment (Dunett's or Dunn's test, $p < 0.05$ after one-way ANOVA). The doted reference lines indicate the control level of each biomarker.

Contrarily to AMX, OTC did not cause any malformation in embryos. However, hatching inhibition was observed (72 h: Kruskal-Wallis: H = 13.98, p = 0.01; 96 h: H = 14.91 with, p = 0.01) (**Fig 1, Table 1**). An effective concentration of OTC at 72 h-EC_{50} of 127.6 ± 3.3 mg/l and at 96 h-EC_{50} of 139.5 ± 4.0 mg/l was estimated. At 72 h, more than 95 % of the embryos in the control, 75 and 100 mg/l treatments had hatched whereas only 30 % of the embryos hatched at 150 mg/l and none hatched at 300 mg/l of OTC. At 96 h the non-hatched embryos from 150 and 300 mg/l presented an opaque and rigid chorion (**Fig 5 E and F**) dying without being able to hatch after 144 h of exposure (data not shown).

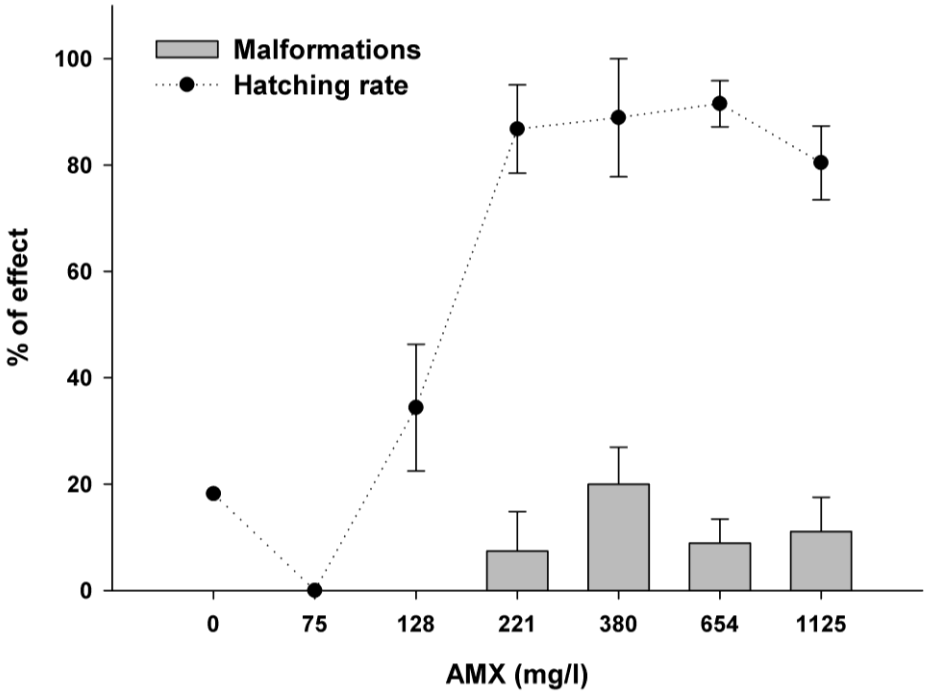


Fig 4 Effects of AMX on zebrafish embryos development after 48 h of exposure. The doted lines (mean values ± standard error) represent cumulative hatching of embryos for each concentration. The grey bars represent embryos that showed malformations, namely edema and tail deformities (mean values ± standard error)

4. Discussion

In the aquatic environment, few studies have reported the effects of AMX and OTC in fish, probably because they are not described as highly toxic in scientific literature (AMX 96 h-LC_{50}: 1000 mg/l and OTC 96 h-LC_{50}: 110.1 mg/l for *Oryzias latipes* according to Park and Choi (2008)). In our study, developmental and biomarkers analysis revealed several effects in zebrafish (embryos and adults) after short-term exposure to AMX and OTC.

The knowledge of basal activities of enzymatic biomarkers is important to assess the health and physiological condition of an organism. In this study, the basal activities obtained under control conditions (**Table 1**) agree with previous assays performed on zebrafish embryos and adults (Coelho et al., 2011). CAT presented higher activities in liver probably due to the elevated activities of hydrogen peroxide in this organ arising as by-product of the catabolism of fatty acids (Tocher, 2003). GSTs presented lower activity in muscle as this organ play a minor role in the detoxification processes within the organism. LDH presented lower activity in liver, which agrees with the results of El-Alfy et al. (2008). These authors investigated LDH activity and the different isozymes in three species of tilapia; the isozyme A4 homotetramer, displayed very high activity of activity and was predominant in skeletal muscle tissues, while heterotetramer of LDH was predominant in liver and presented low activity. Despite the absence of studies describing LDH isoforms in zebrafish, our study showed a low LDH activity in liver corroborating the results found in tilapia species. Altogether, the analysis of biomarkers either in specific tissues of adults or in pools of embryos (whole body), were able to detect effects of AMX and OTC after a short-term exposure.

CAT was highly responsive showing an inhibition pattern following both ABs exposures in adults. AMX strongly inhibited CAT in head and gills samples (**Fig 4 A**). No studies were found in current literature to compare with our results, thus, the inhibition of CAT by AMX remains not yet fully understood. At the highest

treatment of OTC, all tissues sampled also showed CAT activity below the control levels (**Fig 5 A**); particularly in head samples, a clear inhibition was noticed. Ours results agree with previous studies showing a significant inhibition of CAT and other oxidative stress enzymes, due to OTC exposure, in fish (superoxide dismutase, reduced glutathione) and also rats (Enis Yonar et al., 2011; Pari and Gnanasoundari, 2006). *In vitro* studies have shown that CAT activity decreases with increasing OTC concentrations due to molecular interactions or directly binding of OTC with CAT altering enzyme activity (Chi et al., 2010). However, the interaction mechanism between OTC and CAT is not still totally understood.

Table 2 – Percentage of hatched *D. rerio* embryos after AMX and OTC exposure

	Days of exposure				Days of exposure		
AMX (mg/l)	**D2**	**D3**	**D4**	**OTC (mg/l)**	**D2**	**D3**	**D4**
0	18.2 (0)	81.8 (13.9)	93.9 (6.1)	**0**	19.8 (5.3)	97.9 (2.4)	100 (0)
75	0 (0)	100 (0)	100 (0)	**75**	10 (5.8)	100 (0)	100 (0)
128	34.4 (11.9)	68.8 (21.1)	100 (0)	**100**	0 (0)*	100 (0)	100 (0)
221	86.8 (8.3)*	90.7 (4.9)	100 (0)	**150**	6.7 (3.3)	30 (0)	46.7 (3.3)
380	88.9 (11.1)*	93.3 (6.7)	93.3 (6.7)	**300**	0 (0)*	0 (0)	0 (0)*
654	91.5 (4.3)*	100 (0)	100 (0)	**600**	-	-	-
1125	80.4 (6.9)*	100 (0)	100 (0)	**900**	-	-	-

* Designate statically different groups ($p < 0.05$, Dunn's method); Standard deviations values between brackets

- all organisms were dead

One milligram per litre of AMX provoked an increase of GST in muscle and gills of zebrafish adults (**Fig 4B**) whereas no effects in embryos were observed at doses as high as 221 mg/l. The difference of response between the zebrafish life stages might occur due to different metabolism between embryos and adults and/or impossibility of analysing enzymatic activities on embryo specific tissues (dilution of enzyme of interest in the total amount of proteins). The induction in

adults was expected since the role of GST on the conjugation of reduced glutathione with ABs containing electrophilic centres is already described (Park 2012; Park and Choung, 2007). In OTC exposed organisms, an increase of GST activity was found (liver and muscle) also indicating an increase in the detoxification process at concentrations above 10 mg/l (**Fig 5B**). Once again in embryos, GST was not increased as expected; only an inhibition of the enzyme at 100 and 150 mg/l of OTC was observed (**Fig 5D**). An exposure to lower doses could elucidate if the typical pattern of response of this enzyme (bell shaped curve) is present. This phenomenon is already described for fish exposed to different types of contaminants, e.g. metals (Domingues et al., 2010) and might indicate high levels of stress and a collapse of GST enzymatic pathway.

LDH changed after OTC exposure in embryos and adults (liver) (**Fig 5 C and E**). LDH is a glycolytic enzyme that catalyzes the inter-conversion of pyruvate and lactate, respectively using the cofactors, NADH and NAD+ (De Almeida‐Val et al., 2005). It is possible that a metabolic hypoxia due to detoxification process increases the anaerobic pathways causing the LDH changes. Effects of OTC in LDH has been previously reported to Indian Major Carp (*Labeo rohita*) exposed to OTC during 25 days. A pattern of inhibition during the fist 5 days of exposure followed by an induction until the end of the test was observed in different tissues, namely muscle, liver and gills (Ambili et al., 2012). The inhibition trend in head samples and the induction trend in muscle and liver tissues observed in adults would have to be clarified with further studies with further studies. Interestingly, these trends agree in both ABs. Although others studies have successfully applied LDH as a sensitive enzymatic biomarker of chemical stress in zebrafish embryos and adults (Coelho et al., 2011; Sancho et al., 2009) in this study no conclusive results were obtained.

Fish embryo testing has been recommended as a tool to assess the acute toxicity of chemicals, including kanamycin, chloramphenicol and β-diketone antibiotics (Lammer et al., 2009; Song et al., 2010; Wang et al., 2013). Sub-lethal

parameters have been successfully used, detecting chemicals effects at very low doses, however in this study changes in the development and biomarkers of embryos only occurred at much higher concentrations than in adults. In the development, the most remarkable effect for both ABs was the alteration of hatching rates. The different responses might be related by the different mode of action of AMX and OTC which ones belongs to distinct ABs groups β-lactamic and tetracycline, respectively. Inhibition hatching was already described to kanamycin and a mixture of β-diketone antibiotics (Song et al., 2010; Wang et al., 2013). Different toxic mechanisms could justify hatching enhance or failure such as the induction of abnormal function of the enzyme chorionase and/or inability of the emerging larvae to break the egg shell (Yamagami, 1981).

In adults, the analyses of different tissues revealed to be very useful in the assessment of sub-lethal effects because different responses of enzymes were detected according to the tissue sampled. In some cases not all tissues were responsive (e.g. only CAT measured in head responded to OTC exposure) and in other cases biomarkers in different tissues have opposite behaviours (e.g. after AMX exposure, GST activity in muscle increased but showed an inhibition trend head samples). These differences would not be discriminated if whole body homogenates were used. Moreover, tissues with highest basal activities are not necessarily the most responsive (e.g. GST activity in muscle was very responsive towards the testing compounds in spite of its low basal activity).

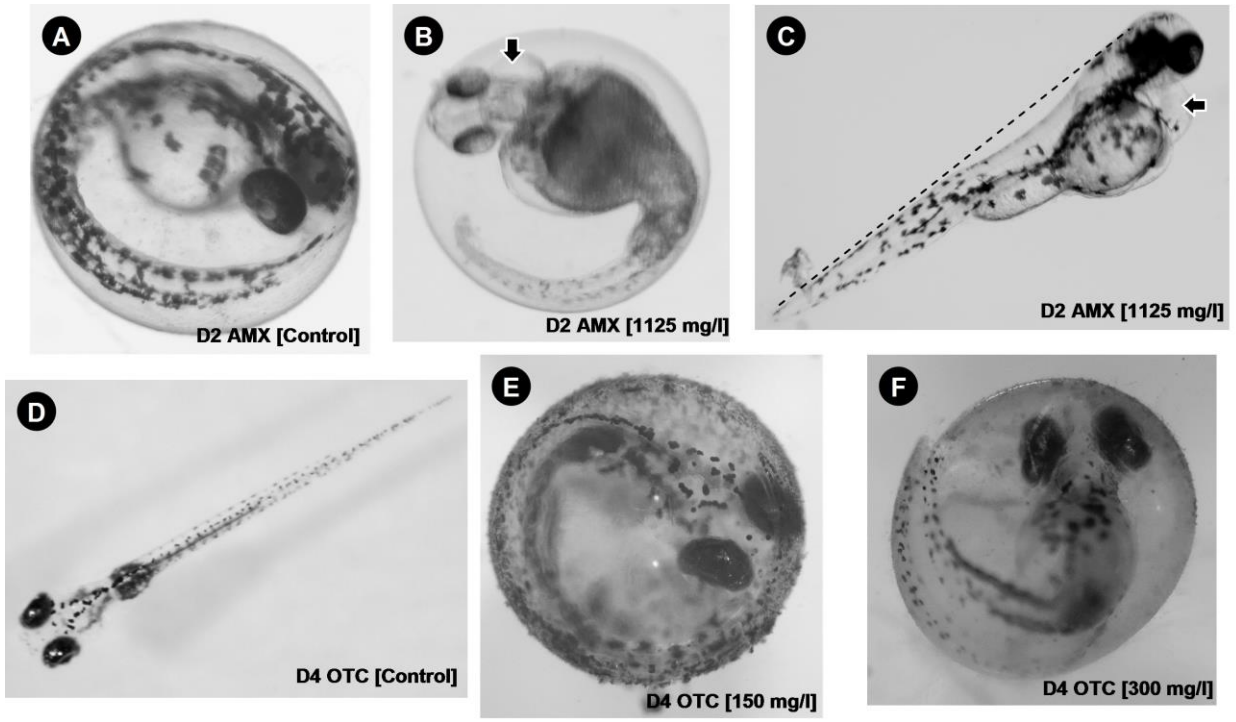


Fig 3 Zebrafish embryos malformations after AMX and OTC exposure: (A) control organism after 48 h; (B) embryo 48 h of exposed to 1125 mg/l of AMX with edema (black arrow); (C) Hatched embryos 48 h exposed to 1125 mg/l of AMX with edema (black arrow) and spine deformity (doted line); (D) Normal hatched embryo from control group after 96 h; (E) not hatched embryo exposed to 150 mg/l of OTC; (F) not hatched embryo exposed to 300 mg/l of OTC.

