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Ano 2009

Rhaul de Oliveira

O peixe-zebra como uma ferramenta para avaliação de ecotoxicidade

Zebrafish early life-stages and adults as a tool for ecotoxicity assessment

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Zebrafish early life-stages and adults as a tool for ecotoxicity assessment

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Drª Paula Inês Borralho Domingues e Prof. Dr. Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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o júri

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palavras-chave

Danio rerio, embryotoxicidade, toxicidade aguda, biomarcadores, genotoxicidade, toxinas Bt, dicromato de potássio, triclosão, peixe-zebra

Resumo

O peixe-zebra (*Danio rerio*) é um vertebrado modelo grandemente utilizado em investigação científica em todo o mundo. Nas últimas décadas, foram desenvolvidos protocolos e técnicas que permitem o uso deste organismo na avaliação de ecotoxicidade. Com o presente trabalho pretendeu-se aplicar múltiplas técnicas para avaliar a toxicidade de diferentes químicos em *Danio rerio* a nível letal e subletal (embriotoxicidade, genotoxicidade e biomarcadores) realizando testes com embriões e adultos. Numa primeira fase foram selecionados químicos representativos de diferentes tipos de contaminação ambiental. O dicromato de potássio (metal pesado), o triclosão (bactericida) e as δ-endotoxinas (toxinas Bt) foram escolhidos com base nos seus diferentes modos de ação, uso e potecial risco ecológico. Foram realizados ensaios ecotoxicológicos utilizando o peixe-zebra nos diferentes estádios do seu ciclo de vida. Os protocolos da OECD "Fish, Acute Toxicity Test" e "Fish Embryo Test" foram adoptados para avaliação da toxicidade aguda, alterações comportamentais e embriotoxicidade (no caso dos testes com embriões). Foram ainda incluidos ensaios para determinação da actividade de algumas enzimas (colinesterase (ChE), glutationa-S-transferase (GST) e lactato desidrogenase (LDH)) e da genotoxicidade (teste do micronúcleo, anomalias nucleares e teste do cometa). Os resultados mostraram que os testes com embriões são muito mais informativos que os testes com adultos visto que incluem uma gama de endpoints mais vasta, permitindo obter um "perfil de toxicidade" específico para cada químico testado. Os efeitos a nível do desenvolvimento embrionário (anomalias e atrasos) e os biomarcadores mostraram ser os endpoints testados mais sensíveis. O teste com embriões de zebrafish mostrou assim ser uma óptima ferramenta para avaliação da toxicidade contribuindo para uma melhor compreensão dos efeitos dos tóxicos abordados e uma melhor perspectiva do seu risco ambiental.

Keywords

Danio rerio, embryotoxicity, acute toxicity, biomarkers, genotoxicity, Bt toxins, potassium dichromate, triclosan, zebrafish

Abstract

The zebrafish (*Danio rerio*) is a model vertebrate extensively used in scientific investigation worldwide. In the last decades, protocols and techniques have been developed in order to evaluate the effects of chemicals at different levels of biological organization of this species and to characterize the lethal and sub-lethal effects of pollutants. The main aim of this study was to assess the toxicity of different classes of chemicals to Danio rerio at lethal and sublethal levels applaying multiple techniques (embryotoxicity, genotoxicity and biomarkers) with adults and embryos. In the first phase of this study was the selection of chemicals in order to contemplate different type of contaminants. Potassium dichromate (heavy metal), triclosan (biocide) and δ-endotoxins (Bt toxins-biopesticide) were selected due to their different modes of action, use and potential environmental risk. Ecotoxicological assays were performed using different life-stages of zebrafish. The OECD protocols “Fish, Acute Toxicity Test” and “Fish Embryo Test” were followed. Acute toxicity, behavioral alterations and embryotoxicity were evaluated. Adaptations in OECD protocols were performed in order to enable tissue sampling to be used in enzymatic activity assays (acetylcolinesterase – ChE; glutatione-S-trasferase – GST, lactate desidrogenase – LDH) in genotoxixity assays (micronuclei and COMET) and in citotoxicity assays (nuclear abnormalities). Results obtained showed that *Danio rerio* early-life stages test is much more informative than test with adult fish as they include a wide range of endpoints allowing the obtaining of a more specific toxicity profile for each chemical tested. Effects on embryonic development (anomalies and delays) and biomarkers seemed to be the most sensitive endpoints. Zebrafish embryo test showed to be an excellent tool for toxicity evaluation contributing for a better understanding of the effects of the selected chemicals and for a more accurate prespective of their environmental risk.

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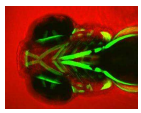

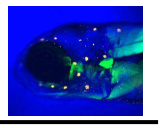

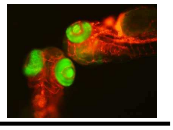

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detachment at 24 and 48 hours; (B); lack of reaction to mechanical stimulus and equilibrium difficulties at 96 hours. "#" means all embryos died at 96h. Asterisks above the plots mean significantly different from control (Dunn's test after Kruskal-Wallis, $p<0.05$)

Chapter IV – Figure 1. General overview of TCS effects on *Danio rerio* embryo and larvae mortality and hatching during the 144 h (6 days) of exposure.

Chapter IV – Figure 2. Embryo (dots) and adults (triangles) mortality at 96 hours (mean value ± standard error)

Chapter IV – Figure 3. Zebrafish embryos and larvae abnormalities during exposure to TCS: (A) 24h old embryo exposed to 0.9 mg/L TCS with weakly pigmented retina and body; (B) Normal embryo 24h after fertilization, body and eyes with adequate pigmentation, (C) 96h larvae exposed to 0.5 mg/L of TCS with abnormal length, spine deformity and cardiac and yolk-sac edema; (D) Normal larvae (96h) with well developed tail and normal body structure.

Chapter IV – Figure 4. Variation of biomarkers activities (mean value ± standard error) on zebrafish larvae and adults after 96 h exposure to TCS: (A) ChE activity, (B) GST and (C) LDH. B. "*" mean significantly different from control treatment (Dunnet test $p<0.05$).

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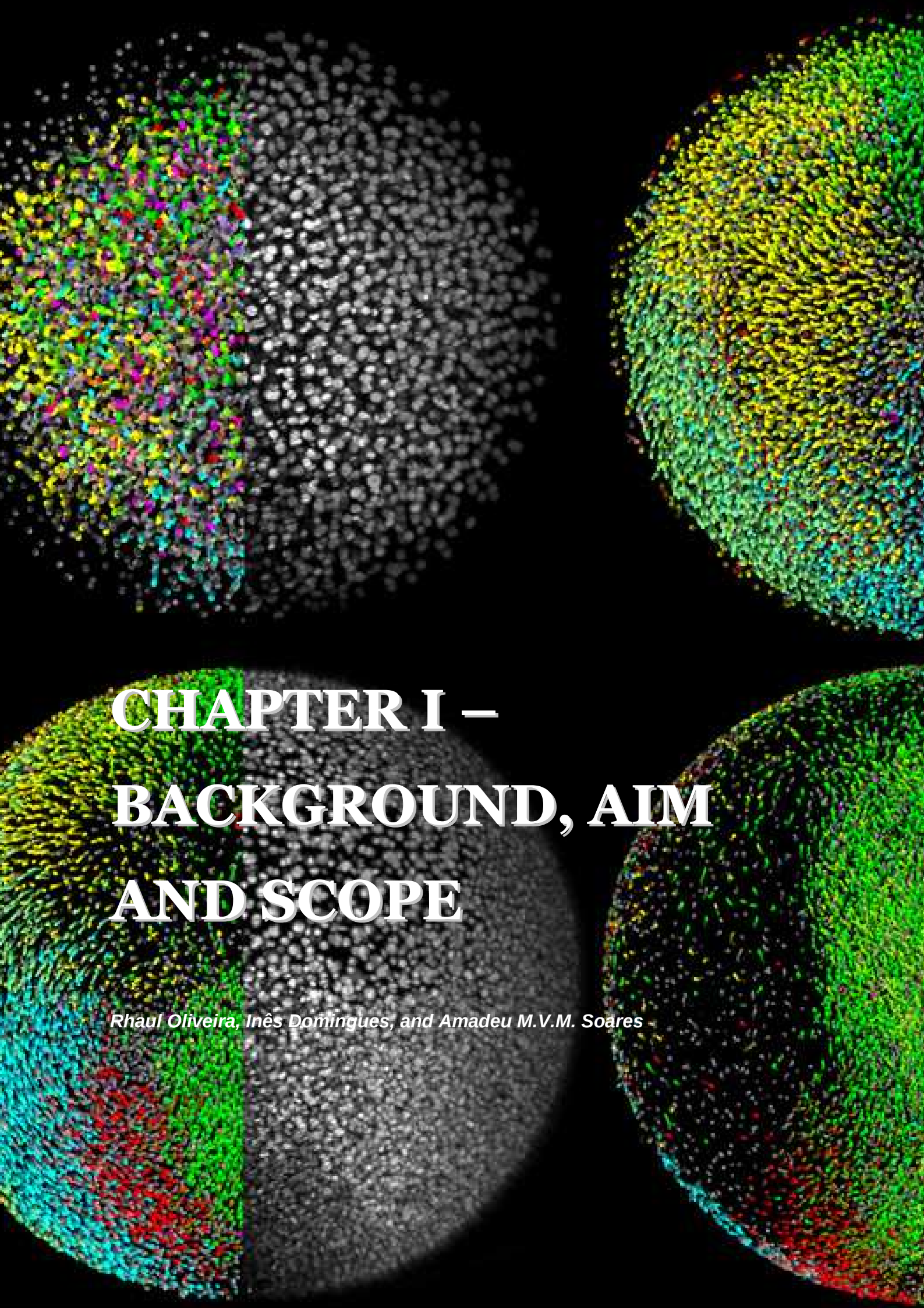


CHAPTER I – BACKGROUND, AIM AND SCOPE

Rhaul Oliveira, Inês Domingues, and Amadeu M.V.M. Soares

1. Background, aim and scope

1.1 *Danio rerio* natural history

The zebrafish (Figure 1) is a tropical freshwater fish, member of the *family Cyprinidae*. It was firstly described by Francis Hamilton Buchanan in 1822 in the book *"An account of the fishes found in the river Ganges and its branches",* together with other ten species of the genera *Danio*. *Danio rerio* is morphologically characterized by five uniformly, pigmented, horizontal stripes on the lateral side of the body, all extending onto the end of caudal fin rays, anal fin distinctively striped, lateral line absent, rostral barbels extending to anterior margin of orbit; maxillary barbels ending at about middle of opercle (Froese and Pauly, 2009). Weber and Beaufort (1916) assigned *Danio rerio* in the subgenus *Brachydanio* based in two morphological characters (1) the short dorsal fins with only seven branched rays and (2) lateral line incomplete or absent. However, in 1991, the unification of the *Danio* and *Brachydanio* was proposed due to diagnostic of characters that did not reliably separate the two groups (Barmam, 1991).

***Danio rerio* (Hamilton-Buchanan, 1822)**

Taxonomy
Plylum Chordata
Class Actinopterygii
Order Cypriniformes
Family Cyprinidae
Subfamily Rasborinidae
Genus *Danio*
Specie *Danio rerio*
Common name: Zebrafish

Adult

Larva

Embrvo

Figure 1. Zebrafish taxonomy and life-cycle stages.

Danio rerio is distributed throughout South and Southeast Asia. The natural occurrence of the species is predominantly related to the Ganges and Brahmaputra river basin localized in north-eastern India, Bangladesh and Nepal (Spence *et al,* 2008; Spence *et al,* 2006). However, others studies confirmed the presence of zebrafish all over the Indian continent (Figure 2). The zebrafish wild

habitats have been frequently described as small water bodies with a range of temperature among 24 to 38 ºC, visibility to a depth of ~30 cm, silt substrate and aquatic vegetation (Engeszer *et al,* 2007). The zebrafish is commonly encountered in shallow ponds and standing water bodies connected with rice-fields. This association is probably related with food availability and absence of predators (Spence *et al,* 2008). The zebrafish is omnivorous, and its natural diet is composed basically by zooplankton, insects and phytoplankton (McClure *et al,* 2006).