Measured environmental concentrations (MEC) of pharmaceuticals/antibiotics in surface waters typically range from nanograms to micrograms per litre, suggesting that aquatic organisms are exposed to low concentrations of ABs during their whole lifecycle (Andreozzi et al., 2004; Ji et al., 2012; Lalumera et al., 2004; Locatelli et al., 2011; Murata et al., 2011; Ok et al., 2011). For instance, Park and Choi (2008) reported MEC of AMX and OTC in surface water of 6 and 340 ng/l, respectively. Our study shows that exposure to OTC and AMX for 96 h leads to oxidative stress and hatching rate alterations in zebrafish only at much higher doses suggesting that physical and physiological impairment of natural fish populations are unlike to occur. However, it is important to note that the reported MEC refer to single ABs concentrations, but the combined concentrations of ABs can be higher than the reported levels. Thus, the potential of ABs to impair fitness and resilience of fish living in long-term impacted aquatic environments deserve further investigation.

5. Conclusions

The short-term zebrafish exposure to AMX and OTC affected enzymatic activity in embryos and adults. Analysis of biomarkers in different tissues of zebrafish adults discriminated effects that could not be detected in embryos. Both ABs showed a general trend of CAT inhibition and GST induction in adults. In embryos no response was observed under exposure to AMX, whereas a GST inhibition and LDH induction was verified under exposure to OTC. These alterations may have an ecological cost for fish that are exposed to environmental contamination. Moreover, studies on the effects of ABs at low concentrations, which focus on the long-term effects and applying supplementary methods such as chronic toxicity tests and mixture exposures is the next step for a better understanding of ABs environmental risks.

Acknowledgements

Authors acknowledge *Fundação para Ciência e Tecnologia* (FCT - Portugal) by the financial support through the grants SFRH/BPD/31752/2006 (Inês Domingues), SFRH/BPD/62561/2009 (Jessica C.L. Ladewig) and SFRH/BD/62605/2009 (Rhaul Oliveira) and University of Aveiro.

Ethical standards

The procedures described in the present paper followed the Portuguese law for animal experiments and the University of Aveiro Animal Welfare Committee - CREBEA guidelines for ethical principles for animal welfare.

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Supporting Information

Table 1 – Biomarkers levels (nmol^{-1}min^{-1}mg of protein) in different adult *D. rerio* samples after AMX and OTC exposure (mean values ± standard deviation)

	AMX (mg/l)					OTC (mg/l)				
	1	**10**	**25**	**50**	**100**	**1**	**10**	**25**	**50**	**100**
CAT										
Gill	7.1 (5.1)	4.4 (2.5)	2.6 (1.9)*	3.2 (2)*	2.5 (1.2)*	5.2 (3.3)	5.8 (1.7)	5.1 (2.8)	5.7 (3.1)	5.8 (1.5)
Head	3.4 (1.6)	3.6 (0.8)	6.1 (2.6)	2.1 (1.2)*	1.1 (0.8)*	7.4 (3.6)	5.3 (1.7)	3.5 (1.6)*	1.7 (1.1)*	0.8 (0.7)*
Liver	211.1 (53.4)	216.5 (92.3)	213.3 (60.7)	183.2 (45.8)	226.9 (76)	146.2 (78.4)	184.5 (46)	184.7 (40.1)	189.7 (88.1)	154.6 (34.3)
Muscle	6.2 (4.5)	4.2 (1.8)	5.4 (1.6)	6 (3.1)	7.1 (2.5)	6.2 (2.8)	3.5 (1.5)	6.3 (3.4)	6.1 (2.6)	4.1 (1.7)
GST										
Gill	27.5 (8.6)	38.3 (9.3)*	26.5 (9)	26.2 (13.4)	32.6 (9.6)	17.5 (3.8)	18.6 (5.2)	18.7 (2.8)	18.5 (3.9)	18.7 (3.2)
Head	19.6 (7.7)*	17.7 (4.2)*	19.7 (4.4)	23.1 (6.2)	19.8 (5.5)	28.1 (4.5)	28.1 (4.2)	29.8 (4.3)	26.3 (2.9)	25 (3.3)
Liver	24.6 (3.5)	24.6 (3.4)	21.1 (3)	20.8 (2.3)	21.5 (4.3)	25.6 (11.1)	33.6 (5.7)*	30.8 (6.6)*	35.3 (5.4)*	31.4 (7.7)*
Muscle	7.3 (2.2)*	7 (2.2)*	6.5 (0.6)*	7.5 (2)*	9.5 (2)*	4.4 (1.7)	6.1 (1.9)*	5.5 (1.7)	6.1 (1.6)*	6.3 (1.7)*
LDH										
Gill	366.8 (46.2)	332.2 (71.7)	338.3 (35)	326.9 (61.6)	321.1 (65)	297.7 (58.4)	310.5 (55.8)	311.7 (7.7)	296.6 (62.7)	327.6 (48.5)
Head	302.6 (64.7)	287.6 (115.9)	313.1 (53.9)	279 (69.3)	329.7 (58.4)	379.2 (73)	359 (89)	346.2 (49.8)	273.9 (78)*	330.2 (87.7)
Liver	198.3 (54.3)	166.9 (88.6)	172.1 (85.9)	137.8 (56.6)	198.8 (74)	187.5 (36.1)	232.6 (89)*	182.6 (69.1)	244.3 (67.7)*	159.6 (48.7)
Muscle	402.9 (43.6)	378.3 (52.3)	431.7 (50.6)	396.7 (93.4)	312 (119.4)	377.7 (65.5)	477.9 (67.5)*	399.8 (75.7)	413.7 (61.6)	415.1 (55.2)

* Designate statically different groups ($p < 0.05$, Dunn's method); Standard deviations values between brackets

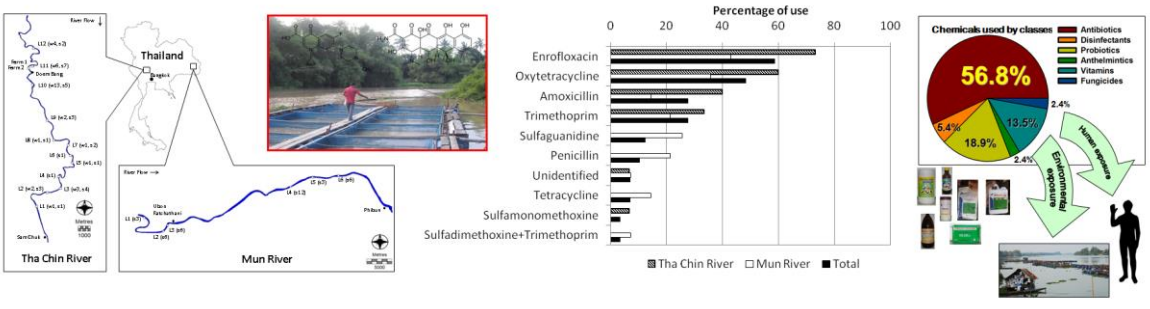
Table 2 – Biomarkers levels ($nmol^{-1}min^{-1}mg$ of protein) in *D. rerio* embryos samples after AMX and OTC exposure (mean values ± standard deviation)

	AMX (mg/l)			**OTC (mg/l)**		
	75	**125**	**221**	**75**	**100**	**150**
GST	11.2 (1.1)	10.7 (2.2)	12.4 (1)	22 (2.7)	18.3* (2.8)	16.1* (1.4)
LDH	167.9 (18.7)	167.9 (26.1)	160 (8.2)	347.4 (29.5)	396 (134)	527* (97.2)

* Designate statically different groups ($p < 0.05$, Dunn's method); Standard deviations values between brackets

Chapter 9

Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand



Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand

Andreu Rico; Rhaul Oliveira; Sakchai McDonough; Arrienne Matser; Jidapa Khatikarn; Kriengkrai Satapornvanit; António J.A. Nogueira; Amadeu M.V.M. Soares; Inês Domingues; Paul J. Van den Brink

This chapter has been submitted as an original article in:
Environmental Pollution

Abstract

The use, environmental fate and ecological risks of antibiotics applied in tilapia cage farming were investigated in the Tha Chin and Mun rivers in Thailand. Information on antibiotic use was collected through interviewing 29 farmers, and the concentrations of the most commonly used antibiotics, oxytetracycline (OTC) and enrofloxacin (ENR), were monitored in river water and sediment samples. Moreover, we assessed the toxicity of OTC and ENR on tropical freshwater invertebrates and performed a risk assessment for aquatic ecosystems. All interviewed tilapia farmers reported to routinely use antibiotics. Peak water concentrations for OTC and ENR were 49 and 1.6 µg/L, respectively. Antibiotics were most frequently detected in sediments with concentrations up to 6,908 µg/kg d.w. for OTC, and 2,339 µg/kg d.w. for ENR. The results of this study indicate insignificant short-term risks for primary producers and invertebrates, but suggest that the studied aquaculture farms constitute an important source of antibiotic pollution.

Capsule Abstract

Antibiotics applied in tilapia cage farming in Thailand are released into surrounding aquatic ecosystems and constitute an important source of antibiotic pollution.

Keywords: antibiotics; ecological risk assessment; tilapia; aquaculture; Thailand

1. Introduction

Aquaculture production has intensified at a rapid pace across Asian countries in order to supply the increasing demand of aquatic products at a national level and in importing regions such as European or North-America (FAO, 2012). As long as aquaculture practices have intensified and the quality of water supplies in aquaculture-clustered areas has deteriorated, the Asian aquaculture industry has been overwhelmed with a wide range of parasitic and bacterial diseases affecting the cultured species (Bondad-Reantaso et al., 2005). In order to prevent or treat such disease outbreaks, farmers often rely on a wide array of veterinary medicinal products such as antibiotics and parasiticides, which are mainly applied during periods of high stress in the cultured species (Rico et al., 2012; Rico et al., Accepted). Residual concentrations of antibiotics used in aquaculture production have been measured in aquatic ecosystems down-stream of aquaculture production areas of Asia (Managaki et al., 2007; Zou et al., 2011; Zhou et al., 2011; Takasu et al., 2011; Shimizu et al., 2013), and due to the importance and the geographical spread of this economic activity throughout this continent, aquaculture production has been considered as one of the main pathways of veterinary medicines into the environment (Managaki et al., 2007).

Thailand is ranked sixth in aquaculture production globally, with tilapias (*Tilapia* spp.) being the most important cultured fish species group (FAO, 2012). About 30% of Thai tilapias are produced at high densities in floating cages placed on rivers or irrigation canals (Belton et al., 2009). Tilapias cultured under such open culturing systems are highly vulnerable to stress produced by water quality fluctuations and can easily be infected by naturally occurring parasites. Particularly, infestations with *Streptococcus* spp. and other bacteria (e.g. *Aeromonas* spp.*, Pseudomonas* spp.*,* and *Vibro* spp.) have been reported to be the main causes of mortality in caged tilapia farming (Belton et al., 2009). In order to prevent mass tilapia mortalities, farmers often apply antibiotics mixed with the fish diet. Large amounts of antibiotics applied in marine cage-based aquaculture production have been reported to end-up in the surrounding ecosystems through leaching or sedimentation of medicated feeds, or via excretion from the cultured

species (Coyne et al., 1994; Capone et al., 1996). Similar situations are expected to occur in freshwater aquaculture, however, studies that report the environmental fate and distribution of antibiotics in rivers impacted by freshwater cage aquaculture are currently unavailable.