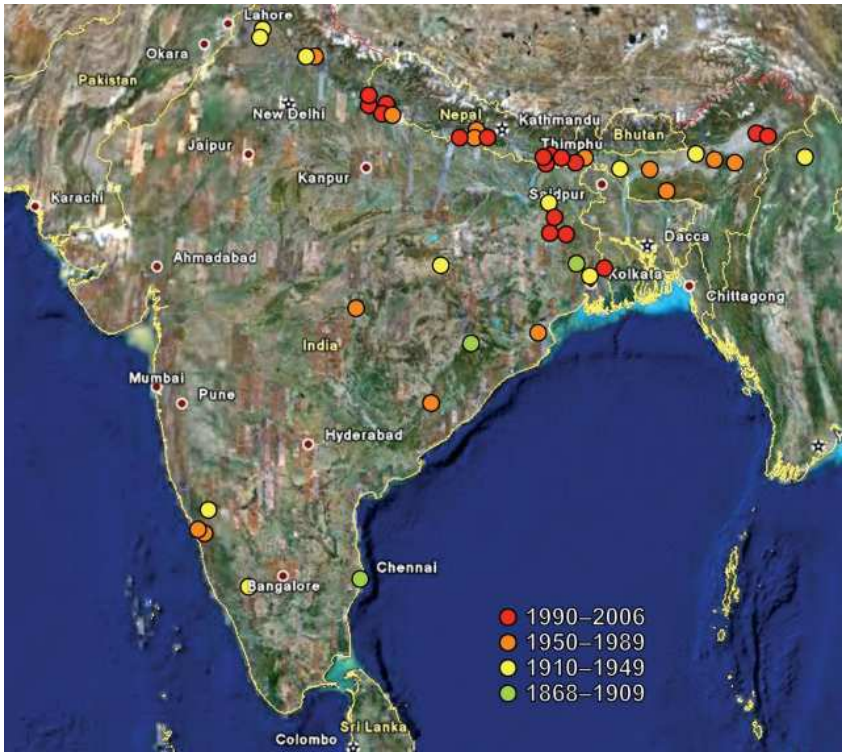


Figure 2. Zebrafish distribution registered from 1868 to 2006 (adapted from Engeszer *et al,* 2007).

Zebrafish has a very short reproduction cycle reaching the maturity age in few months (3 - 6). Field studies described zebrafish as an annual species. The spawning period is induced by temperature and commences at the onset of the monsoon season. The courtship behavior in zebrafish consists of a male chasing the female rapidly, swimming around her and often nudging her flanks. The females are choosy with respect to sites for oviposition. In general the females prefer a gravel substrate in shallow vegetated areas that offer protection for eggs

and larvae. Spawning occurs in a short period at dawn (Spence *et al,* 2008; Lawrence, 2007; Engeszer *et al,* 2007). Despite the popularity of zebrafish as an aquarium fish, nowadays it is one of the most important vertebrate model for genetic, developmental sciences, medicine, pharmacology and ecotoxicology (Spitsbergen *et al*, 2008; Scholz and Fischer 2008; Scalzo and Levin 2004; Rubinstein, 2006). However, the lack of knowledge about zebrafish ecology and natural history is a critical point for interpreting its behavior, physiology, genetic and phenotypic variation, and the evolutionary history of embryonic, larval, and adult (Engeszer *et al,* 2007).

1.2 Zebrafish under laboratory conditions

Zebrafish can be easily maintained in laboratory conditions. The male and female adults are easily indentified (Table 1). One female can produce a mean of 185 eggs per spawning; these enable a high production of organisms for tests and an effortless stock maintenance.

Table 1. Zebrafish male and female morphological characteristics.

Males	Females
 ♂	 ♀
- Yellow especially on the belly - Slimmer - Longer - Large anal fin	- More silvery - Plumper - Small anal fin - Small genital papilla

The zebrafish breeding in laboratory follows some general steps: (1) A mating cage or box with a mesh, grill or marbles in the bottom is prepared (2) one female to two males are then added to the box in the evening. (3) In the following morning, the fertilized eggs fall on the floor of the breeding box, being protected from cannibalism by adults. The most used zebrafish breeding technique in laboratory consists in breeding tanks with the bottom filled with marbles (Mullins *et*

*al,*1994). For experiments purposes, zebrafish eggs should be collected a few minutes after natural mating. Usually, washing and microscope-checking are recommended (OECD, 2006; OECD 1998). The unfertilized eggs and the ones with irregularities during cleavage or injured are discarded.

The most important factors that may affect egg production and quality are: water quality, diet and density of fish in the cage. Reductions in the overall egg production may reflect sub-optimal husbandry parameters. Usually, zebrafish are maintained in flow-thought systems. The water is treated by reverse osmosis filter. It is not clear in the current literature which is the optimal range of physico-chemical parameters for zebrafish maintenance. In the majority of the zebrafish facilities, temperature of 25 ± 3 ºC is the most usual; pH is around 7.0 – 8.0; and hardness is 100 mg/L $CaCO_3$; salinity ranges from 0.25 to 1.0 ppt (Lawrence, 2007) and dissolved oxygen levels are maintained at or just under 80 % of saturation (~ 7.8 mg/L at 28.0 ℃) to ensure fish health.

Feeding practices are also crucial in zebrafish husbandry exerting a profound impact on the feed efficiency, growth rates and gamete production of fish (Lee *et al*, 2000). The amount of food provided at each feeding and the frequency of application are important components of feeding protocols, and are often specific to both species and purposes of the culture. Standard practices for the frequency of feeding and amount of food are scarce for zebrafish but generally, to provide an adequate diet, the following parameters are taken into account: (a) number of organisms per cage; (b) life-stage; (c) environmental conditions (e.g. temperature); (d) supplies of live food as *Artemia sp.* or *Dapnhia*.

Due to the increasing importance of the zebrafish as a research model system, it is imperative that standards for its husbandry (nutrition, breeding, welfare) are developed to improve the reproducibility of experiments and the efficiency of the use of this model in research environment.

1.3 Zebrafish as general model organism

In the early 1970's, scientists of the University of Oregon determined the zebrafish as a vertebrate model for development and genetics. Dr George Streisinger (1927-1984) is considered the founding father of zebrafish research. Together with Dr Charles Kimmel and others researchers of *University of Oregon's Institute of Molecular Biology*, he published several studies on zebrafish development and genetics (Kimmel *et al,* 1995; Streisinger *et al*, 1985). Nowadays hundreds of research centers worldwide use zebrafish in fundamental and applied research.

Among the main characteristics that make zebrafish a model system logistically attractive are: (a) reduced size, (b) short life cycle, (c) short generation time (2-3 months: egg to reproductive adult), (d) good reproduction in captivity (e) possible daily breeding, (f) external fertilization (g) easily available non-adhesive eggs obtained from abundant spawning (h) optically transparent eggs that enable external observation of the embryo delopment (i) rapid embryonic development.

A great amount of information about the physiology, biochemistry and genetics of the zebrafish is available on the literature. The conclusion of the genome sequencing enabled a wide range of applications in advanced genetic and molecular biology (Van der Ven et al, 2005; Van der Ven *et al*, 2006). The transparency of the egg enables microscopic techniques to monitore individual cells in vivo across a broad range of developmental stages or throughout the duration of an experimental exposure (Lele and Krone, 1996; Scholz *et al,* 2008; Kimmel, 1995). *In vitro* techniques, such as cell culture, also permit the monitoring of biological responses rapidly and with low costs (Gosh et al, 1994). On the other hand, *in vivo* (whole animal) studies can provide improved prediction of the biological response in intact systems. Zebrafish has high similarity in cellular structure, signaling processes and physiology with other high-order vertebrate. An overall homology between *Danio rerio* and humans is also observed. This fish shares similarities regarding sense modalities, including vision, olfaction, taste, touch, balance, hearing and cognitive behavioral and their sensory pathways.

Newly developed techniques (e.g. microarrays technology, hybridizations and transgenic organisms) involving large scale screening assays are valious tools that contribute to the increasingly use of Zebrafish. Thus, zebrafish is an emerging model for drugs toxicity screening and biomedicine (Zon and Peterson, 2005; Spitsbergen and Kent, 2003; Langheinrich, 2003; Chico *et al*, 2008; Rubinstein, 2006).

1.4 Zebrafish use in ecotoxicology

1.4.2 Current protocols for ecotoxicological assessment

Fish is an important taxonomic group and traditionally used in toxicity testing of individual substances and effluents (Braumbeck and Lammer 2005). Due to the importance of fish in aquatic pollution biomonitoring, numerous guidelines have been developed for ecotoxicological evaluations. Among the model organisms defined in the guidelines, the species *Danio rerio* is one the most frequently used worldwide. At the International organization level, as OECD (Organization of Economic Co-operation and Development) and ISO (International Organization for Standardization), several protocols for ecotoxicity assessment with zebrafish (see table 2) have been developed in the last years. Initially, the protocols were established for acute toxicity assessment (OECD, 203 and OECD, 210), growth (OECD, 215) and short-term effects on early life-stages (OECD, 212). Nowadays, several modifications in old guidelines have been discussed and new draft guidelines that include more sophisticated endpoints for ecotoxicity assessment have been proposed. The new guidelines proposed by OECD have given more emphasis on specific mode of action of compounds. (eg. Endocrine disruptors), or have also been focusing in full life-cycle studies (OECD, 2008).

Table 2. Current OECD and ISO guidelines for toxicity evaluation using *Danio rerio*.

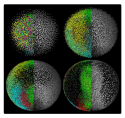
Guidelines	Description	Reference
Fish, acute toxicity test	Fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.	OECD (1992)
Fish short term toxicity test on embryo and sac-fry stage	Embryos are exposed to the test substance, preferably under flow-through conditions, or where appropriate, semi-static conditions. The test begins by placing fertilised eggs in the test chambers and is continued at least until all the control fish are free-feeding. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentrations.	OECD (1998)
Fish juvenile growth test	Juvenile fish in exponential growth phase are exposed for 28 days, after being weighed, to a range of sublethal concentrations of the test substance. Fish are fed daily. The food ration is based on initial fish weights and may be recalculated after 14 days. At the end of the test, the fish are weighed again. Effects on growth rates are analyzed using a regression model in order to estimate the concentration that would cause a x % variation in growth rate, i.e. ECx (e.g. EC10, EC20 or EC30).	OECD (2000)

Continuation table 2. Current OECD and ISO guidelines for toxicity evaluation using *Danio rerio*.

Guidelines	Description	Reference
Fish two-generation test	Parental fish exposure: the test protocol is initiated with adults that have a record of reproductive success as measured both by fecundity (number of eggs) and embryo viability (e.g., hatchability). This is established during a 7 to 21-d pre-exposure period in the same test system as will be utilized for the chemical exposure phase. Exposure of F1 generation: the viability of resultant embryos (e.g., hatching success, developmental rate, occurrence of malformations, etc.) is assessed in animals held in the same treatment regime to which the adults were exposed. Exposure of F2 generation: the viability of resultant embryos from the F1 (i.e., hatching success, developmental rate, occurrence of malformations, etc.) is assessed in animals held in the same treatment regime to which the adults were exposed.	OECD (2008)
Fish embryo toxicity	The Test Guideline is based on chemical exposure of newly fertilized zebrafish eggs for up to 48 hours and it is expected to reflect toxicity in fish in general. After 24 and 48 hrs, four apical endpoints are recorded as indicators of acute lethality in fish: (i) coagulation of fertilized eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heart-beat. Embryos are individually exposed in 24-well microplates to a range of concentrations of the test substance. Lethal effects, as described by four apical endpoints, are determined by comparison with controls to identify the LC50, NOEC and LOEC values.	OECD (2006)

Continuation table 2. Current OECD and ISO guidelines for toxicity evaluation using *Danio rerio*.

Guidelines	Description	Reference
The fish screening assay for endocrine active substances	In the assay, male and female fish in a reproductive status (enabling a clear differentiation of each sex) are exposed together in test vessels. A sex-related analysis of each endpoint is performed, ensuring the evaluation of their sensitivity towards exogenous chemicals. At test termination, sex is confirmed by microscopic examination of the gonads. On sampling at day 21, all animals are killed humanely. Blood samples are collected for determination of vitellogenin.	OECD (2008)
Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio)	Specifies a method for the determination of degrees of dilution or concentrations as a measure of the acute toxic effect of waste water to fish eggs within 48 h. It is also applicable to treated municipal waste water and industrial effluents.	ISO 15088 (2007)
Determination of toxicity to embryos and larvae of freshwater fish	A method for the determination of toxicity on fish embryos and larvae. It is also applicable to treated chemicals, waste water effluents and industrial effluents.	ISO 12890 (1999)
Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)]	Specifies a static method for the determination of the acute lethal toxicity of stable, non-volatile, single substances, soluble in water under specified conditions.	ISO 7346 (1996)



1.4.1 Zebrafish in acute toxicity assessment

The zebrafish, at all stages of life, has been extensively used in acute toxicity test. The acute toxicity data is an essential component in the base set of data requirements for environmental risk assessment and hazard classification of new substances (Braunbeck et al., 2005). Zebrafish is well-established as a model for studying ecotoxicity of solvents, heavy metals, pharmaceuticals and pesticides (see table 3 and 4).

The “Fish acute toxicity test (FATT)” (OECD, 1992) is a method performed routinely to assess ecotoxicity of chemicals (see table 3). The advantages of using acute tests are: (a) they can be applicable to a wide range of chemicals; (b) they tend to be short, reproducible, sensitive, cheap, and with low variability; (c) they are standardized, accepted by the regulatory and scientific community; (d) it is possible to compare the sensitivity of different species or organisms. Despite the simplicity of the FATT, it should be performed strictly in accordance with standard requirements and observing recommended conditions (Fairbrother et al, 2001). The aim of FATT is to identify a dose causing 50% of mortality of exposed organisms. The mortality is evaluated over 96 hours and others major adverse effects may be evaluated. Also, the minimum dose causing lethality in a short period of time (LOEC) and the dose at which are not observed effects (NOEC) are estimated. The lethality data (LC50 value) have been used by authorities in classification and labeling as well in calculation of predicted no-effect concentration of the substances (PNEC). The acute fish test provides only lethal (LC50, NOEC and LOEC) data and, therefore, has only limited pertinence, since sublethal toxicity by definition is excluded. Mortality is the primary endpoint and it is often hypothesized that fish suffer distress and perhaps pain. This method is against by European REACH regulations propose the reduction of the use of live animals’ assays.

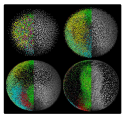
Table 3. Acute toxicity values for *Danio rerio* adults.

Substance	LC50 96h (µg/L)	Reference
1,2,3-Trichlorobenzene (123 TCB)	1850	Roex et al., (2002)
2,4-Dichlorophenol	3900	Pedersen and Petersen, (1996)

2,4-DInitrophenol	11000	Pedersen and Petersen, (1996)
2,3-DInitrophenol	7364	Lange et al., (1995)
2-Nitroanisole (2-Na)	214382000	Lange et al., (1995)

Continuation table 3. Acute toxicity values for *Danio rerio* adults.

Substance	LC50 96h (µg/L)	Reference
3,4-Dichloroanillne (3,4-DCA)	8500	Ensenbach and Nagel, (1995)
3,4-Dichloroanillne (3,4-DCA)	9800	Ensenbach and Nagel, (1995)
3,4-Dichloroanillne (3,4-DCA)	7000	Pedersen and Petersen, (1996)
3,4-Dichloroanillne (3,4-DCA)	8101000	Lange et al., (1995)
Acetone	8100000	Pedersen and Petersen, (1996)
Acroleln	14	Pedersen and Petersen, (1996)
Aldicarb	10064	Gallo et al., (1995)
Allylamine	22100	Pedersen and Petersen, (1996)
Anillne	32000	Pedersen and Petersen, (1996)
Butanol	1730000	Pedersen and Petersen, (1996)
Butyl lactate	75000	Bowmer et al., (1998)
Cadmium (-chloride)	2200	Pedersen and Petersen, (1996)
Carbaryl	6037000	Lange et al., (1995)
Carbaryl	9256000	Gallo et al., (1995)
Copper	121	Palmer et al., (1998)
Copper	127	Palmer et al., (1998)
Copper	102	Palmer et al., (1998)
Copper (II) Carbonate	49	Palmer et al., (1998)
Copper (II) Carbonate	50	Palmer et al., (1998)
Copper (II) Carbonate	40	Palmer et al., (1998)
Copper (II) Carbonate	75	Palmer et al., (1998)
Copper (II) Carbonate	79	Palmer et al., (1998)
Copper (II) Carbonate	64	Palmer et al., (1998)
Copper (II) Sulfate	6	Palmer et al., (1998)
Copper (II) Sulfate	6	Palmer et al., (1998)
Copper (II) Sulfate	5	Palmer et al., (1998)
Copper Hydroxide	4	Palmer et al., (1998)
Copper Hydroxide	4	Palmer et al., (1998)
Copper Hydroxide	3	Palmer et al., (1998)
Copper Hydroxide	30	Palmer et al., (1998)
Copper Hydroxide	31	Palmer et al., (1998)
Copper Hydroxide	25	Palmer et al., (1998)
Copper total	210	Palmer et al., (1998)
Copper total	298	Palmer et al., (1998)
Copper total	262	Palmer et al., (1998)
Diethanolamine (DEA)	3700000	Pedersen and Petersen, (1996)
Diethanolamine (DEA)	3700000	Pedersen and Petersen, (1996)
Di-*n*-butylphthalate	1300	Pedersen and Petersen, (1996)
DNOC	1981000	Lange et al., (1995)
Ethanol	14200	Pedersen and Petersen, (1996)
Ethyl lactate	320000	Bowmer et al., (1998)
Ethylene glycol	50000	Pedersen and Petersen, (1996)
Lactic acid	320000	Bowmer et al., (1998)
Lindane	100	Ensenbach and Nagel, (1995)
Lindane	110	Ensenbach and Nagel, (1995)
Lindane	120	Pedersen and Petersen, (1996)



Continuation table 3. Acute toxicity values for *Danio rerio* adults.

Substance	LC50 96h (µg/L)	Reference
Malathion	19822000	Lange et al., (1995)
Mercury (-chloride)	50	Pedersen and Petersen, (1996)
Methyl–ethyl–ketone	3200000	Pedersen and Petersen, (1996)
Methylparathion	5400	Pedersen and Petersen, (1996)
n-Heptanol	34000	Pedersen and Petersen, (1996)
n-Propanol	5000000	Pedersen and Petersen, (1996)
o-Cresol	24000	Pedersen and Petersen, (1996)
Parathion	1940	Roex et al., (2002)
Pentacholorophenol (PCP)	200	Pedersen and Petersen, (1996)
Pentacholorophenol Sodium Salt	200	Pedersen and Petersen, (1996)
Phenol	28233	Lange et al., (1995)
Tributytin oxide (TBTO)	2,7	Pedersen and Petersen, (1996)
Triethanolamine	11800000	Pedersen and Petersen, (1996)
Triphenyltin ecetate (TPTA)	40	Strmac and Braunbeck, (1999).
Triphenyltin ecetate (TPTA)	15	Strmac and Braunbeck, (1999).

Adapted from (Martins *et al,* 2007)

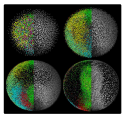
The "*Fish Embryo Test (FET)"* (OECD, 2006) has emerged as an alternative to determine the toxicity of substances and the zebrafish as an excellent model for understanding toxic mechanisms of the chemicals. Initially developed in Germany in 2001, the Fish Embryo Test (FET) has been applied as a mandatory component in routine effluent testing. After national standardization, the protocols were submitted to ISO for standardization at international level. Nowadays, the fish embryo test is a consolidated guideline by ISO 15088 (2007) and has already been submitted with modifications for OECD for acute toxicity determinations in chemical testing. Meanwhile, the scientific community worldwide started to apply the protocols and drafts for ecotoxicity assessment of many substances (table 4).

Table 4. *Danio rerio* embryotoxicity data.

Substances	EC50 48h (µg/L)	Reference
Acetone	13091232	Bachmann (2002)
Acetyl salicylic acid	53327.40	Bachmann (2002)
Acrolein	370	Schulte (1997)
4-Aminophenol	470	Schulte (1997)
Aniline	161200	Schulte (1997)
Atrazine	36800	Wiegand et al. (2000)
Benzoic acid	19539	Bachmann (2002)
4-Bromoindole	4300	Kammann et al. (2006)
5-Bromoindole	5500	ISIS (2003)

Continuation table 4. *Danio rerio* embryotoxicity data.

Substances	EC50 48h (µg/L)	Reference
5-Bromoindole	5310	Kammann et al. (2006)
6-Bromoindole	5310	ISIS (2003)
6-Bromoindole	9210	Kammann et al. (2006)
2-Bromophenol	45120	Kammann et al. (2006)
3-Bromophenol	60660	Kammann et al. (2006)
4-Bromophenol	46320	Kammann et al. (2006)
n-Butylamine	35911	Brust (2001)
sec-Butylamine	95155	Brust (2001)
Butyldiglycol	1281617	Bachmann (2002)
p-tert-Butylphenol	1730	Maiwald (1997)
Carbaryl	4700	Schulte (1997)
2-Chloroaniline	28300	Schulte (1997)
3-Chloroaniline	21000	Schulte (1997)
4-Chloroaniline	21300	Schulte (1997)
4-Chlorophenol	45600	Schulte (1997)
4-Chlorophenol	42800	Stangl (1991)
Colcemide	3417	Bachmann (2002)
Copper(II) sulfate pentahydrate	499	Bachmann (2002)
Cycloheximide	1463	Bachmann (2002)
Cyclohexylamine	63376	Brust (2001)
n-Decylamine	3146	Brust (2001)
2,4-Dibromophenol	7950	Kammann et al. (2006)
2,6-Dibromophenol	41770	Kammann et al. (2006)
Dibutylamine	40454	Brust (2001)
2,4-Dichloroaniline	21500	Schulte (1997)
3,4-Dichloroaniline	2400	Schulte (1997)
3,4-Dichloroaniline	1620	Lange et al. (1995)
Dicyclohexylamine	31187	Brust (2001)
Diethylamine	93254	Brust (2001)
Diethylene glycol	50809218	Bachmann (2002)
Diethylene glycol dimethylether	11056432	Bachmann (2002)
N,N-Diethylmethylamine	69993	Brust (2001)
N,N-Diisopropylethylamine	104563	Brust (2001)
Diisobutylamine	47195	Brust (2001)
Diisopropylamine	91477	Brust (2001)
N,N-Dimethylanilin	54320	Foerster (2008)
N,N-Dimethylbutylamine	50999	Brust (2001)
N,N-Dimethylcyclohexylamine	53055	Brust (2001)
N,N-Dimethylethylamine	82865	Brust (2001)
N,N-Dimethylformamide	10020639	Bachmann (2002)
Dimethylsulfoxide	29100000	Maiwald (1997)
4,6-Dinitro-o-cresol	600	Foerster (2008)
2,4-Dinitrophenol	900	Schulte (1997)
Dipentylamine	42786	Brust (2001)
Dipropylamine	31167	Brust (2001)
D-Mannitol	77768	Bachmann (2002)
Ethanol	11999830	Bachmann (2002)
Ethanol	11100000	Maiwald (1997)
Ethyl acetate	1859036	Bachmann (2002)



Continuation table 4. *Danio rerio* embryotoxicity data.

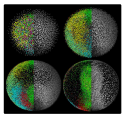
Substances	EC50 48h (µg/L)	Reference
Ethylenediamine	396660	Bachmann (2002)
1-Ethylpiperidine	71318	Brust (2001)
2-Ethylpiperidine	93958	Brust (2001)
α-D-Glucose	77072448	Bachmann (2002)
n-Heptylamine	28459	Brust (2001)
Hexamethyleneimine	115346.34	Brust (2001)
2,5-Hexanedion	4668498	Bachmann (2002)
n-Hexylamine	42297	Brust (2001)
Hydroquinone	7900	Schulte (1997)
Hydroxyurea	2311920	Bachmann (2002)
Isobutylamine	92617.70	Brust (2001)
Isoniazid	42390	Bachmann (2002)
Isopentylamine	59098	Brust (2001)
Isopropylamine	149607	Brust (2001)
Lindane	800	Schulte (1997)
Malathion	6200	Schulte (1997)
Malathion	7600	Schulte (1997)
Methanol	22100000	Ensenbach (1987)
Methoxyacetic acid	53687	Bachmann (2002)
2-Methoxyethanol	23831388	Bachmann (2002)
1-Methoxy-2-propanol	16338756	Bachmann (2002)
3-Methyl-1-butanol	1075674	Bachmann (2002)
N-Methylanilin	75000	Foerster (2008)
N-Methylformamide	22315815	Bachmann (2002)
Methyl mercury (II) chloride	144.62	Bachmann (2002)
1-Methylpiperidine	68332	Brust (2001)
2-Methylpiperidine	102350	Brust (2001)
4-Methylpiperidine	92928	Brust (2001)
Morpholine	601215	Brust (2001)
2-Nitro-4′-hydroxydiphenylamine	540	Liu et al. (2007)
2-Nitroaniline	21500	Schulte (1997)
2-Nitroanisole (2-NA)	30620	Lange et al. (1995)
4-Nitrobenzoic acid	29900	Schulte (1997)
4-Nitrophenol	4900	Schulte (1997)
4-Nitrophenol	43100	Schulte (1997)
4-Nitrophenol	57200	Ensenbach (1987)
n-Nonylamine	11462	Brust (2001)
1-Octanol	15628	Bachmann (2002)
1-Octanol	15400	Schulte (1997)
n-Octylamine	25461	Brust (2001)
Penicillin G (sodium salt)	5452461	Bachmann (2002)
Pentachlorophenol	90	Maiwald (1997)
Pentachlorophenol	1000	Stangl (1991)
Pentachlorophenol	380	Ensenbach (1987)
n-Pentylamine	30856	Brust (2001)
4-tert-Pentylphenol	3500	Baumann (2008)
Phenol	49100	Schulte (1997)
Phenol	49800	Ensenbach (1987)
Piperidine	110504	Brust (2001)

Continuation table 4. *Danio rerio* embryotoxicity data.