The main objective of this study was to investigate the use of antibiotics in tilapia cage farms in Thailand and to assess their environmental fate and risks for tropical aquatic ecosystems. Initially, we performed interviews with tilapia-cage farmers at two Thai rivers with significant aquaculture production. Then, we monitored residues of the most commonly used antibiotics, oxytetracycline (OTC) and enrofloxacin (ENR), in water and sediment samples collected in the environment surrounding the surveyed tilapia cage farms and in a 'non-polluted' reference area, and measured antibiotic concentrations in samples collected during and after antibiotic administration in two reference farms. In order to characterize the ecosystem sensitivity to antibiotics we performed toxicity tests with tropical invertebrates and derived safe environmental concentrations for primary producers and invertebrates. Finally, ecological risks for primary producer and invertebrate communities exposed to antibiotic residues were calculated based on the obtained measured environmental concentrations. To our knowledge, this is the first study describing the use and potential ecological risks of antibiotics applied in freshwater cage aquaculture production.

2. Material and methods

2.1 Study areas and antibiotic use data collection

This study was conducted in the Tha Chin River and in the Mun River (Fig. 1; see Supporting Information for a description of the study areas). Both rivers are subject to monsoon climate, with the rainy season lasting from May to October. The Tha Chin and the Mun rivers significantly contribute to the total cage-based production volume of tilapia in Thailand. In these rivers, mono-sex Nile (*Oreochromis niloticus*) or (hybrid) red tilapias (mainly *O. mossambicus* x *O. aureus*) are cultured in 3x3 m (1.5-2 m depth) floating cages composed of steel

frames and polypropylene mesh. Tilapias are fed with commercial pelleted feeds for a period of 4 months, until they reach a weight of 600-1000 g. Farms are formed by several tilapia cages (4-100) placed in parallel to the banks of the river, which normally operate in batches throughout the year.

Information on antibiotic use was collected by structured interviews conducted with 29 tilapia farmers (15 in the Tha Chin River and 14 in the Mun River) between November, 2010, and April, 2011. Information collected included names of antibiotic ingredients, dosages, and modes and frequencies of application. Additional information on farmer perceptions on water quality and disease occurrence was also collected.

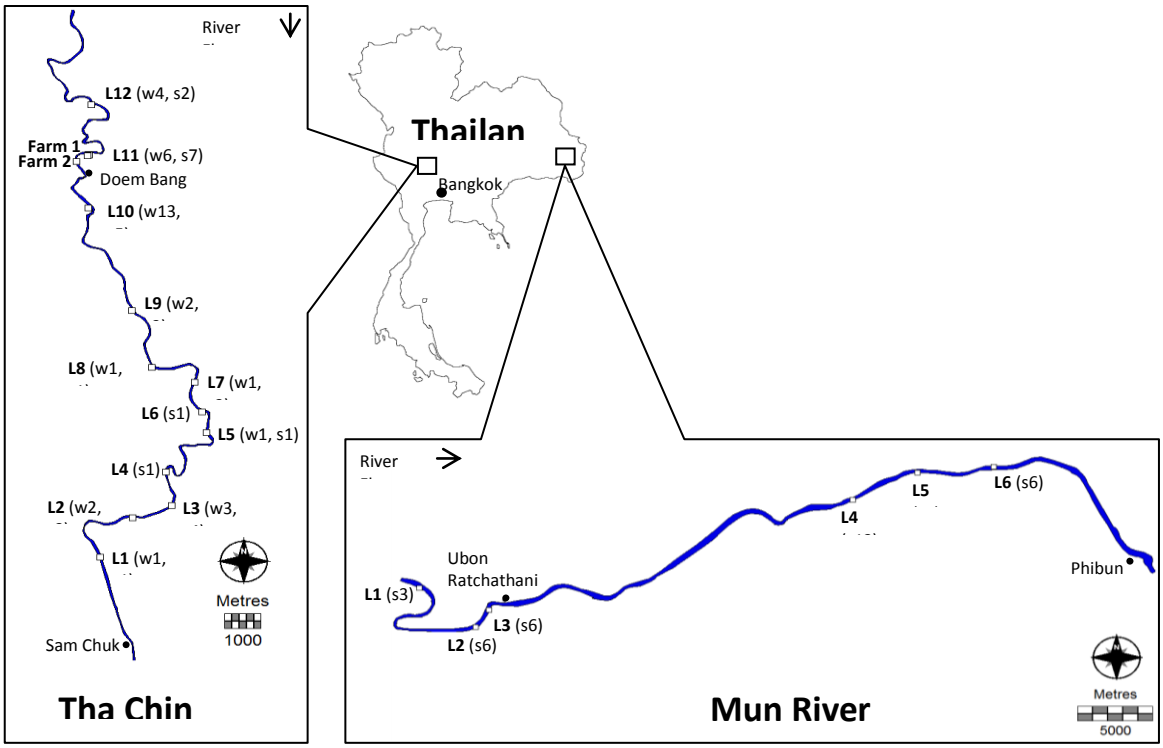


Fig 1 Sampling locations (L) in the Tha Chin River and in the Mun River (Thailand), and number of water (w) and sediment (s) samples collected in each location. In Tha Chin River: L1,2,3 and 4 were sampled in the dry season; L5,6,8 and 9 were sampled in the wet season; and L7,10,11 and 12 were sampled in both seasons. The location L3 in the Mun River was considered as the reference site.

2.2 Sample collection

Water samples were collected in the Tha Chin River during the dry season (March; n=24) and in the wet season (June; n=10) (Fig. 1). Sediment samples were collected during the dry season in the Tha Chin River (March; n=19) and in the Mun River (January-February; n=30), and during the wet season in the Tha Chin River (June; n=12) (Fig. 1). In addition, six reference sediment samples were collected from a location in the Mun River isolated from the aquaculture farms. This area was assumed not to be impacted by anthropogenic activities and was considered as the 'non-polluted' reference site (Fig. 1). For a description of the antibiotic sample collection methods see the Supporting Information.

In order to assess the fate and dissipation of the studied antibiotics during and after an antibiotic administration period, extra samples were collected in two tilapia farms located in the Tha Chin River. In the first farm (FARM 1), OTC was administered mixed with feed at a dose of 40 mg/kg fish body weight (b.w.). Six cages containing 600 fish per cage (approximate weight 300 g/fish) were treated. Water samples were taken in duplicate inside the cages and next to the cages at 15 min, 1h and 15h after the antibiotic administration. In the second farm (FARM 2), OTC and ENR treatments were applied to 14 tilapia cages with 600 fish per cage (approximate weight 600-700 g/fish). Both antibiotic treatments had a duration of 7 days and overlapped in time (Fig. S1). Antibiotics were applied once a day at a dose of 14 and 8 mg/kg b.w. for OTC and ENR, respectively. Sediment samples were collected on day 6, 8, 10, 13 and 18 after the first OTC application. Water and sediment samples were collected in the afternoon (15-30 min after antibiotic administration) on day 1, 3, 5, 8, 13 and 25 after the first ENR administration. Samples were collected next to the tilapia cages, and at 30 m and 60 m downstream the cages (see Fig. S1 for a synopsis).

2.3 Toxicity experiments

The acute toxicity of oxytetracycline (OTC) and enrofloxacin (ENR), was assessed on five tropical freshwater invertebrate species: a worm (*Limnodrilus hoffmeisteri)*, two molluscs (the snails *Physella acuta* and *Melanoides tuberculata*), an insect (*Micronecta* sp.), and a crustacean (i.e., the shrimp *Macrobrachium lanchesteri*). The toxicity of OTC and ENR to invertebrates was assessed by

means of static laboratory toxicity tests with a duration of 48 h. Detailed characteristics of the test organisms and experimental set-up are provided in Table 1. Test operating procedures were based on the OECD standard protocol for toxicity testing with *Daphnia magna* (OECD, 2004), however the temperature and the photoperiod (12 h of natural light) were adapted to match the environmental conditions of tropical ecosystems (Table 1).

The toxicity tests were performed with the commercial products OXYBAC 50 (oxytetracycline-HCl 50%) and EnFlocin (enrofloxacin-HCl 20%), which were typically used by tilapia farmers in the study area. Since tetracycline antibiotics have been reported to attach strongly to glass (Ciarlone et al., 1990), the test vessels used for the OTC toxicity experiments were previously rinsed with a solution of Na-EDTA in methanol (0.2% v/v). In order to assess the potential effects of rinsing the glass vessels with Na-EDTA solution, controls with rinsed and non-rinsed glass vessels were used in the OTC experiments.

In order to verify the antibiotic exposure concentrations, a sample of test media (10 mL) was taken from one replicate per treatment level at 1h and 48h after the start of the experiments. The effects of the antibiotics on the evaluated endpoints were recorded 24h and 48h after the start of the experiment. The studied endpoints were immobility, for snails and worms, and mortality, for the arthropod species (Table 1). Animals were considered immobile when no movement was observed after repeated (three times) tactile stimulation with a laboratory needle. The tests were accepted only when immobility or mortality in the controls did not exceed 20%. Finally, the Effect or Lethal Concentration for the 10% and 50% of organisms (EC10/50 or LC10/50) after an exposure period of 48h, and their 95% confidence interval (CI), were calculated. Calculations were performed with the ToxRat Professional Version 2.07 program by fitting the data to a log concentration – probit regression model. The EC and LCs were calculated with the 48h-averaged measured antibiotic concentrations.

Table 1. Characteristics of the tested organisms, experimental set-up, measured temperature (T), dissolved oxygen (DO) and pH in the test media, and endpoints evaluated in the toxicity tests (mean±SD).

Species	Individual's characteristics			Experimental set-up				Oxytetracycline			Enrofloxacin			Endpoint
	Length[a] (mm)	Weight[a] (mg)	Origin[b]	Water volume (mL)	Replicates (*n*)	Number per replicate	Aeration[c]	T (°C)	DO (mg/L)	pH	T (°C)	DO (mg/L)	pH	Immobility/ Mortality
Limnodrilus hoffmeisteri	26±5.5	NM	A	250	3	10	Yes	28.8±0.7	6.1±0.2	7.4±0.6	28.1±0.7	7.2±0.2	7.6±0.6	Immobility
Macrobrachium lanchesteri	31±4.3	329±145	A	500	6	5	Yes	28.7±0.4	5.8±0.4	7.3±0.5	28.1±0.4	5.4±0.4	7.3±0.5	Mortality
Melanoides tuberculata	17±0.7	356±67	B	250	3	10	Yes	27.6±0.7	5.8±0.2	7.2±0.6	27.5±0.7	5.5±0.2	6.5±0.6	Immobility
Micronectinae sp.	2.1±0.4	NM	B	250	3	10	No	28.7±0.7	NM	6.9±0.6	29.4±0.7	NM	7.5±0.6	Mortality
Physella acuta	7.5±1.2	63±21	B	250	3	10	Yes	27.4±0.7	5.8±0.2	7.0±0.6	27.3±0.7	5.6±0.2	7.2±0.6	Immobility

NM: not measured.

[a] *n* = 10

[b] A: purchased from fish retailers; B: collected from unpolluted freshwater tanks located in the Ornamental Fish Facilities of Kasetsart University (Bangkok, Thailand)

[c] The aeration system consisted of an air pump with plastic tubes connected to glass pipettes bubbling air into the test media. Since *Micronectinae sp.* are air breathers, aeration was not installed in these tests and dissolved oxygen levels in the test media were not measured.

2.4 Antibiotic analysis

The methods used for the extraction and analysis of the antibiotics in the water and sediment samples are described in the Supporting Information.

2.5 Ecological risk assessment

Ecological risks of OTC and ENR were calculated for the water layer and for the sediment. Ecological risks for the water layer were based on the measured antibiotic concentrations. The ecological risks for sediment were calculated by converting the measured sediment concentrations into their corresponding pore water concentration ($C_{pore\ water}$) according to the following equation:

$$C_{pore\ water}\ (\mu g/L) = \frac{C_{sediment}}{K_{oc} \cdot f_{om}}$$

where $C_{sediment}$ is the measured concentration in sediment, K_{oc} is the organic carbon partitioning coefficient, and f_{om} is the fraction of organic matter in the sediment. The K_{oc} values for OTC (26,134 L/kg) and ENR (186,342 L/kg), were obtained from Jones et al. (2005) and Nowara et al. (1997), respectively, and were selected based on experimental data from soils with similar characteristics (i.e., clay content, organic matter content) as the sediments we sampled. The f_{om} values were based on the mean measured value in each monitoring campaign (Table S1).