Substances	EC50 48h (µg/L)	Reference
Prochloraz	2900	Baumann (2008)
2-Propanol	10138870	Bachmann (2002)
n-Propylamine	79150	Brust (2001)
Quinone	472	Groth et al. (1993)
Retinoic acid	1090	Bachmann (2002)
all-trans-Retinol	610	Bachmann (2002)
Saccharin sodium salt hydrate	20947857	Bachmann (2002)
Salicylic acid	24585	Bachmann (2002)
Salicylic acid	22790	Bachmann (2002)
Sodium chloride	12009420	Lange et al. (1995)
2,4,6-Tribromophenol	4420	Kammann et al. (2006)
Tributylamine	301210	Brust (2001)
Triclosan	360	Oliveira et al. (2009)
Triethylamine	60398	Brust (2001)
Triethylene glycol	53900000	Maiwald (1997)
Tripropylamine	188833	Brust (2001)
Urea	22360338	Bachmann (2002)
Urea	22462440	Lange et al. (1995)
Urea	22900000	Schulte (1997)
Valpromide	1131509	Bachmann (2002)
Valproic acid	20189	Bachmann (2002)

Adapted from (Lammer *et al,* 2009)

The use of the maintenance of zebrafish for ecotoxicity assessment is simple. The female of zebrafish produce hundreds of eggs every week, so, large samples are easily achieved for statistically powerful dose-response studies. Zebrafish are easy to breed in the laboratory and due to the transparency of both eggs and post-hatch larvae, development can be continuously monitored and effects identified by light microscopy. In addition the FET allows the monitoring of a wide range of endpoints, allowing the detection of sublethal effects, which provide more complete information about the mode of action and ecological risk of substances. Hence, effects of chemicals on, e.g., heartbeat, pigmentation or development of the eyes and the brain may be revealed. The test can potentially be prolonged to 96 h or even 120 h to detect effects on additional sublethal endpoints such as hatching rate and larval abnormalities (OECD, 2006). Due to these many practical attributes, the FET is an important alternative for replacement or reduction of the use of adult organisms for ecotoxicity assessment, as well as for the refinement of the current toxicity tests. (Braunbeck et al. 2005).



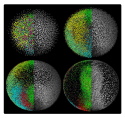
The use of animals for scientific proposes in the European Community is a subject under regulatory restrictions by the Directive 86/609/EEC. However, the embryos, including zebrafish embryos, are not classified as animals, and for that are not subjects to welfare issues described by this directive (Nagel 2002). This point is another attribute of fish embryos testing. The FET, when performed over 48h, is completely excluded from the referred regulations as this is the well defined embryo period before hatching. When FET is extended for more days (totalizing at the maximum of seven days) for evaluation of teratology and hatching or in case of the delay of toxicity of substances, post hatc larvae may be exposed to chemicals. In this case, these animals may be subjected to the mentioned regulatory directive. However, this discussion is complex. The transition from embryo, pro-larva and larvae stages are not clearly defined and different juridical interpretation may be applied. Despite the juridical aspects related to the use of zebrafish embryos in scientific experimentation, there are the ethical concerns. Even the 48 hours embryo when observed, presents all characteristics of a heathy fish and, when dechorioned, is capable of surviving (Braunbeck et al. 2005). In addition, information about the ability of the early-life stages of zebrafish to feel pain or passion is still unclear.

1.4.2 Zebrafish in sublethal effects assessment

Despite the wide use of fish acute tests in ecotoxicological evaluations, new methodologies have been developed in order to achieve a more refined and precise evaluation of chemical ecotoxicity. The new guidelines include tools for assessment of relevant effects in several levels of biological organization. The focus of new guidelines has been the identification of a greater number of sublethal effects in much lower concentrations, emphasizing the importance of methodological development in order to identify more specific modes of action of the pollutants (Braunbeck et al. 2005). In zebrafish studies, several tools have been developed for assessment of contamination in environmental samples such as identifications of DNA damage, differential gene expression evaluation, enzymatic activity alterations and teratogenic potential.

Many studies have been using zebrafish eggs and adults for the assessment of contamination of river and tropical lakes sediment by in situ and laboratorial assays (Kosmehl et. al, 2007). The same group that has developed field techniques with zebrafish adults and early life stages has also elaborated the protocols for genotoxicity assessment in embryos by adaptation of established techniques as micronuclei and comets assay (Nagel, 2002; Kosmehl et. al, 2007). However, the majority of studies have used only zebrafish adults for genotoxicity assessment (Grisolia *et al*, 2009; Oliveira *et al*, *in press*). The difference relies basically in the fact that for adults an adequate amount of blood for erythrocytes analyses is available, as in case of embryos, others cell type has been chosen for application of the techniques (Nagel, 2002). In adults and embryos assays, authors have been focusing in the evaluation of the potential of chemical and/or metabolites to cause genetic damage. In general, genotoxicity assays address different interactions between chemicals and the DNA. In tha past few years, a wide variety of genotoxicity assays using adult fish have been used in ecotoxicological evaluations of environmental pollutants. The majority of the approaches involve the detection of DNA strand breaks that are produced by the chemical exposure. As a second instance, few studies about fish chronic exposure to genotoxic chemicals are available. The long-term exposure to a genotoxic chemical may induce a cascade of events resulting in changes in gene frequency in populations and mutational events (Oost *et al*, 2003).

Moreover, at molecular level, the zebrafish is one the most important vertebrate model for ecotoxicogenomic evaluation. This relatively new scientific field, derived from a combination of the fields of ecotoxicology and genomics, is concerned with the identification of potential environmental toxicants and their mechanisms of action (MoA), through the use of genomic resources. One of the most used resources are the DNA microarrays or "chips," that allow the monitoring of the expression of thousands of genes simultaneously and that can be used as a highly sensitive and informative marker for toxicity (Lettieri, 2006; Neumann and Galvez, 2002). It has been supported that gene expression arrays may significantly enhance our ability to assess both the characteristics of exposure and the subsequent effects induced by contaminants. Genomic approaches using fish



(especially zebrafish, whose the genome has already been totally sequenced) promise increased investigative power, and have already provided insights into the mechanisms that underlie short-term and long-term environmental adaptations (Park *et al,* 2005). Others studies by (Weil et al. in press, Scholz et al. 2008) have made use of reverse transcriptase polymerase chain reaction, gel electrophoresis and densitometric analysis of the gels for monitoring potential markers genes. These studies are based on the hypothesis that analysis of gene expression could be used to predict chronic fish toxicity. The increment of FET with a gene expression analysis is a promising tool with applications in regulatolry context.

Many important enzymes in fish metabolic pathways may be induced or inhibited upon exposure to xenobiotics. Conventionally called as biomarkers, has been used on *Danio rerio* in several studies. The enzymes Cholinesterases (ChEs), lactate dehydrogenase (LDH), cytochrome P450 1A (CYP1A), oxidative stress enzymes, and the proteins metallothioneins (MTs) and vitellogenin (VTG) are the most investigated biomarkers for ecotoxicological assessment of pollutants on *Danio rerio* (Table 5).

Table 5. Principal biomarkers used in ecotoxicological evaluations with *Danio rerio* model.

Protein	Function description	Some studies with *Danio rerio*
Cholinesterases (ChEs)	ChEs are a group of enzymes belonging to the family of esterases that hydrolyze carboxylic esters and have been divided in two types: acetylcholinesterase (AChE) and butyrylcholinesterase. AChE has a key role in the maintenance of normal nerve function and is inhibited by neurotoxic compounds like organophosphate insecticides. Inhibition of acetylcholinesterase leads to an accumulation of the neurotransmitter acetylcholine in the synapse resulting in a disruption to the nervous system function.	• Pacheco et al. (2007) decribed effects of ethanol on acetylcholinesterase activity and gene expression in zebrafish brain. • Kuster (2005) characterized cholinesterase in developing zebrafish embryos and their potential use for insecticide hazard assessment. • Roex et al. (2002) described alterations on AChE activity by 2,3-trichlorobenzene and parathion exposure.
Lactate dehydrogenase (LDH)	LDH is an important enzyme in the muscular physiology. Increased LDH activity levels have been observed in conditions of chemical stress when high levels of energy are required constituting a marker for tissue damage.	• Sancho et al. (2009) showed effects of tricyclazole on LDH activity zebrafish (*Danio rerio*) and post-exposure recovery • Klyachko and Ozernyuki (2004) explained about the different functional and structural properties of lactate dehydrogenase isozymes at different stages of *Danio rerio* ontogenesis

Continuation table 5. Principal enzymatic biomarkers used in ecotoxicological evaluations with *Danio rerio* model.

Protein	Function description	Some studies with *Danio rerio*
Cytochrome P450 1A (CYP1A)	Member of the family of Cytochrome P450 the CYP1A is a liver microsomal enzyme used as biomarker for organic xenobiotics (e.g. Polychlorinated Biphenyls (PCBs), dioxins, polycyclic aromatic hydrocarbons (PAHs) Induction of CYP1A is the best studied biomarker for environmental contamination in aquatic ecosystems.	• Jonsson et al. (2009) showed effects of benzo[a]pyrene and β-naphthoflavone exposure on CYP1A levels. • Billiard et al. (2006) toxicity of polycyclic aromatic hydrocarbons to zebrafish using CYP1A activity as biomarker.
Oxidative stress	The production of reactive oxygen species (ROS) can induce oxidative damage and may be a mechanism of toxicity for aquatic organisms living in environments receiving water-borne contaminants. Environmental contaminants such as herbicides, heavy metals and insecticides are known to modulate antioxidant defensive systems and to cause oxidative damage in aquatic organisms by ROS production, which can be measured by using superoxide dismutase, catalase, glutathione peroxidase and Glutathione S-transferase (GST) activities as well as GSH and lipid peroxidation as biomarkers.	• Hu et al. (2008) study the biological toxicity effect of Tetrabromobisphenol A (TBBPA) by zebrafish embryo toxicity assays in combination with three biomarkers, including superoxide dismutase (SOD), lipid peroxidation (LPO), and heat shock protein (Hsp70). • Jimena et al. studied effects microcystin toxicity in embryos-benefits and costs of microcystin detoxication using CAT, GST, Gpx enzymatic assays. • Wiengand et al. (2001) realized GST assays for understanding toxicokinetics of atrazine in zebrafish embryos.

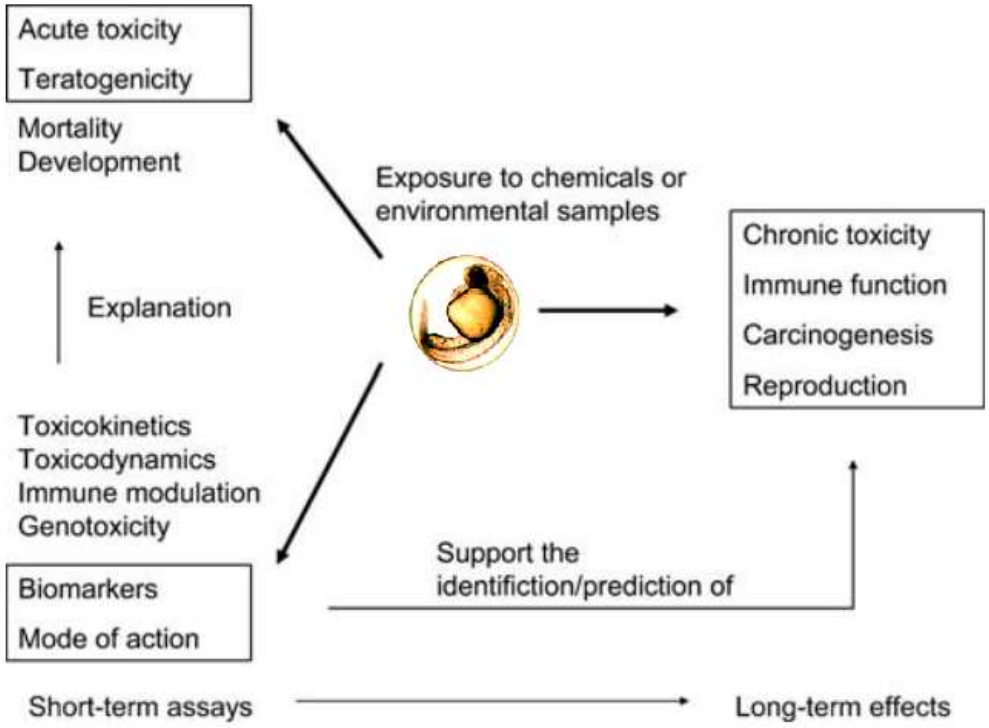
Continuation table 5. Principal enzymatic biomarkers used in ecotoxicological evaluations with *Danio rerio* model.