The ecological risk assessment was performed by following a Risk Quotient (RQs) approach and by using Species Sensitivity Distributions (SSDs). RQs were calculated by dividing the maximum measured water and pore water concentrations by Predicted No Effect Concentrations (PNECs) for primary producers and invertebrates. PNECs were derived by using toxicity data for standard test species and assessment factors as proposed in the international guidelines for the risk assessment of veterinary medicines (VICH, 2004). SSDs were separately built for primary producers and invertebrates (for rationale see Posthuma et al., 2002). The datasets used to build the SSDs were a combination of the toxicity data generated in the current study and toxicity values (EC50 and LC50) collected from the literature (see Table S2). SSDs were built by fitting the toxicity data to a log-normal distribution using the ETX 2.0 software (Van

Vlaardingen et al., 2004). The fit of the toxicity datasets to the log-normal distribution was assessed by the Anderson-Darling goodness of fit test (p = 0.05). The median Hazardous Concentration for the 5 and 50% of species (HC5 and HC50, respectively) and their 95% confidence interval (CI) were calculated according to Aldenberg and Jaworska (2000). In order to compare the primary producers and invertebrate sensitivities to the studied antibiotics, the significant differences between the distributions of toxicity data were evaluated by the two-sample Kolmogorov-Smirnov test (p = 0.05) using the GenStat 15th Edition software (VSN International Ltd, Hemel Hempstead, UK). For each SSD, the Potentially Affected Fraction (PAF) of species was conservatively calculated based on the maximum measured antibiotic concentration in the water and the estimated maximum sediment pore water concentration.

3. Results

3.1 Antibiotic use

All the interviewed tilapia farmers reported to apply antibiotics. Ten different antibiotic ingredients were identified (Fig. 2), belonging to 5 different classes (β-lactams, quinolones, sulfonamides, tetracyclines, diaminopyrimidines). Overall, the most commonly used antibiotics were enrofloxacin (59% of the interviewed farmers declared to use it), followed by oxytetracycline (48%), amoxicillin (28%), and sulfadiazine potentiated with trimethoprim (28%). On average, farmers reported to use two different antibiotics per farm, however, some farmers (24%) applied between 4 and 7 different antibiotic ingredients. Antibiotic use patterns of the interviewed farmers in the Tha Chin River and in the Mun River were found to be very similar. In all cases antibiotics were reported to be applied mixed with feed (once or twice a day) for a period ranging between 3-10 days or longer until tilapia mortalities decreased. Dosages reported in product labels ranged between 10-100 mg/kg b.w. per day, depending on the active ingredient. Farmers reported to use antibiotics to treat disease outbreaks between 1 and 3 times per production cycle (usually 4 months). In addition, about 70% of the interviewed farmers reported to apply antibiotics routinely to prevent diseases associated to the stress generated during the stocking of fingerlings. Changes in river water quality were reported to

be associated to fish disease, particularly dissolved oxygen drops during the dry season and heavy water runoff events occurring during the beginning of the rainy season. Farmers also reported that high loads of organic material and traces of pesticides from the surrounding agricultural fields (mainly rice crops) could be the cause of the mortalities observed after runoff events. Besides antibiotics, farmers also reported the use of parasiticides (e.g. trifluralin, praziquantel), salts (e.g. sodium chloride, potassium permanganate), probiotic enzymes and yeasts, and other feed additives such as vitamin C, proteins, and polysaccharides.

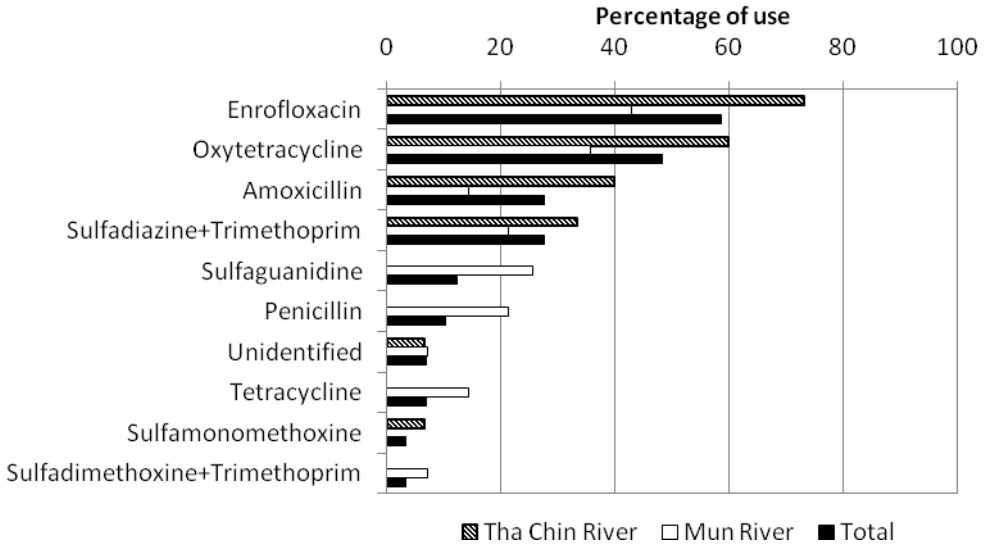


Fig 2 List of reported antibiotics and percentage of use amongst the interviewed tilapia farmers.

3.2 Antibiotic contamination

OTC was detected in 25% of the water samples collected during the dry season and in 100% of the water samples collected during the wet season in the Tha Chin River, with a maximum measured antibiotic concentration of 3.1 µg/L (Table 2). ENR was only detected in the water samples collected during the wet season, and the maximum measured concentration was 1.6 µg/L (Table 2). Both antibiotics were detected indistinctively in the majority of the sampling locations (Fig. S2). OTC and ENR were measured in all the sediment samples collected in the aquaculture-impacted areas of the Tha Chin River (dry and wet season) and

Mun River. Sediment concentrations of antibiotics ranged between 4.5 and 4,062 µg/kg d.w. for OTC, and between 1.4 and 2,339 µg/kg d.w. for ENR. Overall, measured antibiotic concentrations in the Mun River were found to be higher than those measured in the Tha Chin River, and antibiotic concentrations in the wet season were found to be lower than in the dry season, especially for OTC (Fig. 3). The highest sediment concentrations in the Mun River were measured in locations 1, 2, and 4 (Fig. S2), which coincided with the river locations that held the highest density of tilapia cages. ENR was not detected in the sediments of the reference site sampled in the Mun River, and OTC was detected at low concentrations (5.4-6.0 µg/kg d.w.) in 4 out of the 6 collected samples.

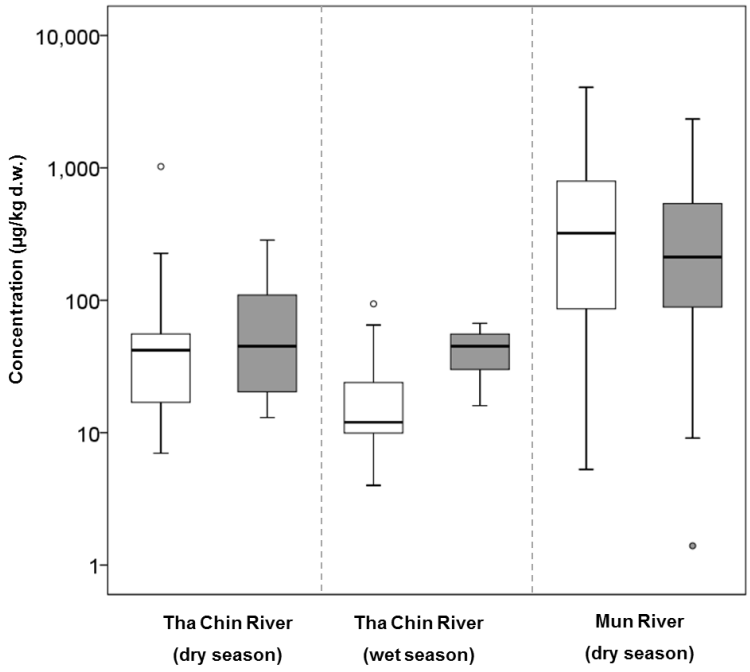


Fig 3 Measured concentrations of oxytetracycline (white boxes) and enrofloxacin (grey boxes) in the sediment samples. Each box shows the median, the 25th and 75th percentiles, the lowest datum within 1.5 times the interquartile range of the lower quartile, and the highest datum within 1.5 times the interquartile range of the upper quartile. Dots represent outliers (values between 1.5 and 3 times the range between the lower and upper interquartile).

OTC concentrations in the water samples collected 15 min after antibiotic administration in FARM 1 were 37 (26-49) µg/L inside the cages, and 6.4 (1.6-11) µg/L next to the cages (mean; minimum-maximum). Mean OTC concentrations dropped to 0.4 µg/L inside the cages and 0.1 µg/L next to the cages 1h after administration, and fell below the LOD 15h after antibiotic administration (Fig. 4A).

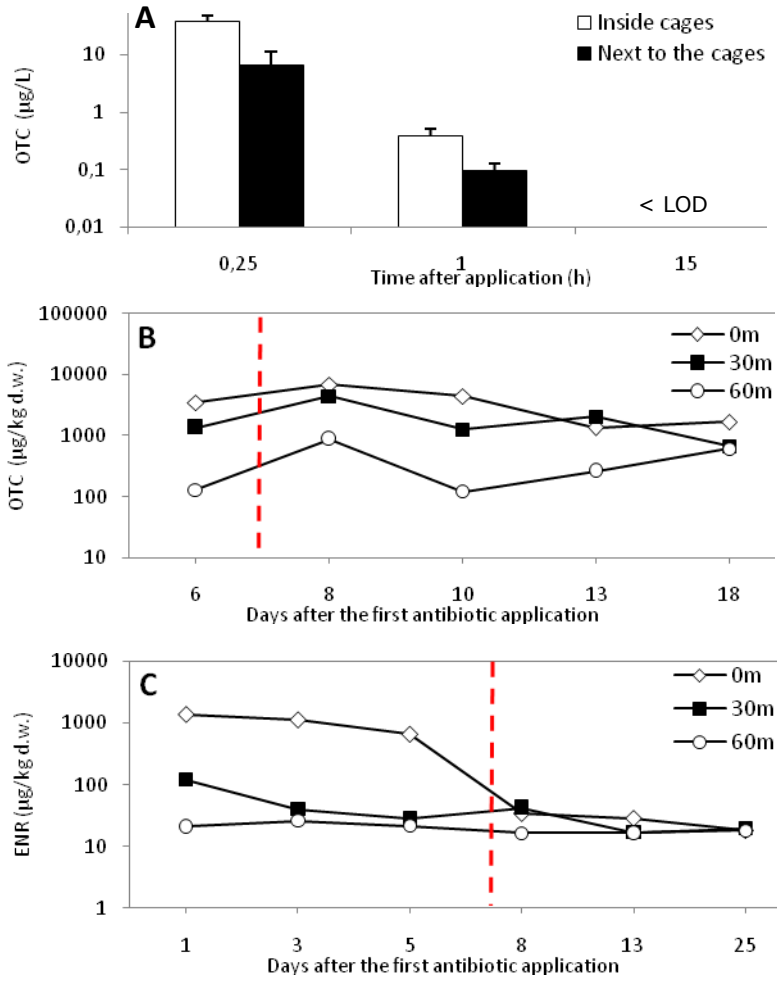


Fig 4 Measured oxytetracycline concentrations in water samples of FARM 1 (**A**), and measured oxytetracycline (**B**) and enrofloxacin concentrations (**C**) in sediment samples of FARM 2. The dashed lines indicate the end of the antibiotic administration period.

Maximum OTC concentrations in the sediments of FARM 2 (1 day after the end of the treatment) were 6,908 µg/kg d.w. in the sampling points located next to the treated cages, and 4,372 and 880 µg/kg d.w. at 30 and 60 m down-stream the cages (Fig. 4B). OTC concentrations dropped to about 20% of the peak sediment concentrations 11 days after administration, and the estimated first-order half-dissipation time in the sediment ($DT50_{sed}$) was 6.6 d. Measured ENR concentrations in the water samples collected next to the cages ranged between 0.10-0.71 µg/L during the administration period, and could not be detected in the sampling points further down the river, nor in the water samples collected after the administration period. Maximum ENR concentrations in the sediments were 1,398 µg/kg d.w. next to the cages, and 121 and 26 µg/kg d.w. at 30 and 60 m down-stream the monitored cages (Fig. 4C). Enrofloxacin concentrations in sediments next to the tilapia cages decreased sharply after the administration period, but remained stable down-stream from the sampled tilapia cages (Fig. 4C). The estimated $DT50_{sed}$ was 9.3 d (calculated for a period of 18 days after antibiotic administration).

Table 2. Summary of measured oxytetracycline (OTC) and enrofloxacin (ENR) concentrations in the collected water and sediment samples from the Tha Chin and Mun rivers.