Protein	Function description	Some studies with *Danio rerio*
Metallothioneins (MTs)	MTs are low-molecular-mass, highly conserved, and cysteine-rich proteins devoted to the binding, regulation, and detoxification of transition-class metals. Most animal species possess several MT isoforms, which may differ from each other by the position of only a few amino acids. Metals are specifically bound to the protein backbone by means of the S atoms of its cysteine residues, forming characteristic metal–thiolate clusters. Metallothioneins can carry out many different functions, such as Zn-mediated gene regulation, detoxification of nonessential metals, and homeostasis/regulation of essential trace elements.	• • • Chen et al. (2006) studied metallothioneins (MTF-1 nuclear) activity induced by zinc and cadmium. • Riggio et al. (2003) studied changes in zinc, copper and metallothionein contents during oocyte growth and early development of zebrafish. • Yan and Chan et al. (2001) described a method for characterization of metal induction of metallothionein gene, using transient expression assays in a zebrafish caudal fin cell line, SJD.1

Continuation table 5. Principal enzymatic biomarkers used in ecotoxicological evaluations with *Danio rerio* model.

Protein	Function description	Some studies with *Danio rerio*
Vitellogenin (VTG)	VTG is a serum precursor protein of the egg yolk. The induction of VTG has been widely used as an effective and sensitive biomarker for the detection of endocrine disrupting chemicals (substances that induce an estrogenic response by mimicking the action of endogenous estrogens) in the aquatic environment.	• Van der Ven et al. (2003) applied several techniques (histochemistry, immunohistochemistry, and in situ mRNA hybridization) for vitellogenin expression evaluation. • Orn et al. (2003) evaluate effects of ethinylestradiol and methyltestosterone vitellogenin production. • Van den Belt et al. (2003) compared the vitellogenin responses between zebrafish and rainbow trout following exposed to estrogens.

In summary, the Danio rerio represents a model organism with multiple applications in environmental sciences, particulary in early life-stages of development (see figure 3). Furthermore, the validation of the use of early-life stages of zebrafish in risk assessment procedures will probably promote a change at international scale on traditional methodologies adopted for environmental risk evaluation of aquatic ecosystems.



1.4.

Figure 3. Summary approaches and applications of zebrafish embryo in ecotoxicological evaluations (adapted from Scholz et al. 2008).

2. Research plans and goals

Acute fish toxicity and embryo toxicity tests are two important approaches to understand risk of chemicals. In this study, knowledge was acquired in order to better understand the effects of the selected chemicals on zebrafish, using eggs and adults bioassays associated with various sublethal endpoints.

The main aim of this study was the use of multiple techniques to assess the toxicity of different classes of chemicals to *Danio rerio* at lethal and sublethal levels (embryotoxicity, genotoxicity and enzymatic alterations) performing an integrative analysis of the tested endpoints at the different phases of *Danio rerio* life-cycle (Figure 4). This allowed the identification of an individual toxicity profile for each chemical tested.

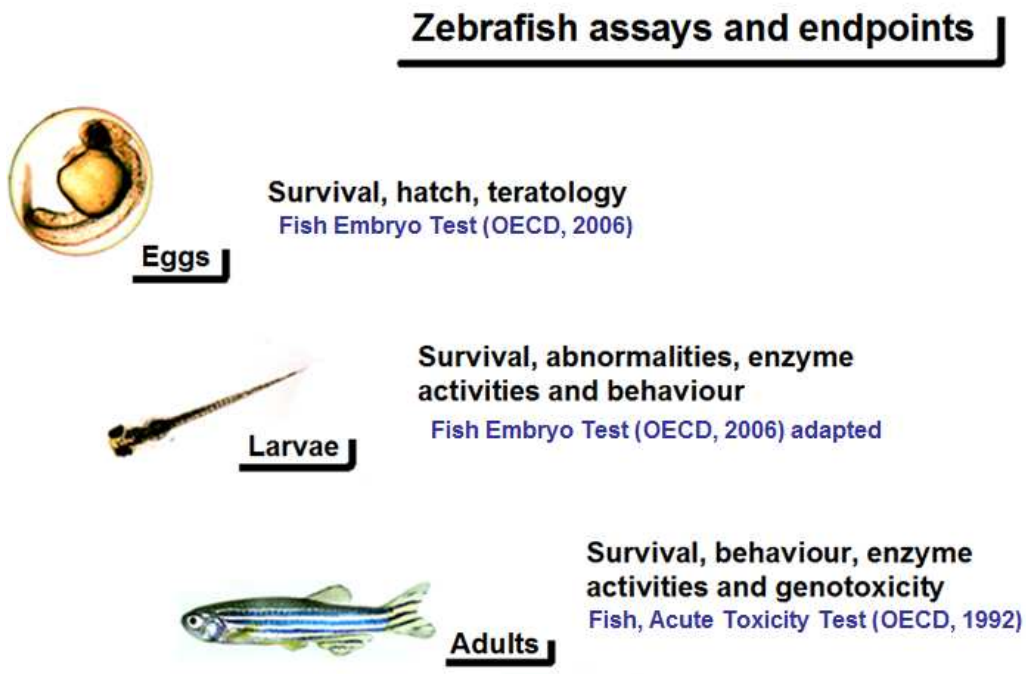


Figure 4. *Danio rerio* bioassays and measured endpoints.

The first phase of this study comprised the selection of chemicals in order to contemplate different type of contaminants. Potassium dichromate (heavy metal), triclosan (antimicrobial agent) and δ-endotoxins (biopesticide) were selected due to their different modes of action, use and potential risk. Preliminary assays were performed to determine the adequate dose levels for assays.

In the second phase, exposure of the fertilized zebrafish eggs to different ranges of the selected toxicants was performed following the guideline Fish Embryo Test (OECD, 2006). Embryo assays provide information on the effects of the chemicals at developmental level, detecting delays and anomalies in the development of specific organs. Enzymatic analyses were performed on post-

hatched larvae (96 hours) exposed to triclosan and potassium dichromate, providing information on effects at biochemical level. The results obtained in this part provided a strong knowledge about the toxicity of selected compounds on zebrafish early-life stages.

In the third part, experimental approaches with zebrafish adults under standard conditions were conduced. After preliminary assay with adults and based in embryotoxicological data, assays following the guideline 203 (OECD, 1992) were performed. Acute toxicity tests were adapted in order to allow genotoxic and enzymatic analysis. The results provided information on the effects of the selected compounds on zebrafish survival, nuclear and DNA integrity, enzymatic biomarkers and behavior.

3. Dissertation structure

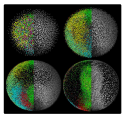
The present dissertation is structured in six chapters:

Chapter II – Toxicity of Chromium VI to Zebrafish Early Life-Stages and Adults

The objective of this chapter is to compare the toxicity of potassium dichromate (PD) in zebrafish early-life stages and adults. Several lethal and sub-lethal endpoints were analyzed in organisms exposed to PD such as mortality, embryo development and behaviour, hatching, micronuclei, DNA damage and biochemical markers (cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH));

Chapter III – Genotoxic Evaluation of Different Δ-Endotoxins from Bacillus Thuringiensis on Zebrafish Adults and Development in Early Life-Stages

The objectives of this chapter were (i) to evaluate the effects of the δ-endotoxins cry 1Aa, cry 1Ab, cry 1Ac, and cry 2A on *Danio rerio* (zebrafish) early



stages and adults and (ii) to explore the toxicological consequences of gene pyramiding in transgenic Bt-crops by testing of binary mistures;

Chapter IV – Effects of Triclosan on Zebrafish Early Life-Stages and Adults

In this chapter, the toxicity of TCS in zebrafish (*Danio rerio*), embryos and adults, was studied. Several lethal and sub-lethal endpoints were analysed in organisms exposed to TCS such as mortality, embryo development and behaviour, hatching, micronuclei and biochemical markers (cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH));

Chapter V – General Discussions: an Integrative View

In this chapter, general conclusions were taken and linkages among different studies were made. Moreover, sensitivity of the different endpoints used was compared. An integrative view about use of zebrafish as model for chemicals risk evaluation is presented.

Chapter VI – Recommendations and Perspectives

This chapter contains recommendations and perspectives on the use of *Danio rerio* in ecotoxicology.

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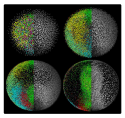

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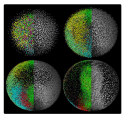

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CHAPTER II – TOXICITY OF CHROMIUM VI TO ZEBRAFISH EARLY LIFE-STAGES AND ADULTS

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For submition to Aquatic Toxicology (2009)

Abstract

Hexavalent chromium is very often an issue in ecotoxicity studies due to its importance as a pollutant that threats especially aquatic ecosystems and due to its alue as a reference toxicant in standard toxicity tests. Hexavalent chromium (Cr VI) is used in several industries including wood preservation, leather tanning, metal finishing, pigments, refractory, etc, whose waste waters can be highly contaminated with this metal. Cr VI is used as a reference substance because its ability to provide reproducible test results, its universal toxicity, its persistence, water solubility and easy quantification. This work aimed at comparing the toxicity of potassium dichromate (PD) in zebrafish early life-stages and adults. Several lethal and sub-lethal endpoints were analyzed in organisms exposed to PD such as mortality, embryo development and behaviour, hatching, micronuclei, DNA damage and biochemical markers (cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH)).

Embryo/larvae assay followed the OECD guideline on Fish Embryo Toxicity Test. Embryos were exposed at measured concentrations of 0, 50, 118, 183, 225, 281 and 290 mg/l of PD for 6 days and were inspected daily with the help of a stereomicroscopy for mortality, developmental parameters (egg coagulation, otolith formation, eye and body pigmentation, somite formation, heart-beat, tail circulation, detachment of the tail-bud from the yolk sac) and hatching. A similar test was run to obtain larvae for ChE, GST and LDH analysis. The adult test followed the OECD Guideline TG 203 in semi-static conditions. Adult zebrafish were exposed to concentrations of 0, 60, 90, 120, 150 and 180 mg/l of PD for 96 h and were daily inspected for mortality and behavior alterations. A second test was run to obtain organs for biomarkers analysis: Heads, muscles and gills were isolated and snapfrozen in eppendorfs and used for ChE, LDH and GST determinations, respectively. Adult zebrafish testing also comprised a third test for micronucleus and nuclear abnormalities analysis in which the concentrations of 0, 95 and 135 mg/l were use; and a fourth test for assessment of DNA damage through the “comet” assay in which the concentrations of 0, 60, 90 and 120 mg/L

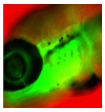
of PD were used.

PD showed low toxicity for embryos while inside the egg being observed low levels of mortality and no anomalies or delays on embryo development. Once hatched mortality increased, larvae with malformations were observed at 72 h and larvae with swimming and equilibrium disturbances were observed at 96 hours. Biomarkers measured at 96 h showed to be very sensitive: ChE and GST activities decreased in a dose dependent pattern while LDH increased in the lowest concentrations (50 and 118 mg/L) and decreased in the highest concentration (281 mg/L of PD). A 96h-LC50 of 362.4 and 112.8 mg/L was calculated for early life-stages and adults respectively, however, biomarkers measured on adults did not seem to be as sensitive as early life-stages since only GST was affected (dose dependent inhibition). High frequency of micronucleated erythrocytes was observed in the concentration of 135 mg/L while evidences of DNA damage were found for the concentration of 60, 90 and 120 mg/L where higher frequencies of categories 3 and 4 “comets” were observed compared to control.

Dissimilar 96 h-LC50 values found for D. rerio embryos and adults may result from the protection of the egg shell in the pre-hatching period. Embryo/larvae assay was more informative than adult assay, showing important effects on biomarker levels, larvae malformations and behavior. PD showed to be genotoxic for adult fish but induced biochemical alterations only on GST.

The importance of zebrafish early life-stages assays in the assessment of chemicals effects is reinforced with this work. The wide range of endpoints that can be used along with the higher sensitivity of biochemical parameters, provide information at very different levels, contributing to a better understanding of chemicals mode of action and being a valid substitute of adult assays.