		Water		Sediment	
		OTC	ENR	OTC	ENR
Tha Chin River (dry season)	Number of samples	24	24	19	19
	Detection rate	25%	n.d.	100%	100%
	Geometric mean concentration [a]	0.22	n.d.	42.4	51.2
	Maximum concentration [a]	3.05	n.d.	2119	285
Tha Chin River (wet season)	Number of samples	10	10	12	12
	Detection rate	100%	100%	100%	100%
	Geometric mean concentration [a]	0.50	0.49	12.9	45.4
	Maximum concentration [a]	1.76	1.59	94.9	67.3
Mun River (dry season)	Number of samples	NM	NM	30	30
	Detection rate	NM	NM	100%	100%
	Geometric mean concentration [a]	NM	NM	245	198
	Maximum concentration [a]	NM	NM	4062	2339
Mun River (reference site)	Number of samples	NM	NM	6	6
	Detection rate	NM	NM	67%	n.d.
	Geometric mean concentration [a]	NM	NM	5.57	n.d.
	Maximum concentration [a]	NM	NM	5.95	n.d.

[a] Concentrations are expressed in µg/L for water samples and µg/kg d.w. for sediment samples.

NM: not measured; n.d.: not detected

3.3 Toxicity experiments

The results of the toxicity experiments are shown in Table 3. Average antibiotic dissipation in the test media 48-h after the start of the experiment ranged between 18 and 33% for OTC, and between 6 and 21 % for ENR. No significant differences were observed in the recorded effects between the controls that were rinsed with the Na-EDTA solution and the non-rinsed ones in the OTC experiments. Calculated EC10 and EC50 values were in the order of mg/L, indicating that the studied antibiotics do not show a high acute toxicity to the tested invertebrate species. *L. hoffmeisteri* was found to be the most sensitive species to OTC (EC50 = 217 mg /L), whereas *M. lanchesteri* and *P. acuta* showed the highest sensitivity to ENR (LC50 = 202 and EC50 = 281 mg/L, respectively). *M. tuberculata* showed the lowest sensitivity to both antibiotics, probably due to their ability of covering their soft body with the operculum during chemical stress events. All the tested species showed higher tolerance to the tested antibiotics than *Daphnia magna*, the standard test species used as invertebrate surrogate in the risk assessment of antibiotics (Fig. 5).

3.4 Ecological risk assessment

The risk assessment based on RQs indicated potential risks (RQ>1) for the growth of primary producer communities exposed to OTC in the water layer and river sediments, and for primary producers exposed to water concentrations of ENR. The calculated risks of both antibiotics following the RQ approach for invertebrates were found to be insignificant (Table 4).

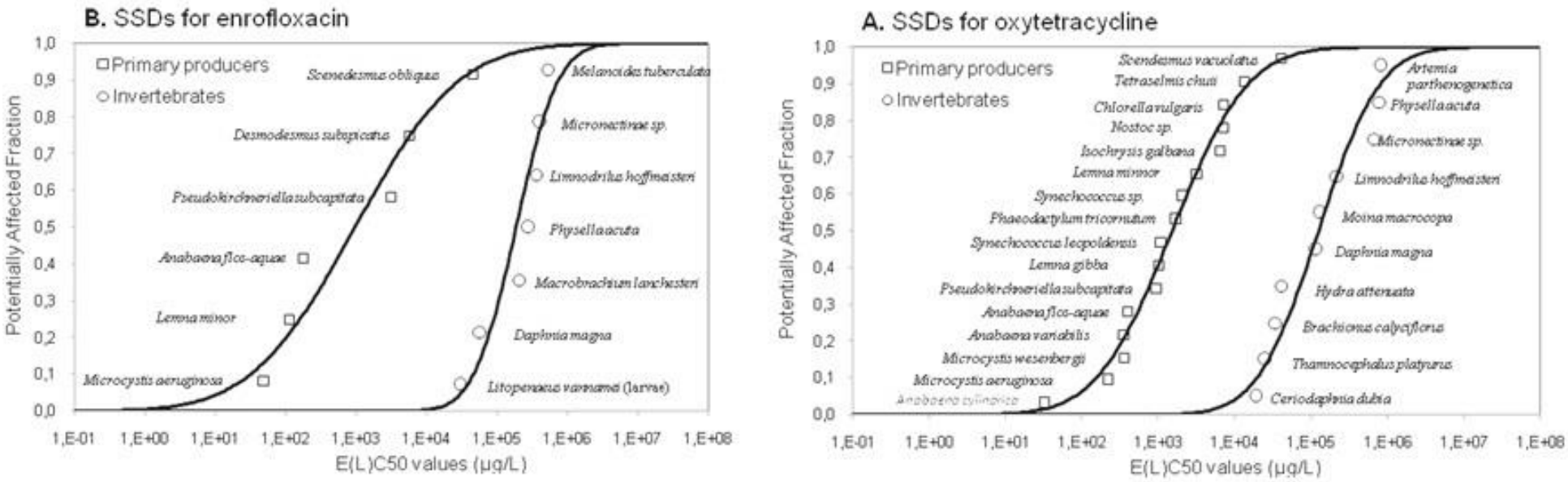


Fig 5 Species Sensitivity Distributions (SSDs) for oxytetracycline (**A**) and enrofloxacin (**B**) based on acute toxicity data. The parameters of the SSDs are shown in Table 4.

The SSD curves for OTC and ENR are shown in Fig. 5. The Anderson-Darling goodness of fit test was accepted for the four SSD curves. The SSDs indicate that, for OTC, blue-green algae (*Anabaena* sp. and *Microcystis* sp.) are more sensitive than the other taxa represented within the primary producer community, and microinvertebrates invertebrates are generally less tolerant than macroinvertebrates. The calculated median HC5 values for OTC were 75 µg/L for primary producers and 10,405 µg/L invertebrates. For ENR, blue-green algae (*Microcystis aeruginosa*) and macrophytes (represented by *Lemna minor*) showed a higher sensitivity compared to green algae species, and the larval stage of *Litopennaeus vanamei* was found to be more sensitive than *D. magna* and the rest of macroinvertebrate species tested in this study. The median HC5 values for ENR were 8.8 and 28,190 µg/L for primary producers and invertebrates, respectively. The results of the Kolomogorov-Smirnov test indicated that primary producers are significantly more sensitive than invertebrates for both OTC (ks=0.69, n_1=16, n_2=10, p=0.003) and ENR (ks=0.86, n_1=6, n_2=7, p=0.009). The ecological risk assessment based on the maximum antibiotic exposure concentrations and the SSDs indicated minimal risks for both investigated antibiotics on the studied aquatic communities (PAFs < 5%) (Table 4).

4. Discussion

A total of ten different antibiotics were reported to be used for disease treatment and prevention by farmers in the Tha Chin and in the Mun rivers. The frequency of antibiotic use in the interviewed farms is markedly higher than the frequencies reported in pond-based tilapia farms of Thailand (Rico et al., Accepted), probably due to the higher incidence of disease outbreaks associated to the fluctuating environmental and water quality conditions, and the high vulnerability to parasites and bacterial infestations (Belton et al., 2009). In addition, the limited disease diagnostic capacity denoted by the interviewed farmers combined with the promotional sales by the feed distribution companies are main factors contributing to the regular antibiotic use. The batched production system and the antibiotic use patterns in each batch (i.e., characterized by long treatment durations and several applications per culture cycle) is resulting in a continuous discharge of antibiotics in the rivers in which tilapias are cultured.

Table 3. Results of the oxytetracyline and enrofloxacin toxicity tests: measured exposure concentrations at the start of the experiment, dissipation of the antibiotics in the test media, effect concentrations, and slope of the fitted dose-response curve.

	Oxytetracycline					Enrofloxacin				
Species	Exposure concentrations (mg/L)	Dissipation (%) after 48h (mean±SD)	EC10-48h in mg/L (CI 95%)	EC50-48h in mg/L (CI 95%)	Slope (L/mg)	Exposure concentrations (mg/L)	Dissipation (%) after 48h (Mean±SD)	EC10-48h in mg/L (CI 95%)	EC50-48h in mg/L (CI 95%)	Slope (L/mg)
Limnodrilus hoffmeisteri	80, 136, 265, 467, 829	18±17	95.5 (NC)	217 (NC)	3.59	32, 64, 135, 293, 503	10±6	285 (238-342)	360 (317-409)	12.7
Macrobrachium lanchesteri	13, 32, 89, 167, 300	29±16	> 300 [a,b]	> 300 [a,b]	NC	147, 270, 366, 514, 634	11±8	85.2 (27.0-269) [b]	202 (128-319) [b]	3.41
Melanoides tuberculata	112, 277, 461, 868, 1052	33±13	> 958 [a]	> 958 [a]	NC	79, 113, 207, 370, 658	11±5	98 (56-173)	520 (337-801)	1.77
Micronectinae sp.	138, 330, 519, 677, 937	29±9	148 (59-369) [b]	647 (440-952) [b]	1.99	46, 91, 185, 352, 603	6±5	335 (292-384) [b]	408 (367-455) [b]	14.9
Physella acuta	142, 315, 631, 1072, 1409	29±7	528 (376-742)	791 (676-925)	7.31	58, 137, 424, 524, 749	21±7	40.1 (13.3-121)	281 (141-558)	1.51

NC: not calculated.

[a] Not clear dose-response.

[b] The evaluated endpoint was mortality.

The maximum OTC and ENR water concentrations measured in the Tha Chin River (49 and 1.59 µg/L, respectively) are one or two orders of magnitude higher than the concentrations monitored in other Asian aquatic environments receiving aquaculture pollution (Managaki et al. 2007; Zou et al., 2011; Yang et al., 2010; Takasu et al., 2011; Shimizu et al., 2013). The measured concentrations were as high as antibiotic concentrations monitored in pig farm effluents, hospital waste waters, sewage waters, and urban drainage systems of Asian countries (Managaki et al., 2007; Takasu et al., 2011; Shimizu et al., 2013), indicating that tilapia cage farming is an important source of antibiotic pollution. The results of this study revealed that peak antibiotic concentrations in the river water occur during antibiotic administration in the tilapia cages. This is in accordance with the study conducted by Duis et al. (1995), which demonstrated that the leaching of several antibiotics from oil-coated fish-feed pellets might range between 18-67% within 15 min after administration, and in the case of the studied tilapia cage farms this amount can be higher since only water was reported to be used as a coating agent. The water peak concentrations of OTC (49-26 µg/L) measured inside the tilapia cages of FARM 1 are in the order of magnitude of the peak concentrations measured during OTC administration in fish hatcheries (Dietze et al., 2005) and raceways (Bebak-Williams et al., 2002). However, the antibiotic dissipation from the surroundings of the administration area was found to be much quicker than in these semi-closed systems, as antibiotics are rapidly transported down-stream and diluted with the river water. This shows that water exposure is characterized by short-lasting antibiotic pulses. The higher detection rate of antibiotics in the water samples collected in the beginning of the wet season might be explained by several factors. First, the higher measured concentration of particulate organic matter during the rainy season (Table S1), together with the potential higher concentration of humic substances, are positively correlated to an increased water turbidity and, according to the study by Ge et al. (2010), are likely to contribute to a higher stability of antibiotics in the water column. Second, the increase in the water flow-rates during the wet season (Thaipichitburapa et al. 2010) are likely to contribute to a higher antibiotic desorption and re-suspension from river sediments. Finally, farmers reported higher antibiotic application rates during this season due to the observed higher fish stress, potentially caused by more frequent

runoff events and the varying environmental and water quality conditions during the monsoon period.

Our analysis of the sediment samples demonstrated that antibiotics are retained in the river sediments and may reach concentrations up to several mg/kg. The OTC and ENR sediment concentrations measured in the Tha Chin and Mun rivers are within the order of magnitude of the antibiotic concentrations reported in sediments collected under marine fish cages after chemotherapy (Coyne et al., 1994; Capone et al., 1996), and are comparable or even higher than antibiotic concentrations measured in sediments of other large Asian rivers impacted by urban or agricultural pollution (Yang et al., 2010; Zhou et al., 2011; Xue et al., 2013). The results of the monitoring performed in FARM 2 show that antibiotics tend to accumulate underneath or next to the tilapia cages, suggesting that not only water-sediment sorption, but also faeces and uneaten feed sedimentation, might play a substantial role in the antibiotic's transport to the sediment. Antibiotics were found to be widely distributed along the sampled river areas and to persist for several weeks in the river sediments (Fig. 4). Factors such as seasonal water flow variation, suspended solid concentrations in river water, river morphology, and farm density are expected to influence the distribution and occurrence of antibiotics in river sediments. For instance, higher water flow and the resulting dilution during the wet season might explain the lower OTC concentrations in the sediment, as tetracyclines have higher water solubility and lower sediment-sorption potential than fluoroquinolone antibiotics (Thiele-Bruhn, 2003). Overall, the higher magnitude of the antibiotic concentrations measured in the sediment samples from the Mun River compared to the Tha Chin River could be related to the lower relative distance of the sampling points to the tilapia cages and the higher concentration of cage farms in the sampled locations. Low OTC concentrations were detected in the sediment samples collected in the reference area sampled in the Mun River. This could be explained by the possibility that tetracycline antibiotics naturally occur in the environment, as a product of *Streptomyces* spp. bacteria (Chopra and Roberts, 2001), or because of other possible sources of contamination (e.g. transport from up-stream farms).

Table 4. Maximum measured exposure concentrations (MECs), predicted acute no effect concentrations (PNECs), calculated parameters of the species sensitivity distributions, results of the risk assessment expressed as risk quotients (RQs) and median potentially affected fractions (PAFs) based on acute toxicity data for water and sediments

	Exposure concentrations		Effect assessment				Risk assessment		
	MEC water in µg/L	MEC pore water in µg/L [a]	PNEC in µg/L	HC5 in µg/L (CI 95%)	HC50 in µg/L (CI 95%)	SD of log SSD data	RQ water	RQ sediment	PAF (%) water (CI 95%)	PAF (%) sediment (CI 95%)
Oxytetracycline										
Primary producers	49	5.08	2.18 [b]	74.5 (16.4-199)	1504 (686-3295)	0.78	22.4	2.33	3.05 (0.46-11.7)	0.09 (0.002-1.70)
Invertebrates			117 [c]	10405 (1790-28173)	123857 (53270-287982)	0.63	0.41	0.04	NC [f]	NC [f]
Enrofloxacin										
Primary producers	1.59	0.29	0.49 [d]	8.80 (0.05-91.5)	948 (105-8559)	1.16	3.24	0.60	1.25 (0.01-17.1)	0.23 (<0.001-9.81)
Invertebrates			57.5 [e]	28190 (4613-68012)	184683 (83219-409855)	0.47	0.03	0.005	NC [f]	NC [f]

[a] Pore water concentrations corresponding to a measured sediment concentrations of 6908 and 2339 µg/kg d.w. for oxytetracycline and enrofloxacin, respectively.

[b] Based on the geometric mean of the EC50 values reported by Ando et al. (2007) and Holten Lützhøft et al. (1999) for *Microcystis aeruginosa*, and an assessment factor of 100.

[c] Based on the geometric mean of the EC50 values reported by Park and Choi (2008), Isidori et al. (2005), and Kołodziejska et al. (In press) for *Daphnia magna,* and an assessment factor of 1000.

[d] Based on the EC50 value reported by Robinson et al. (2005) for *Microcystis aeruginosa*, and an assessment factor of 100.

[e] Based on the geometric mean of the EC50 values reported by Park and Choi (2008) and Kim et al. (2010) for *Daphnia magna,* and an assessment factor of 1000.

[f] Not calculated. The exposure concentration falls below the lower limit used in the PAF calculations, indicating an insignificant risk.

The RQ-based ecological risk assessment performed for OTC and ENR indicated potential risks for primary producer communities (particularly for blue-green algae), mainly in the water layer. However, the calculated risks were found to be negligible when the more realistic SSD approach was used. Knapp et al. (2005) did not find significant effects on water quality or microbial community structure in freshwater microcosms exposed to a single-pulse of 25 µg/L of enrofloxacin. Wilson et al. (2004) found dose-response effects on phytoplankton communities exposed to a mixture of four tetracyclines for 35 days, with significant effects starting at an approximate concentration of 120 µg/L. Regarding the results of the experiments performed by Knapp et al. (2005) and Wilson et al. (2004), together with the risk assessment calculations performed in this study, the monitored antibiotic concentrations in the waters of the Tha Chin and Mun rivers are not expected to exert direct toxic effects on non-target aquatic communities. However, more experiments should be performed with long-term exposure regimes and with benthic and sediment dwelling organisms, since antibiotic exposure in sediments was demonstrated to be chronic. These experiments should also include microorganisms and their related functional endpoints. Moreover, the nature of the antibiotic use practices and the results of the environmental monitoring show that aquatic ecosystems are exposed to antibiotic mixtures. Therefore, refined ecological risk assessments must consider the potential consequences of combined antibiotic toxicity (and other stressors) in aquatic organisms.

Despite the low ecological risks calculated in this study, the regular antibiotic administration and the prevalence of antibiotics in river sediments is expected to exert selective pressure on the sediment bacterial communities, leading to development of (multiple) antibiotic resistance (Cabello et al., 2006). The development of multi-drug resistance in the environment and the horizontal gene transfer to human pathogens has become a serious problem in the recent years, particularly in Asia (Suzuki and Hoa, 2012). It is estimated that antimicrobial resistance is responsible for more than 30,000 deaths annually in Thailand (Pumart et al. 2012). Despite the majority of the acquired drug resistance in humans is thought to be a consequence of irrational antibiotic consumption (Sumpradit et al., 2012), the contribution of the regular antibiotic use in tilapia cage farming to the health of the tilapia farmers and riverine populations, relying on water resources and

fish from antibiotic polluted rivers, remains largely uncertain. Furthermore, the development of resistant bacteria, which are usually more virulent than non-resistant strains, might pose a threat for the wild fish populations in the polluted rivers and for consumers capturing wild fish stocks exposed to antibiotic residues. Therefore, further attention must be paid by local authorities to monitor antibiotic pollution and resistant bacteria in these rivers, and to assess their impacts to the ecosystem and human's health.

5. Conclusions

This study demonstrated that the intensive use of antibiotics in tilapia cage production in Thailand is an important source of contamination for freshwater ecosystems. Ecosystems are regularly exposed to antibiotic pulses and to antibiotic mixtures. Regarding the risk assessment calculations performed in this study, which is based on a single-compound approach, the measured antibiotic concentrations of OTC and ENR are not expected to result in short-term toxicity to primary producers and invertebrates. However, further research is needed in order to assess the effects of long-term exposure and mixtures of antibiotics to tropical aquatic ecosystems. Such experiments should include sediments and benthic organisms, and ecosystem functional endpoints associated to microorganism communities such as nutrient cycling and organic matter decomposition. On the other hand, the repeated antibiotic use is expected to result in the development of antibiotic-resistant bacteria, making antibiotics actually ineffective against the target pathogens. In this way, farmers are forced to increase doses and continuously change antibiotic ingredients, compromising the (environmental) sustainability of this aquaculture practice. Further attention must be paid by local farmers and Thai authorities to control the use of antibiotics in tilapia cage-farm production, and to monitor the development and potential consequences of multi-drug resistance for human's and environmental health.

Acknowledgements

The present study was mainly funded by the EU-FP7 Sustaining Ethical Aquaculture Trade (SEAT) project (contract 222889). The work of Rhaul Oliveira was supported by a PhD grant (SFRH/BD/62605/2009) and the work of Inês Domingues

by a Post-Doc grant (SFRH/BPD/31752/2006) provided by the Portuguese Science and Technology Foundation (FCT). The authors would like to thank Frederieke Knopperts for collaborating during the interviews and field sampling, Steven Crum and Laura Buijse for their support on the antibiotic analysis, and to the farmers for their kind responses and for allowing us to take samples from their farms.

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SUPPORTING INFORMATION

Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand

Andreu Rico [a,*], Rhaul Oliveira [b], Sakchai McDonough [c], Arrienne Matser [d], Jidapa Khatikarn[a,e], KriengkraiSatapornvanit[e], António J.A. Nogueira[b], Amadeu M.V.M. Soares[b], InêsDomingues[b], Paul J. Van den Brink [a,d]

[a]Department of Aquatic Ecology and Water Quality Management, Wageningen University,Wageningen University and Research centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[b] Department of Biology and CESAM, University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal
[c] Aquaculture and Aquatic Resources Management, Asian Institute of Technology, P.O. Box 4, KlongLuang, Pathumthani 12120, Thailand
[d] Alterra, Wageningen University and Research centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[e] Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Chatuchak, 10900 Bangkok, Thailand

*Corresponding author: andreu.rico@wur.nl

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Description of the study areas

The Tha Chin River (Central Thailand) is a distributary of the Chao Phraya River that drains part of the Central Plains of Thailand and mouths into the Gulf of Thailand. The investigated area of the Tha Chin River was located in the SuphanBuri Province, at a river transect of approximately 15 km situated in the Sam Chuk and Doem Bang Nang Buat districts. River width at the study area was 25-45 m, water depth 5.3-11.5 m, and water flow 0.7-1.2 m/s (Thaipichitburapa et al., 2010).

The Mun River (North-East Thailand) drains the waters of the Khorat Plateau and is one of the main tributaries of the Mekong River. The area investigated was located in the UbonRatchathani Province, at a river transect of approximately 40 km length and 200-250 m width, and encompassing a high density of tilapia cage farms.

Sample collection and preservation methods

A long-tail boat was used for transportation in order to collect the water samples in the Tha Chin river. Water samples were collected approximately 1 m below the surface with a water grab sampler and stored in pre-cleaned plastic bottles. The collected samples were placed in a cooler during transportation to the laboratory, and were stored in the fridge (4°C) for a maximum period of 24h until further extraction.

All sediment samples were collected on board a boat or from the cage's steel frames. In all cases, sediment samples (400-800 g) were collected from approximately the top 5 cm layer using an Ekman grab sampler (surface area: 225 cm^2) and were immediately introduced into zip lock plastic bags. The plastic bags were placed in a cooler during transportation and stored in a freezer (-20°C) until further analysis.

During the sampling campaigns, basic water quality parameters were monitored *in situ* (temperature, dissolved oxygen, pH, and conductivity). Furthermore, extra water and sediment samples were taken in order to characterise the water (hardness, suspended solids, chlorophyll-a) and sediment (water, organic matter, texture) of the sampled areas. All water and sediment quality measurements were done according to the protocols described in APHA (1996) and are shown in Table S1.

Antibiotic extraction methods and analysis

Antibiotic extraction from the toxicity test media and the water samples collected in the Tha Chin River were done with solid phase extraction (SPE) OASIS HLB cartridges (3cc, Waters, USA), preconditioned with 5 mL of MeOH and 5 mL of distilled water. For the analysis of the experimental media, 10 mL were passed through the SPE cartridges by means of a plastic syringe. For the analysis of the monitoring samples, a water volume of approximately 1 L was passed through the SPE cartridges by using a vacuum pump (average speed: 10 mL/min). Before extraction, the water

samples collected during the field monitoring were filtered through a 1.2 µm pore size glass microfiber filter (Whatman GF/C), and the pH was adjusted to 5.5-6.0 by drop adding acetic acid/acetate buffer solution, in order to improve the antibiotic retention in the SPE cartridges. After SPE the cartridges were stored in a freezer at -20°C until elution. Elution was performed with 5 mL (5x1 mL) of 0.01 M NaOH-acetonitrile (75:25, v/v) solution. The extracts were transferred into 2 mL plastic vials and stored at -20°C until analysis.

Antibiotic extraction from the sediment samples was based on the method described by Yang et al. (2010). Approximately 20 g of sediment were introduced into plastic centrifuge tubes. Subsequently, 100 µL of internal standard (tetracycline and enrofloxacin d-5) were spiked into the centrifuge tubes and kept in dark for 30 min. Twenty mL of a mix of 0.2 M citric acid buffer (pH4) and acetonitrile (50:50; v/v) were added to the centrifuge tubes and shaken manually. Subsequently, the tubes were sonicated for 15 min and shaken for 30 min (150 rpm). After shaking, the samples were centrifuged at 25 °C for 10 min at 12,000 rpm. The obtained supernatant was placed in 30 mL glass tubes and evaporated at 55°C under a gentle stream of air to remove the organic solvent. The aqueous sediment extract was diluted in 200 mL of Milli-Q water with 0.2 g of Na_2-EDTA. The aqueous sediment extracts were then cleaned-up and enriched by means of SPE Oasis HLB cartridges (500 mg, 6 cc, Waters, USA), preconditioned with 10 mL of MeOH and 10 mL of Milli-Q water. After all the extract was loaded completely, the glass bottle containing the aqueous extract and the cartridge were rinsed with 10 mL of Milli-Q water, and the cartridges were vacuum dried for 1 h. Next, the antibiotics were eluted from the cartridges with 10 mL of MeOH (5x2mL) and the eluents were evaporated to dryness at 55°C. The pellet was dissolved by adding 400 µL of acetonitrile and 1600 µL of Milli-Q water, with vortex and sonication (10 min). Finally, the extracts were transferred into 2 mL plastic vials and stored at -20 °C until analysis.