Keywords: zebrafish, potassium dichromate, genotoxicity, embryotoxicity, biomarkers, *Danio rerio*



1. Introduction

The heavy metal Chromium has several oxidation states but it occurs predominantly in two forms: trivalent (Cr3+) and hexavalent (Cr6+) states. Unlike trivalent chromium, the hexavalent form rarely exists naturally being originated by anthropogenic sources. Hexavalent chromium (Cr (VI)) is very often an issue in ecotoxicity studies for two main reasons: (i) due to its importance as a pollutant that threats especially aquatic ecosystems and (ii) due to its value as a reference toxicant in standard toxicity tests.

Cr (VI) is used in industry (wood preservation, leather tanning, metal finishing, pigments, refractory, etc) (Barnhart, 1997). Very often wastes from such industries are used as a fill material at numerous locations where Cr (VI) easily reaches groundwater by leaching and seepage from the soils. The tanning industry especially contributes to chromium aquatic pollution, being estimated that in India alone about 2000 to 3200 tone of elemental chromium escape into the environment annually (Zayed and Terry, 2003). Table 1 shows studies where effects of Cr (VI) on fish were investigated. Generally these works show that Cr (VI) acts as a strong oxidizing agent (leading to alteration of levels of oxidative stress enzymes such as catalase and glutathione reductase), it causes histological and morphological alterations on kidneys, liver and gills and it is genotoxic (inducing micronucleus, nuclear abnormalities and “comets”). Alterations on the metabolism and hematological indices as well as behavioral disturbances were also noticed in many studies.

Table 1. Literature dealing with effects of hexavalent chromium on several fish species (tests performed with adult fish).

Species	Cr (VI)	Conditions	LC_{50}	Endpoint	Response	Ref
Pimephales promelas	$K_2Cr_2O_7$	7d, 2.5 mg/L		• Genotoxicity	• Induction of micronucleus	(De Lemos et al., 2001)
Oreochromis niloticus	$K_2Cr_2O_7$ (intraperitoneal)	24h, 1.78 mg/Kg		• Biomarkers	• Increase in SOD, and lipid peroxidation, no changes on CAT or Cytochrome P450 or b5	(Tagliari et al., 2004)
Cyprinus carpio	$K_2Cr_2O_7$	2-42 d, 0.001-0.1 mg/L		• Genotoxicity	• Induction of micronucleus	(Zhu et al., 2002)
Hypostomus plecotomus	$K_2Cr_2O_7$	15d, 12 mg/L		• Genotoxicity	• Induction of micronucleus	(Normann et al., 2008)
Oreochromis niloticus	Cr effluent	6d, 20% effluent		• Genotoxicity	• Induction of micronucleus and nuclear abnormalities (binucleated erythrocytes) •	(Cavas and Ergene-Gozukara, 2005)
Poecilia reticulate	$K_2Cr_2O_7$	7 d, $4.27x10^{-4}$ - $6.40x10^{-4}$ mol/L	7d-LC_{50}= $3.42x10^{-4}$ mol/L	• Life cycle	• Increased life span	(Perez-Benito, 2006)

Continuation table 1. Literature dealing with effects of hexavalent chromium on several fish species (tests performed with adult fish).

Species	Cr (VI)	Conditions	LC_{50}	Response	Ref
Carassius auratus	$K_2Cr_2O_7$	96h, 10 and 50 mg/L		• Biomarkers • Oxidation of lipids and proteins, SOD activity was decreased in liver and kidney and CAT only in liver; GST was not affected.	(Lushchak et al., 2008)
Oncorhynchus mykiss	$Na_2Cr_2O_7$	28 d, 10 mg/L (LC_{10})	96h-LC_{50}= 69.5 mg/L	• Biomarkers • MT induction, lipid peroxidation, SOD • Histology • Histological changes on gills and liver. • Behavior • Decreased feeding behavior • Growth • Decreased growth	(Roberts and Oris, 2004)
Channa puctatus	$K_2Cr_2O_7$	96h, 20 and 40 mg/L	96h-LC_{50}= 41.75 mg/L	• Behavior • Erratic swimming and lethargy • Histology • Histological changes in gills, kidney and liver, alteration in ovarian histology.	(Mishra and Mohanty, 2008)
Labeo rohita	$K_2Cr_2O_7$	96h, 80-140 mg/L	96h-LC_{50}= 111.45 mg/l	• Physiology • Secretion of mucus, loss of scales, discoloration. • Behavior • Surfacing and darting movements, lethargy and erratic swimming suggesting loss of equilibrium (highest conc.) • Metabolism and blood indices • Decreased metabolic rate (oxygen consumption) and changes in hematological indices (hemoglobin, total erythrocyte count and mean cell hemoglobin) at LC_{50}. • Biomarkers • Glycogen, total lipid and total protein were affected at LC_{50} concentration.	(Vutukuru, 2005)

Continuation table 1. Literature dealing with effects of hexavalent chromium on several fish species (tests performed with adult fish).

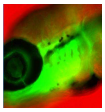
Species	Cr (VI)	Conditions	LC_{50}		Response	Ref
Oreochromis sp.	Cr (VI)	96h, 43.7 mg/L	96h-LC_{50}= 43.7	• Genotoxicity	• Molecular analysis (RAPD fingerprinting pattern) revealed Polymorphic bands on treated fish.	(Abbas and Ali, 2007)
				• Biomarkers	• Glycogen, total lipid and total protein were affected at LC_{50} concentration.	
				• Histology	• Vacuolar degeneration in the liver.	
Oreochromis niloticus	K_2CrO_4	Up to 30d, 0.05 mg/L;	No mortality	• Biomarkers	• Concentration of blood urea nitrogen decreased (10d exposure) and cholesterol increased.	(Oner et al., 2008)
Fundulus heteroclitus	K_2CrO_4	7 d, 0, 1.5, or 3 mg/L		• Gene expression	• altered the expression of 10 genes in adult liver (involved in energy metabolism)	(Roling et al., 2006)
Pimephales promelas	CrO_3	48h	22.46 µg/L			(Baral et al., 2006)
Gambusia affinis	CrO_3	28d, 77.62 mg/L (LC_{10})	96h-LC_{50}= 151.95 mg/L	• Biomarkers	• Induction of SOD and CAT, Lipid peroxidation product, malondialdehyde was enhanced in the viscera tissue.	(Begum et al., 2006)
				• Hystology	• Alterations in gill morphology like hypertrophy and hyperplasia in secondary lamellae followed by detached epithelium with severe necrosis	
				• Behavior	• Perturbation of locomotor behavior (decrease in distance travelled and in swimming speed). They became lethargic and secreted copious amount of mucus from the whole body	
Odonthestes bonariensis	$K_2Cr_2O_7$	96h, 0.5-80 mg/L	96h-LC_{50}= 1.45-8.20 mg/L			(Carriquiriborde and Ronco, 2002)

Continuation table 1. Literature dealing with effects of hexavalent chromium on several fish species (tests performed with adult fish).

Species	Cr (VI)	Conditions	LC_{50}		Response	Ref
Odonthestes Bonariensis	$K_2Cr_2O_7$	134 d, 0-266 µ/L		• Biomarkers • Growth • Histology	• Elevated products of lipid peroxidation, • reduced growth and survival, • Gross and microscopic abnormalities: necrosis (death), fibrosis (scarring) and dilation of tubular lumina affected kidneys, The gill and spleen had subtle microscopic changes, Microscopic changes in the liver and pancreas provided evidence of differences in energy storage and metabolism.	(Carriquiriborde and Ronco, 2008)
Oreochromis niloticus	Tannery effluents	72h, 0.05mg/L		• Genotoxicity	• Increase on nuclear abnormalities (2.53%) of the erythrocytes (blebbed, notched and lobed nuclei) and MN (0.45%); comet assay showed DNA damage.	(Matsumoto et al., 2006)
Oryzias javanicus	$K_2Cr_2O_7$	96h, 24-131 mg/L	96h-LC_{50}= 7.4 mg/L			(Koyama et al., 2008)
Carassium auratus	$Na_2Cr_2O_7$	96h	96h-LC_{50}= 4.89 mg/L			(Velma and Tchounwou, 2008)

Continuation table 1. Literature dealing with effects of hexavalent chromium on several fish species (tests performed with adult fish).

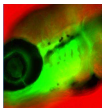
Species	Cr (VI)	Conditions	LC_{50}	Response	Ref
Oncorhynchus mykiss, ***Gasterosteus aculeatus,*** ***Rutilus rutilus,*** ***Perca fluviatilis*** ***Leuciscus leuciscus***	$K_2Cr_2O_7$	96h, 24-133 mg/L	96h- LC_{50}= 28.5, 38.3, 49.3, 33.1 and 71.7 mg/L respectively.		(Svecevicius, 2006)
Channa punctatus	$K_2Cr_2O_7$		96h- LC_{50}= 41.75 mg/L		(Mishra and Mohanty, 2008)



Cr (VI), especially potassium dichromate has been recommended for use as a reference toxicant because of its ability to provide reproducible test results, its universal toxicity, it is persistent and non-degradable, it is water soluble and easily quantified. Reference toxicant tests provide a means of monitoring animal health, laboratory precision and aspects of the overall quality of the toxicity test (Dorn et al., 1987). In several standard toxicity tests such as Daphnids (OECD, 2004) and rainbow trout, fathead minnow, Daphnia spp, Ceriodaphnia dubia, Selenastrum caprocornutum and threespine stickleback (EC, 1990) potassium dichromate has already been adopted as a reference toxicant. With the increasing use of zebrafish, (Danio rerio, Hamilton-Buchanan 1822) as a model organism in studies of developmental biology, physiology, molecular genetics and toxicology, it is important to evaluate Cr (VI) as a reference substance for this species, studying the effects at several levels of biological organization and at different life stages. A deep knowledge on effects on early life-stages (embryos) is particularly important since tests with embryos have been increasingly used to assess the toxicity of chemicals and waste waters and have been replacing, mainly for ethical reasons, tests with adult organisms. In fact, tests with embryos show numerous advantages, first of all the high egg yield oz zebrafish allows the obtaining of large quantities of eggs that can be used in a simple experimental set up (using 24-wells microplates) involving low volumes of test substances. Secondly, eggs are transparent, allowing the monitoring of the entire organogenesis which is completed within the first 48h of development. Finally, embryo tests have been showing to be very informative allowing the study of a wide range of endpoints including developmental (anomalies and delays of embryo development including hatching) and biochemical (biomarkers) parameters (eg. Oliveira et al., in press).

Table 2. Literature dealing with effects of hexavalent chromium on early life-stages of several fish species.

Species	Cr (VI)	Conditions	LC_{50}/ NOEC	Endpoint	Response	Ref
Cyprinus carpio (8hpf eggs)	$K_2Cr_2O_7$	372-545 mg/L	120h-LC_{50}= 464.91 mg/L; 48h (post hatching)- LC_{50}= 458.94 mg/L	• Hatching • Development	• No effect. • Bowing of vertebral column, deformities of the head, missing fin margins; strangulation and enlargement of the yolk sac, swelling of the heart area.	(Krejci and Palikova, 2006)
Clarias gariepinus (immediately after fertilization)	$K_2Cr_2O_7$	5 d, 11-114 mg/L	LOEC= 36 (embryos) and 20 (larvae) mg/L	• Hatching • Development	• No effect. • Body length reduced at 11 mg/L, body weight at 64, abnormal body axis (stunted body) from 36 mg/L.	(Nguyen and Janssen, 2002)
Danio rerio (immediately after fertilization)	$K_2Cr_2O_7$	12 d, up to 86 mg/L	LOEC= 86 (embryos) and 43 (larvae) mg/L. LC_{50}= 47.7 mg/L	• Hatching • Development • Growth	• No effect. • No abnormalities found, • LOEC= 43 mg/L	(Nguyen and Janssen, 2001)
Fundulus heteroclitus (larvae within 48 h of hatching)	K_2CrO_4	30 d, 0 - 24 mg/L	NOEC=1.5, LOEC=3 mg/L (growth)	• Gene expression	• Differentially expressed 16 genes in a dose-dependent manner (involved in energy metabolism or growth)	(Roling et al., 2006)
Brachydanio rerio (blastula stage)	$K_2Cr_2O_7$	16 d, 188-240 mg/L	NOEC=15mg/L LOEC= 60 mg/L	• Hatching	• No effect, LOEC>240 mg/L	(Dave et al., 1987)
Oncorhynchus tshawytscha (eyed egg)	Contaminated groundwater.	98 d, 0.79-260 µg/L	No mortality	• Development • Growth • No effect	• No effect	(Patton et al., 2007)
Lates calcarifer (11 and 24 mm frys)	$K_2Cr_2O_7$	96h, 10 - 45 mg/L	96h-LC_{50}= 27.9 and 33 mg/L	• Histology	• Separation of layers of retina, edematous degeneration of kidney tubules, and depletion of cells in the tubular interstitium were noticed, while, gill showed extensive lamellar Degeneration.	(Krishnani et al., 2003)



In this work, we aim at performing a comparative study of the effects of Cr (VI) as potassium dichromate on early life-stages and adults of zebrafish using mortality and biomarkers as endpoints. Moreover, effects on embryonic development will be recorded and genotoxicity will be evaluated in the adults through the monitoring of micronucleus (MN), nuclear abnormalities (NA) and through the comet assay.

Biomarkers have been widely used in aquatic environment to detect stressors such as pollutants or changes in environmental factors. Their sensitivity allows the identification of a potential hazard before it is verified at higher levels of biological organization, being an important tool in the prevention of risk. The biomarkers selected for this work include cholinesterase (ChE), an important enzyme in the maintenance of normal nerve function (Olsen et al., 2001); glutathione S-transferase (GST), a family of enzymes with a key role in the biotransformation of xenobiotics and endogenous substances (Hyne and Maher, 2003) and lactate dehydrogenase (LDH) which is involved in the carbohydrate metabolism (Diamantino et al., 2001).

Regarding the endpoints of genotoxicity, micronuclei are cytoplasmatic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Therefore, their presence in the cells is indicative of structural and /or numerical chromosomal aberrations. Nuclear abnormalities, other than micronuclei, are also considered to be indicators of genotoxic damage and may complement MN scoring (Cavas and Ergene-Gozukara, 2005). In its turn, the Comet assay (single cell gel electrophoresis (SCGE) assay) is a sensitive method able to detect DNA strand breaks at single cell level (Hartmann et al., 2003) indicating a loss of DNA integrity that can be induced by various contaminants that are capable of interacting with the DNA (Matsumoto et al., 2006; Nogueira et al., 2006).

In this way, in this work, the test with zebrafish adults and embryos exposed to potassium dichromate will allow a comparison of responses between life stages and the selection of the most sensitive and informative test to assess effects of chemicals. Moreover, toxicity parameters obtained (LC50) can be used as indicative values for other tests with zebrafish where potassium dichromate is used as reference substance.

2. Materials and methods

2.1 Chemical

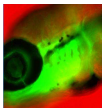
Potassium dichromate (PD) from Riedel-de Haen was used in this work.

2.2 Test organisms

Zebrafish (Danio rerio) from a culture established at the Department of Biology, University of Aveiro are maintained in carbon-filtered water at 27.0 ± 1 ºC; under a 16:8h light: dark photoperiod cycle. Conductivity is kept at 550 ± 50 µS, pH at 7.5 ± 0.5 and dissolved oxygen at 95% saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

2.3 Early stages assay

The assay was based on the OECD guideline on Fish Embryo Toxicity (FET) Test (OECD, 2006) and on the embryo test described by (Fraysse et al., 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 eggs or with irregularities during cleavage or injured were discarded. 72 eggs per treatment were used (distributed in 12 replicates). Eggs were placed in each well individually with 2 ml of test solution. The following measured concentration of PD (K2Cr2O7) were used: 0,



50.4, 118.3, 182.8, 225.4, 281.2 and 290.9 mg/l. Test solutions were prepared by dilution of stock solution in water (pH 7.00 ± 0.5 and conductivity 500 ± 50 µS). The temperature during the test was 26.0 ± 2 ºC. Embryos and larvae were daily observed with the help of a stereomicroscopy. Magnification used for observations of eggs was 70 x and larvae was 40 x. Test run for 6 days. In the embryo phase the following parameters were evaluated: egg coagulation, otolith formation, eye and body pigmentation, somite formation, heart-beat, tail circulation, detachment of the tail-bud from the yolk sac and hatching. After hatching, the following parameters were evaluated in the larval phase: heart-beat, oedemas, tail malformation, larval behaviour and mortality. A second test was performed for collection of larvae for biomarkers analyses. Test ended at day 4 when clusters of eight larvae were snap-frozen in eppendorfs with 0.4 ml of the adequate buffer (described ahead). Concentrations used were the same except for the last concentration that was skipped. Samples were stored at - 20 ºC until enzymatic analysis.

2.4 Adults assay

The assay with adult fish followed the OECD Guideline TG 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. All test solutions were prepared by dilution of stock solutions in water (pH 7.00 ± 0.5 and Conductivity 500 ± 50 µS). The normal water values of dissolved oxygen were maintained above 80 % of air saturation: a 16:8-h light:dark photoperiod cycle was used and the temperature was set to 26.0 ± 2 ºC. During the 96-h acute toxicity experiment, test solutions were daily exchanged to maintain water quality and PD concentration. The following concentrations of PD were used: 0, 60, 90, 120, 150 and 180 mg/l. Groups of eight Danio rerio were exposed per treatment. The fish were not fed during the test period. Mortality was recorded each 24 h.

A second test using a similar design was run to allow the use of organs for biomarker analysis. Four treatments were used: control and PD treatments at nominal concentrations of 60, 90 and 120 mg/l. Groups of 12 Danio rerio were

used per treatment. At the end of the test, the number of dead fish was recorded and the living fish were sacrificed. Heads, muscles and gills were isolated and snap-frozen in eppendorfs with 0.5 ml of the adequate buffer and used for ChE, LDH and GST determinations, respectively. Samples were stored at - 20 ºC until enzymatic analysis.

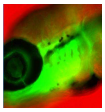
2.5 Biomarkers assay for adults and larvae

Assays were performed to analyse Cholinesterase (ChE), Glutathione S-transferase (GST) and lactate dehydrogenase (LDH) activities on larvae and adults of Danio rerio. All collected samples were immediately frozen at -80 ºC in adequate buffer until analysis. On the day of enzymatic analysis, samples were defrosted on ice and the Ystral GmbH D-7801 was used for homogenisation.

For ChE analyses, heads of adults or clusters of eight larvae per concentration were homogenised on ice on 0.4 ml of potassium phosphate buffer (0.1 M, pH 7.2). The supernatant obtained after the centrifugation of the homogenate (4 ºC, 6000 rpm, and 4 min) was removed and used as enzyme extract for ChE activity determination. Total ChE activity was performed at 414 nm according to the Ellman's method (Ellman et al., 1961), adapted to microplate (Guilhermino et al., 1996).

For the LDH determinations the dorsal muscle of adults or clusters of eight larvae were homogenised in 0.5 and 0.4 ml of Tris–NaCl buffer (0.1 M, pH 7.2) respectively, on ice. Samples were centrifuged (4 ºC, 6000 rpm, 3 min) and the supernatant was removed and used to determine LDH activity. LDH activity was measured following the methodology described by Vassault et al. (1983) with the modifications introduced by Diamantino et al. (2001).

For the GST determinations a pair of gills or clusters of eight larvae was homogenised in 0.5 and 0.4 ml of potassium phosphate buffer (0.1 M, pH 6.5). After centrifugation (4 ºC, 8367 rpm, 30 min) the supernatant was used to determine GST activity. GST activity was measured following the general



methodology described by Habig and Jakoby, (1981) and adapted to the microplate reader by Frasco & Guilhermino (2002).

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute per mg of protein. Protein concentration in the samples was determined in quadruplicate by the Bradford method (Bradford, 1976b), at 595 nm, using γ-globulin as standard. A Labsystem Multiskan EX microplate reader was used for all biochemical determinations.

2.6 Micronucleus (MN) and nuclear abnormalities (NA) assay

The zebrafish micronucleus assay followed the same procedure of the adults assay but with fewer treatments: 0, 95 and 135 mg/l and only 6 replicates per treatment. The micronucleus test was carried out as described by Hooftman and Raat (1982) for fish erythrocyte cells. Peripheral blood was obtained by cardiac puncture with a heparinized syringe and immediately smeared. After fixation in ethanol for 15 min, slides were left to air-dry and then were stained GIEMSA at concentration of 5%. Three thousand erythrocyte cells with complete cytoplasm were scored by fish. 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 must not touch the main nuclei, (c) MN must be a nonrefractive, circular or ovoid chromatin body showing the same staining pattern as the main nucleus (Al-Sabti and Metcalfe, 1995). Nuclear abnormalities (NAs) were classified according to Carrasco et al. (1990). Cells with two nuclei were considered as binuclei (BN). Blebbed nuclei (BL) present a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than the blebbed nuclei which could have several lobes are classified as lobed nuclei (LB). Nuclei with vacuoles and appreciable depth into a nucleus that does not contain nuclear material were recorded as notched nuclei (NT). From each fish, two slides were prepared and 1,000 cells were scored under 1000×

magnification after fixation in ethanol for 15 min and stained by with 5% GIMSA solution for 20 min.

2.7 Comet assay

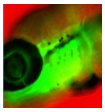
Fish were exposed similarly to the adult assay but with fewer treatments: 0, 60, 90 and 120 mg/l.

The assay was conducted with whole blood under yellow light, to prevent UV-induced DNA damage, and performed as described by Nogueira et al. (2006).

Visual scoring of cellular DNA on each slide was based on the categorization of 100 randomly-selected cells. The comet-like formations were visually graded into five classes, depending on DNA damage level adapted from García et al. (2004): undamaged – no tail visible (class 0); low damage – tails with low fluorescence and head still round and bright (class 1); medium damage – head and tail equally bright (class 2); high damage – small head, and a long and very bright tail (class 3); extreme damage – very long tail, while head is no longer round (class 4). Each cell was assigned a value (from 0 to 4) according to its comet class, and the overall score for 100 cells ranged from 0 (100% of comets being in class 0) to 400 (100% of comets in class 4) (Garcia et al., 2004). The number of comets in each class was counted, and average DNA damage (DD) was calculated as follows:

$$DD = \frac{1n_1 + 2n_2 + 3n_3 + 4n_4}{\Sigma / 100}$$

Formula 1: where n1–n4 is the number of comets in class 1–4 and Σ is the sum of all counted comets (n1 + n2 + n3 + n4). DD is expressed in arbitrary units (Collins et al., 1995).



Positive controls were always included, and consisted of cells exposed for 1h to H_2O_2 before the layering of cells.

2.8 Statistical analysis

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS, 2004). One-way ANOVA was performed except when data did not pass the Kolmogorov Smirnov normality test and so a Kruskal-Wallis test was performed. If significant results were found, the Dunett or Dunn's test was used to verify differences between tested concentrations and control. Lethal concentrations at 50% (LC50) for embryo, larvae and adults were calculated using ToxRat Professional (Ratte, 2009) All statistical analyses were performed with a significance level of 0.05.

3. Results

3.1 Embryotoxicity of potassium dichromate

In the present study fertilized zebrafish eggs were exposed for 144 hours to several concentrations of PD. The control group presented a normal embryo development as described by Kimmel et al. (1995). Post-hatching success in the control was above 70% (81.9%) as it is required for test validity. PD showed low toxicity for embryos while inside the egg. Figure 1 shows mortality of organisms during the exposure period and it is evident that effect of PD was very slight on the first days (where a LC50 could not be calculated) and increased drastically after hatching (see Table 3 for LC50 values). Embryo development parameters (otolith formation, eye and body pigmentation, somite formation, heart-beat, tail circulation, detachment of the tail-bud from the yolk sac) where neither affected. Embryos started to hatch at day 2 (10-29% of the eggs) and on day 3 100% of the embryos had hatched. No statistically significant differences were observed on the hatching rate between concentrations (day 2: One-way ANOVA, $F_{6, 83}=2.051$, $P=0.069$; day 3: One-way ANOVA on Ranks, $H=8.494$, $P=0.204$).