The antibiotic analysis of the water and sediment extracts was made by LC-MS/MS. Prior to injection, the extracts obtained from the toxicity test experimental media were diluted. An extract volume of 50 µL was injected into the chromatographic system by means of an Agilent 1200 series (Agilent Technologies, Germany). Separation was done on a Zorbax XDB-C_{18} column (4.6 x 150 mm, 5 µm), set to a temperature of 25 °C, using binary gradient elution. Mobile phase A consisted of formic acid solution in Milli-Q water (0.01% v/v) and mobile phase B consisted of formic acid solution in acetonitrile (0.01% v/v). The mobile phase lasted for 20 min and was performed at a constant flow rate of 0.7 mL/min according to the following elution gradients: held at 10% B for 10 min, then moved to 80% and held for 4 min, and then moved to 20% and held for 6 min. In order to prevent contamination of the MS, part of the eluent was sent to the waste (0-5 min and after min 16 for water samples, and 0-7 min and 12-20 min for sediment samples). The mass spectrometry analysis was conducted with a triple quadrupole mass spectrometer (Agilent Technologies 6410) equipped with an ESI^+. The nebulizer pressure was set to 35 psi and the flow rate of drying gas (nitrogen) was 8 L/min. The capillary voltage was 3000 V and the dry temperature 350 °C. Sample acquisition was performed in the multiple reaction monitoring (MRM) mode. Calculated recoveries of the method used for the toxicity test media (with an antibiotic concentration of 500 mg/L) were 97±11% for OTC, and 72±6%

for ENR (Mean±SD, *n*=3). The calculated recoveries for OTC and ENR following the method used for the environmental samples were 48±2% and 112±13% (*n*=4), respectively, for an antibiotic concentration of 10 µg/L. The calculated recoveries in the sediment samples collected from the reference site were 78±3% and 99±6% for OTC and ENR, respectively. When the method recovery was below 70% the final measured antibiotic concentrations were re-calculated. The limit of detection (LOD) and limit of quantification (LOQ) in the water samples were 0.02 and 0.05 µg/L for OTC, and 0.01 and 0.02 µg/L for ENR. The LOD and LOQ in the sediment samples were 0.8 and 2.6 µg/kg d.w. for OTC, and 0.3 and 1.1 µg/kg d.w. for ENR, respectively.

Table S1. Water and sediment quality parametersmeasured in the samples collected during the monitoring campaigns in the Tha Chin and in the Munrivers(Thailand). Data are expressed as mean ± SD.

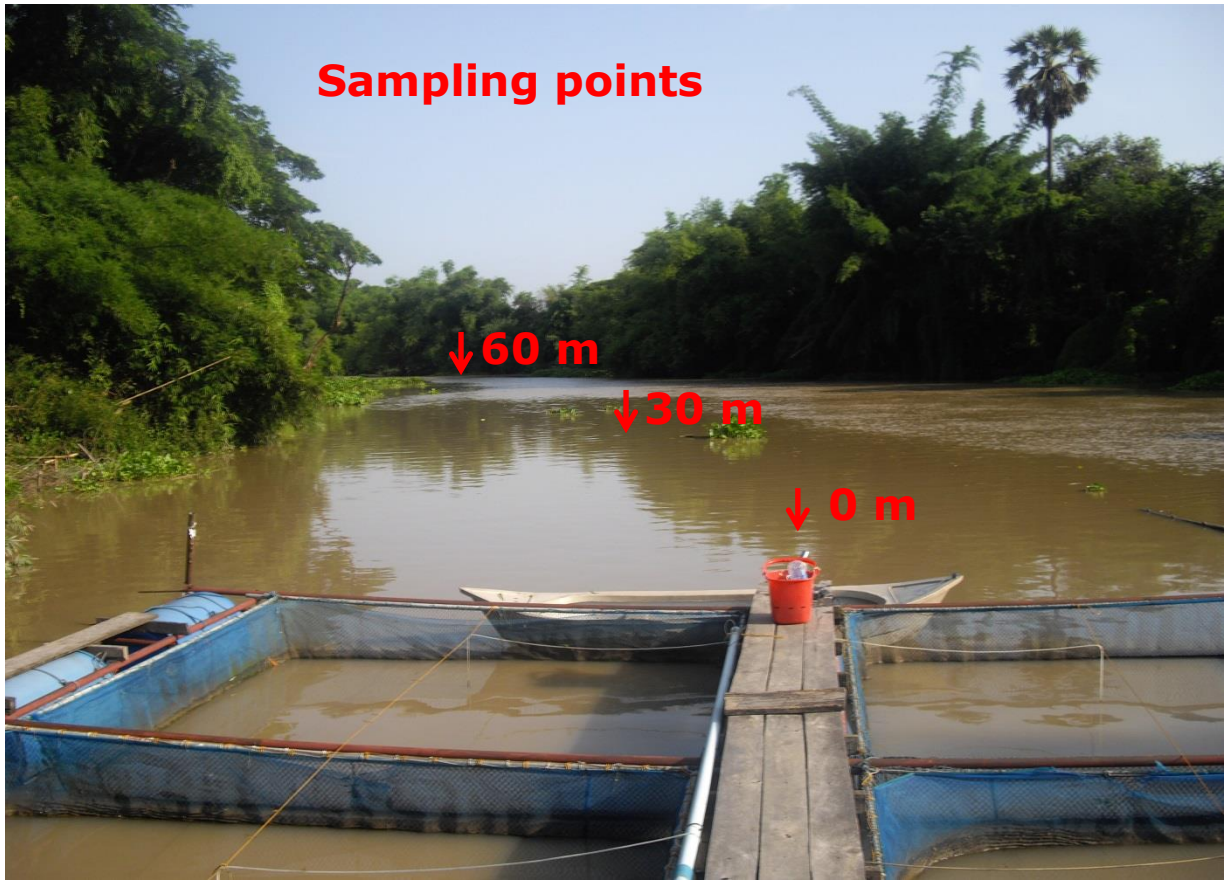
	Tha Chin River (dry season)	Tha Chin River (wet season)	Mun River (dry season)	Mun River (reference site)
Water				
Temperature (°C)	31 ± 1.2	30 ± 0.1	26 ± 1.9	26 ± 1.4
Dissolved oxygen (mg/L)	2.7 ± 1.2	2.8 ± 0.3	5.2 ± 1.6	6.7 ± 0.2
pH	6.8 ± 0.1	6.9 ± 0.5	6.9 ± 0.3	6.9 ± 0.2
Conductivity (µS/cm)	203 ± 5.1	194 ± 2.2	203 ± 33	201 ± 18
Hardness (mg $CaCO_3$/L)	69 ± 1.8	59 ± 3.5	NM	NM
Total suspended solids (mg/L)	16 ± 2.8	70 ± 16	27 ± 11	35 ± 1.1
Chlorophyll-a (µg/L)	1.9 ± 0.7	0.9 ± 0.2	NM	NM
Sediment				
Water content (%)	33 ± 8.3	41 ± 8.6	33 ± 13	36 ± 20
Organic matter (%)	7.5 ± 1.8	5.2 ± 1.4	4.3 ± 1.9	1.6 ± 2.2
Clay (%)	26 ± 12	62 ± 22	13 ± 6.5	3.4 ± 5.5
Sand (%)	34 ± 15	30 ± 23	37 ± 26	80 ± 32

NM: not measured.

Table S2. Toxicity data used to build the species sensitivity distributions. Only EC50 or LC50 values calculated for a exposure duration of 1-7 days for primary producers and 1-4 days for invertebrates were selected. The geometric mean was calculated when more than one toxicity value was available for a species.

Species name	Species group	Evaluated endpoint	Toxicity value (mg/L)	Reference
Oxytetracycline				
Anabaena cylindrical	Blue-green algae	EC50-6d (growth inhibition)	0.032	Ando et al. (2007)
Anabaena flos-aquae	Blue-green algae	EC50-6d (growth inhibition)	0.39	Ando et al. (2007)
Anabaena variabilis	Blue-green algae	EC50-6d (growth inhibition)	0.36	Ando et al. (2007)
Microcystisaeruginosa	Blue-green algae	EC50-6d (growth inhibition)	0.23	Ando et al. (2007)
Microcystisaeruginosa	Blue-green algae	EC50-72h (growth inhibition)	0.207	HoltenLützhøft et al. (1999)
Microcystiswesenbergii	Blue-green algae	EC50-6d (growth inhibition)	0.35	Ando et al. (2007)
Nostoc sp.	Blue-green algae	EC50-6d (growth inhibition)	7	Ando et al. (2007)
Synechococcusleopoldensis	Blue-green algae	EC50-6d (growth inhibition)	1.1	Ando et al. (2007)
Synechococcus sp.	Blue-green algae	EC50-6d (growth inhibition)	2	Ando et al. (2007)
Chlorella vulgaris	Green algae	EC50-72h (growth inhibition)	7.05	Eguchi et al. (2004)
Scendesmusvacuolatus	Green algae	EC50-24h (growth inhibition)	40.4	Kołodziejska et al. (In press)
Isochrysisgalbana	Green algae	EC50-96h (growth inhibition)	6.43	De Orte et al. (In press)
Pseudokirchneriellasubcapitata	Green algae	EC50-72h (growth inhibition)	0.342	Eguchi et al. (2004)
Pseudokirchneriellasubcapitata	Green algae	EC50-72h (growth inhibition)	4.5	HoltenLützhøft et al. (1999)
Pseudokirchneriellasubcapitata	Green algae	EC50-72h (growth inhibition)	0.17	Isidori et al. (2005)
Pseudokirchneriellasubcapitata	Green algae	EC50-96h (growth inhibition)	3.1	Zounková et al. (2011)
Pseudokirchneriellasubcapitata	Green algae	EC50-48h (growth inhibition)	1.26 [a]	Christensen et al. (2006)
Tetraselmischuii	Green algae	EC50-72h (growth inhibition)	13.16	Ferreira et al. (2007)
Phaeodactylumtricornutum	Diatom	EC50-96h (growth inhibition)	1.73	De Orte et al. (In press)
Lemnagibba	Macrophyte	EC50-7d (wet weight)	1.01	Brain et al. (2004)
Lemnaminor	Macrophyte	EC50-7d (growth inhibition)	4.92	Pro et al. (2003)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	2.1	Zounková et al. (2011)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	3.26	Kołodziejska et al. (In press)
Daphnia magna	Crustacean	EC50-48h (immobilization)	621.2	Park and Choi (2008)
Daphnia magna	Crustacean	EC50-48h (immobilization)	114	Kołodziejska et al. (In press)
Daphnia magna	Crustacean	EC50-48h (immobilization)	22.64	Isidori et al. (2005)
Moinamacrocopa	Crustacean	EC50-48h (immobilization)	126.7	Park and Choi (2008)
Artemiaparthenogenetica	Crustacean	LC50-48h (mortality)	806	Ferreira et al. (2007)
Ceriodaphniadubia	Crustacean	EC50-48h (immobilization)	18.65	Isidori et al. (2005)
Thamnocephalusplatyurus	Crustacean	EC50-48h (immobilization)	25	Isidori et al. (2005)
Brachionuscalyciflorus	Rotifer	EC50-48h (immobilization)	34.21	Isidori et al. (2005)
Hydra attenuata	Cnidarian	EC50-96h (morphology)	40.13	Quinn et al. (2008)
Limnodrilushoffmeisteri	Worm	EC50-48h (immobilization)	217	This study
Micronectinae sp.	Insect	EC50-48h (mortality)	647	This study
Physellaacuta	Mollusc	EC50-48h (immobilization)	791	This study
Enrofloxacin				
Microcystisaeruginosa	Blue-green algae	EC50-5d (growth inhibition)	0.049	Robinson et al. (2005)
Anabaena flos-aquae	Blue-green algae	EC50-72h (growth inhibition)	0.173	Ebert et al. (2011)
Desmodesmussubspicatus	Green algae	EC50-72h (growth inhibition)	5.57	Ebert et al. (2011)
Pseudokirchneriellasubcapitata	Green algae	EC50-72h (growth inhibition)	3.1	Robinson et al. (2005)
Scenedesmusobliquus	Green algae	EC50-72h (growth inhibition)	45.1	Qin et al. (2011)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	0.114	Robinson et al. (2005)
Lemnaminor	Macrophyte	EC50-7d (growth inhibition)	0.107	Ebert et al. (2011)
Daphnia magna	Crustacean	EC50-48h (immobilization)	58.3 [a]	Kim et al. (2010)
Daphnia magna	Crustacean	EC50-48h (immobilization)	56.7	Park and Choi (2008)
Litopenaeusvannamei (larvae)	Crustacean	EC50-48h (mortality and morbidity)	29.4	Williams et al. (1992)
Macrobrachiumlanchesteri	Crustacean	EC50-48h (mortality)	202	This study
Limnodrilushoffmeisteri	Worm	EC50-48h (immobilization)	360	This study
Melanoidestuberculata	Mollusc	EC50-48h (immobilization)	520	This study
Micronectinae sp.	Insect	EC50-48h (mortality)	408	This study
Physellaacuta	Mollusc	EC50-48h (immobilization)	281	This study

[a] Geometric mean of the values reported in this study.



Date	8/5	9/5	10/5	11/5	12/5	13/5	14/5	15/5	16/5	17/5	18/5	19/5	20/5	21/5	22/5	23/5	24/5	25/5	26/5	27/5	28/5	29/5	30/5	31/5	1/6	2/6	3/6	4/6	5/6	6/6
Day after start of OTC treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
OTC treatment																														
OTC sediment samples						x		x		x			x					x												
Day after start of ENR treatment	-	-	-	-	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
ENR treatment																														
ENR water and sediment samples						x		x		x			x					x												x

Fig. S1. Synopsis of the sampling points and sampling days in relation to the oxytetraycline (OTC) and enrofloxacin (ENR) treatment applied in FARM 2.

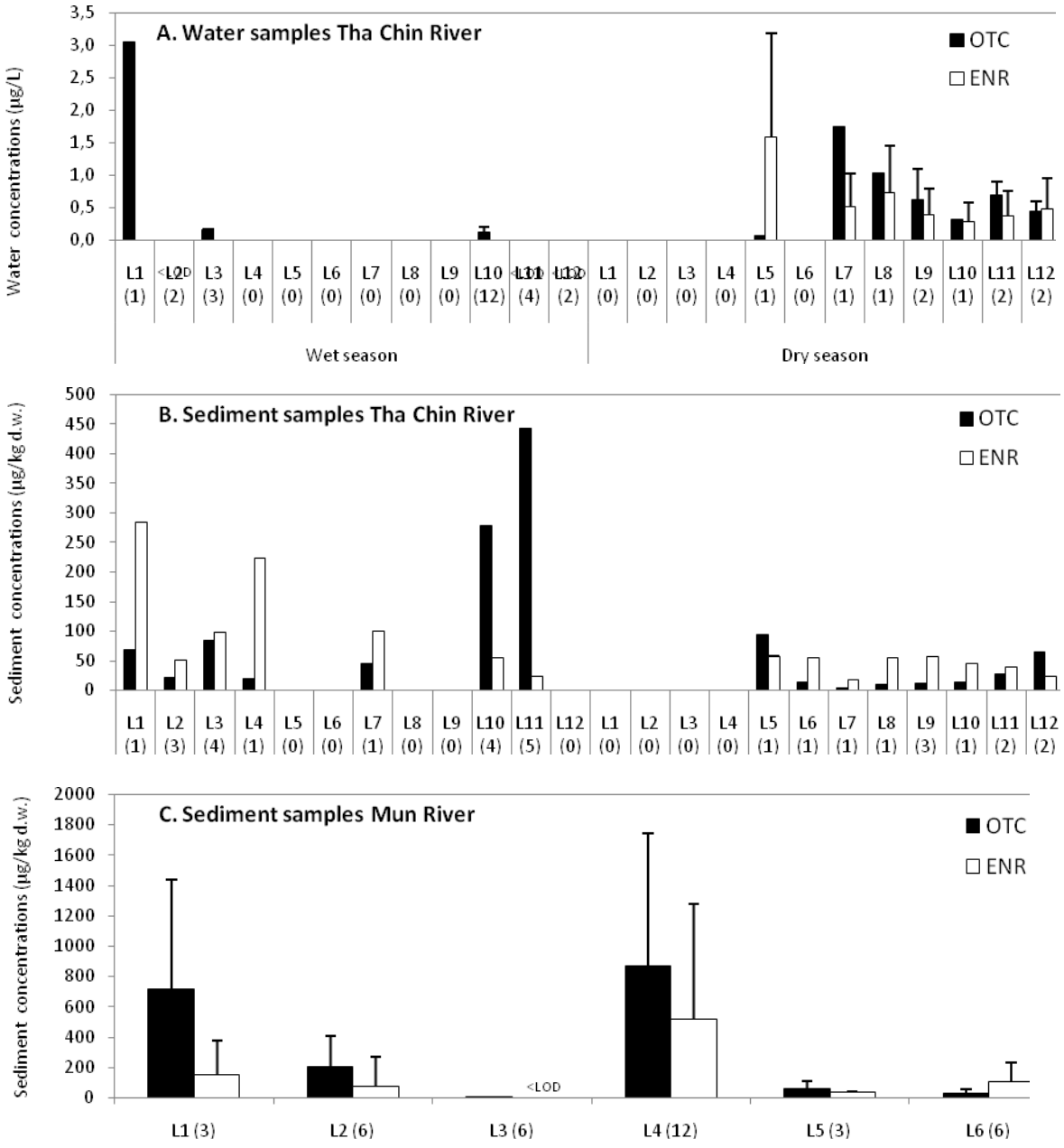


Fig S2. Measured water and sediment oxytetracycline (OTC) and enrofloxacin (ENR) concentrations in the different sampling locations of the Tha Chin and Mun rivers (mean ± SD). The number between brackets indicates the number of analysed samples in each sampling location.

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Chapter 10

Final Remarks

1. Final Remarks - The tip of the iceberg

As detailed previously the aquaculture is one the most promising agricultural activity of this century. However, aquaculture causes environmental impacts: eutrophication, deforestation of coastal areas, introduction of invasive species in aquatic ecosystems and chemical pollution. Dealing with the impacts of aquaculture is if the best interest of government, farms and consumers. A sustainable model of aquaculture will guarantee in a long-term perspective the employment and income for farmers and high-quality food for consumers. In the chapters of this thesis were addressed and discussed the results of short-term and long-term ecotoxicity tests, biomarkers, species sensitivity distribution and risk analysis, filed surveys and monitoring campaigns focused on aquaculture chemicals.

Even focusing only in one of the multiple aspects of aquaculture environmental impact – the chemicals pollution – is difficult to address issues essential for promote an environmental friendly aquaculture. Understanding the effects of aquaculture chemicals on aquatic ecosystem is a multidisciplinary task. The data presented in this work is a small contribution on the aquatic toxicology of aquaculture chemicals. Many major questions on the chemicals pollution from aquaculture activities still open. One first point to be right lighted is the lack of information on the toxicology of many others chemicals used in aquaculture. In a study by Rico et al., (2013) were recorded sixty different veterinary medicinal active ingredients used in aquaculture including 26 different antibiotics, 19 disinfectants, and 15 parasiticides. This study summarize a result of a survey performed between 2010 and 2011 in Bangladesh, China, Thailand and Vietnam and considering tilapia, catfish, prawn and shrimp farms.

The chemicals here studied are between the most used disinfectants, pesticides, and antibiotics used in Asian aquaculture (Rico et al. 2012; Rico et al. 2013; Rico and Van den Brink 2014). The toxic effects of aquaculture chemicals can be severe (e.g., chlorosis, mortality and immobilization) but at the same time a

wide range of sub-lethal effects on aquatic organisms might occur (e.g. behavioral, neurological, and oxidative stress.). Most of the tested chemicals showed to compromise at least a particular group of organisms (e.g. Trichlorfon highly toxic for daphnids) or triggered responses at a sub individual level (e.g. antibiotic induced oxidative stress). Both lethal and sub lethal effects on non-target organisms suggest the potential of aquaculture chemicals to harm the ecosystems causing biodiversity loss in the aquatic ecosystems and consequently compromising the services provided by the ecosystems in a near future. Since the aquaculture relies directly on these services (e.g. high water quality) to succeed, there is an urgent need to understand the effects of the aquaculture chemicals on ecosystems in other to control correctly its use and impacts.

In the context of tilapia aquaculture, both cage and ponds farms can pose risk to human and aquatic environment. The antibiotics oxytetracycline and enrofloxacin and the hormone 1,7α-metiltestosterona where detected in environmental samples. Rico et al., (2014) suggest that about 25% of the mass of veterinary medicines applied in the aquaculture farms is released to the environment. In this way, others chemicals may reach the environment after application in farms. For example, calculated a peak of predicted environmental concentration for BKC = 44 µg/l in a scenario of pond application (Rico et al. 2013). Despite being likely to occur in the environment, highly toxic and used in several countries no monitoring studies of BKC concentrations in aquaculture surrounding areas are found in the literature. The same is observed for many antibiotics, such as sulfonamides and ciprofloxacin, or pesticides such are ivermectin and trifluralin. Monitoring the fate of chemicals used in the farms is an essential step towards a better understanding of the adverse impacts of aquacultures in natural ecosystems.

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ANNEX

AIT – Asian institute of Technology
AARM - Aquaculture and Aquatic Resources Management
UA – University of Aveiro

A team from the University of Aveiro in collaboration with the Asian Institute of Technology has developed this study – AARM partners. The main objective of this survey is to obtain information about current Tilapia cage aquaculture practices that can pose a risk to human and aquatic environment health. Out puts of the data collected in this survey, tools and guidelines will be developed for the promotion of sustainable practices of fish production in Thailand.

1. Personal data and socio-economic aspects

Name: _____ **Phone: ( )** _____

Sex: _____ **Age:** _____

Place of birth: _____ **Marital status:** _____

Nº of family members: _____ **Schooling history:** _____

Position: _____ **How many years experience in aquaculture:** _____

Others professional activities: _____ **Income (THB per month):** _____

2. Farm characterization

Farm Code: _____ **Age of the farm:** _____

Nº of owners: _____ **No. of workers:** _____

Farm area (sq m2): _____ **Species cultivated:** _____

Target market for sale: _____

Frequency of cage cleaning: _____

Any integrated farming practices: _____

3. Types of cultivation:

Fingerlings

	Nº of cages
	Density of organisms
	Amount of food /per cage/day
	Size of fish for sale
	Price of fish for sale
	How many cycles completed per year
	Time taken for grow-out

Grow out

	Nº of cages
	Density of organisms/cage
	Amount of food /per cage/day
	Size of fish for sale
	Range of price of fish for sale
	How many cycles completed per year
	Time taken for grow-out (per cycle)

4. Chemical use:

1. Never	2. Rarely	3.Frequetly	4. Every day

	Antibiotic: _____
	Disinfectants: _____
	Vitamins: _____
	Pesticides: _____
	Anesthetics: _____
	Probiotics: _____

Most common products

Product	Dose	Route of application	How do you know this product?
1_____	_____	_____	_____
2_____	_____	_____	_____
3_____	_____	_____	_____
4_____	_____	_____	_____
5_____	_____	_____	_____
6_____	_____	_____	_____
7_____	_____	_____	_____
8_____	_____	_____	_____
9_____	_____	_____	_____
10_____	_____	_____	_____

Usually from which suppliers do you purchase chemicals? _____

Are you advised to take any type of caution when handling the products (e.g. gloves mask)? _____ YES or _____ NO

Do you use protection equipment when handling the products (e.g. gloves mask)? _____ YES or _____ NO

Do you make use of mask when handling Formaldehyde? _____ YES or _____ NO

Do you use gloves when mixing the chemicals with food? _____ YES or _____ NO

Does the seller provide any type of printed advice? _____ YES or _____ NO

Did you receive any information or training from DFO? _____ YES or _____ NO

What are your criteria to choose among various products? (Suggest: Price, personal experience, expert opinion)

_____

Do you know some of these chemicals?

- ☐ Gentamycin
- ☐ Erythromycin
- ☐ Nitrofurazone
- ☐ Ciprofloxacin
- ☐ Enrofloxacin
- ☐ Norfloxacin
- ☐ Oxolinic acid
- ☐ Sulfamethazine
- ☐ Sulfamonomethoxine

- ☐ Chlortetracycline
- ☐ Oxytetracycline
- ☐ Chloramphenicol
- ☐ Trimethoprim
- ☐ Amoxicillin
- ☐ Saponin
- ☐ Malachite Green
- ☐ Formaldehyde
- ☐ Sodium hypochlorite

- ☐ Acetic acid
- ☐ Benzalkonium chloride
- ☐ Calcium hypochlorite
- ☐ Trichlorfon
- ☐ Trifluralin
- ☐ fentin acetate
- ☐ Rotenone

5. Waste and Water management

Fate of container of used chemicals: _____

Any treatment for waste of chemical solutions: _____

Are there problems with water quality (smell, color etc…):_____

Is the farm water of good quality (0 - 10): _____

Is there a problem with water scarcity: _____

Others uses for the water

- ☐ Fishing
- ☐ Irrigation
- ☐ Watering animals
- ☐ Drinking
- ☐ Domestic use (cooking, sewerage)
- ☐ Recreation

6. Infestations and Disease (incidence/treatment/prevention)

The last time disease was encountered on the farm (date, week, month): _____

How many times per year is there a problem with disease: _____

Is there a prophylactic use of chemicals (change in the dose): _____

_____

Did you notice some resistance effect of diseases or parasites to used products: _____

_____

Most common diseases and treatment

Name of disease	_____
Product	_____
Method of Application	_____
Dose	_____
Frequency	_____

Name of disease	_____
Product	_____
Method of Application	_____
Dose	_____
Frequency	_____

Name of disease	_____
Product	_____
Method of Application	_____
Dose	_____
Frequency	_____

Name of disease	_____
Product	_____
Method of Application	_____
Dose	_____
Frequency	_____

Thank you for your collaboration!!!
If you have any questions about this document, please contact us by e-mail at
rhaul.oliveira@gmail.com