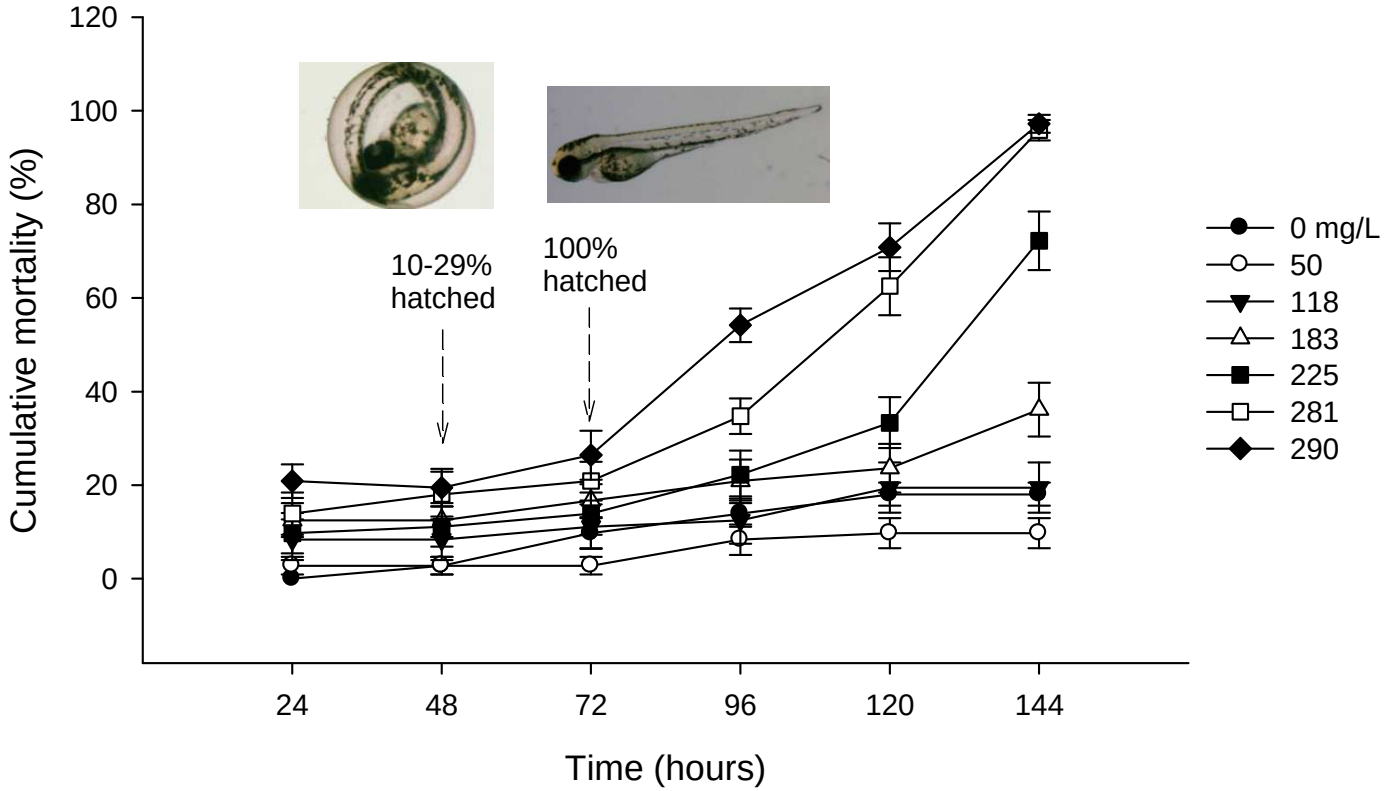


Figure 1. Cumulative mortality observed for the several concentration of potassium dichromate tested over the exposure period 6 days (144 hours) of the test. Values are mean ± standard error. Percentages of hatching at 48 and 72 hours are signed in the graph.

72 hours after the beginning of the exposure, some larvae presented general malformations including shorter body size and spine deformities (Fig 2 B). This effect was higher at concentrations of 225 and 290 mg/L (9.6 and 14.1 % respectively) although statistical significance was not observed (One-way ANOVA on Ranks, H=7.390, P=0.286). At 72 hours mortality at highest concentration was 26 %. At 96 hours larvae presented a series of behavioural disturbances characterized by a difficulty in keeping a normal upright position in water; larvae were constantly moving the fins in order to avoid lying in one side, they were floating upside down and were unable to swim down to bottom (Fig 2 D). This behavior was significantly different from control at concentrations higher than 50 mg/l (One-way ANOVA on Ranks, H=39.264, P<0.001). Mortality at highest concentration was 54% and and a LC50 of 362.4 mg/L (95% CI= 253.4-14758.0) was calculated (Table 3, Fig 1).

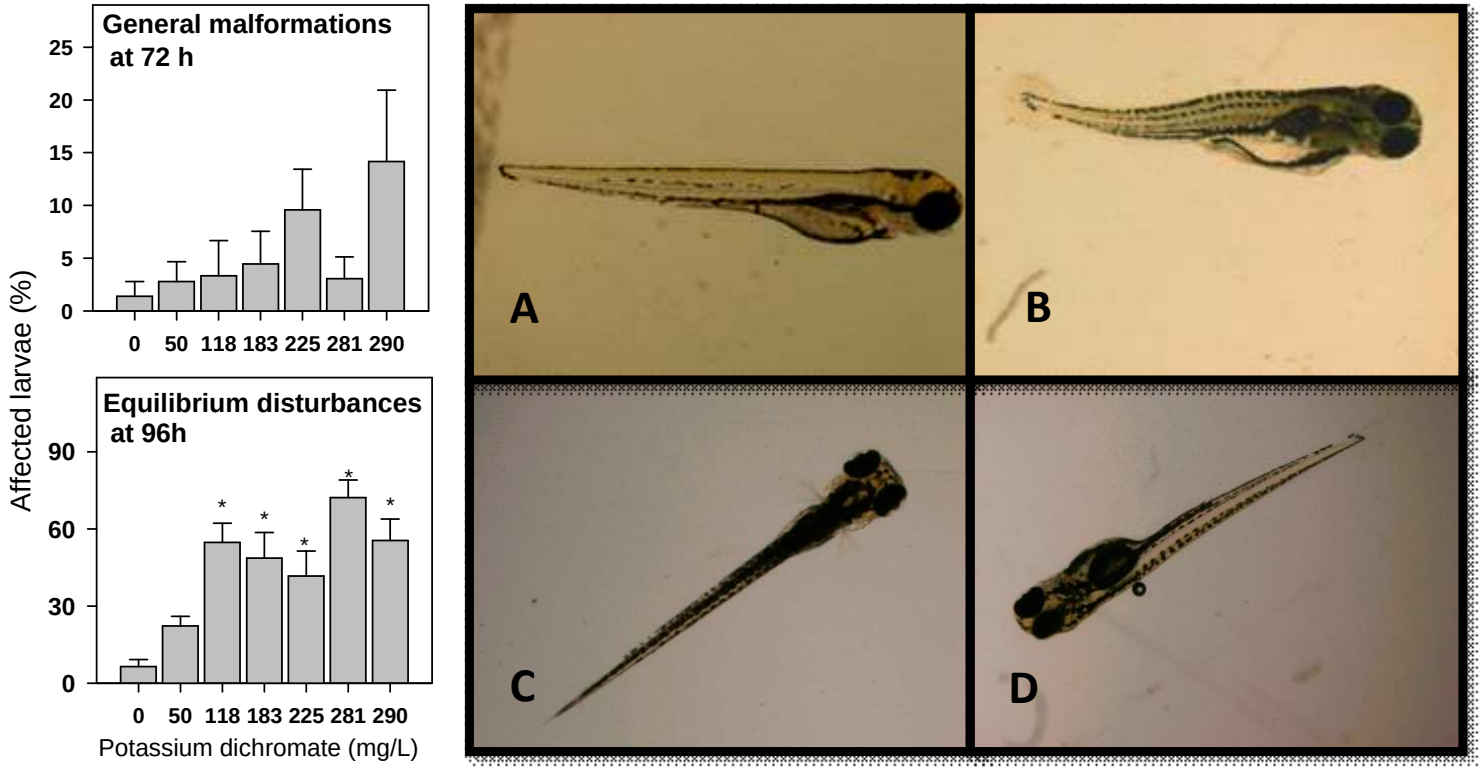


Figure 2. Developmental and behavioral anomalies observed during the test. Plate A shows a larvae from control at 72 h of exposure while plate B shows a larva from the concentration of 183 mg/L with general malformation. Upper graph illustrates percentage of general malformations at 72h. Plate C shows a larva from control at 96 h of exposure while plate B shows a larva from the concentration of 118 mg/L with equilibrium disturbances. Lower graph illustrates percentage of larvae with behavioral anomalies at 96 h. An asterisk (*) represents significant differences from control (Dunn's method).

The effects of PD on the activities of the biomarkers ChE, LDH and GST of larvae are presented in Fig. 3 (d, e and f): a dose dependent ChE activity inhibition was observed although results were only statistically different from control in the last concentration (one-way ANOVA: F5, 38 = 6.13; P <0.001) (Fig. 3d); GST activity was drastically inhibited in the 3 last concentrations when compared to control (Kruskal-Wallis: H = 35.83; P< 0.001) (Fig. 3e) and LDH activity was increased in larvae exposed to the concentrations of 50 and 118 mg/L and inhibited in the highest concentration tested (281 mg/L) (one-way ANOVA: F5, 40= 50.35; P <0.001) (Fig. 3f).

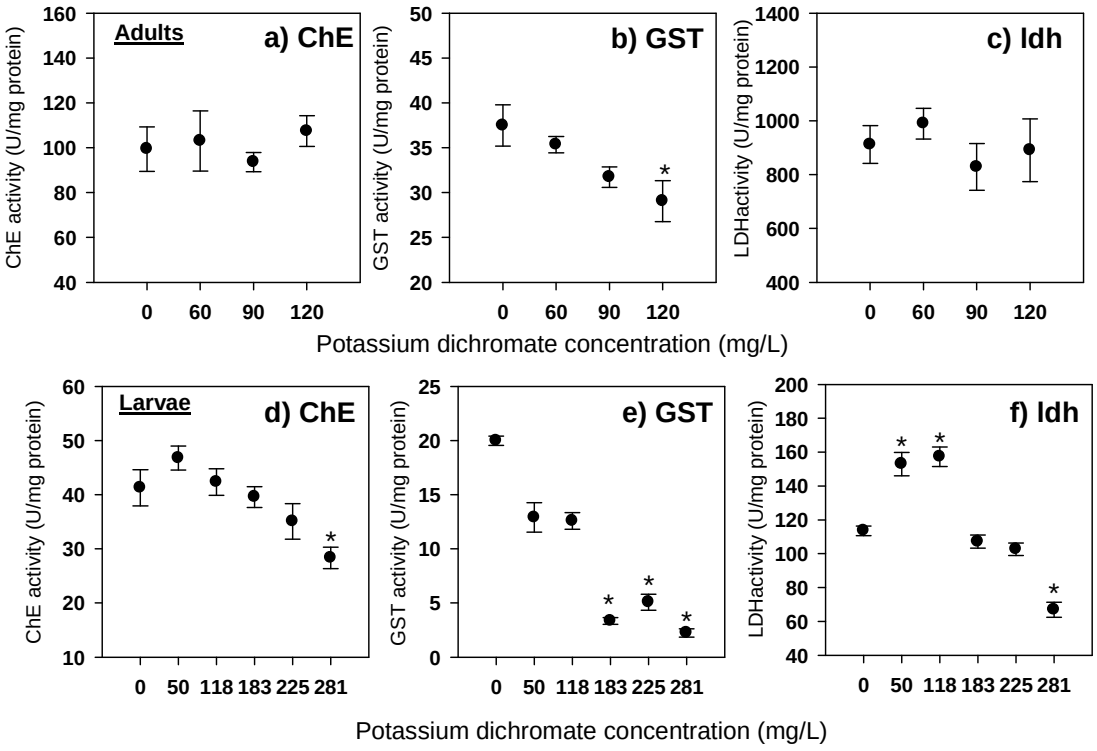


Figure 3. Variation of biomarkers activities (mean value ± standard error) on zebrafish larvae and adults after 96 h exposure to potassium dichromate: a, b and c: ChE, GST and LDH activities on adults; d, e and f: ChE, GST and LDH activities on larvae. Asterisks mean significantly different from control treatment (Dunett test P<0.05 after 1-way ANOVA, or Dunn's test after One-way ANOVA on Ranks).

3.2 PD toxicity for adults

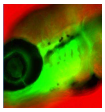
The 96 h-LC50 value for adult Danio rerio was 112.8 mg/L (95% CI: 86.1-129.9) (Table 3).

Table 3. LC_{50} and LC_{10} values for zebrafish early life-stages and adults exposed to potassium dichromate using Weibul model on ToxRat. n.d.: not determined due to mathematical reasons or inappropriate data.

Early life stages	LC_{50} (mg/L)	95% CI (confidence interval)	LC_{10} (mg/L)	95% CI
24h	n.d.	n.d	98.48	26.9-146.8
48h	n.d.	n.d.	96.31	33.1-139.9
72h	680.33	441.1-2549.6	127.16	65.0-164.8
96h	362.42	253.4-14758.0	88.42	0.1-145.4
120h	245.17	177.0-430.5	79.56	2.6-130.6
144h	176.15	85.1-218.4	88.46	7.9-134.9
Adults				
48h	141.38	130.3-149.1	126.37	101.6-135.0
96h	112.76	86.1-129.9	70.94	31.1-90.6

In vivo effects of PD on ChE, LDH and GST activities are presented in Figure 3 (a, b and c). No alterations on the enzymatic levels were observed for ChE or LDH (ChE: one-way ANOVA: F3, 21 = 0.32; P= 0.81, see Fig. 3a and LDH: ANOVA: F 3, 21=0.7; P=0.59, see Fig 3c). GST activity was dose dependent inhibited although only at last concentration activity was significantly different from control (one-way ANOVA: F3, 20 = 4.29; P= 0.02, see Fig. 3b).

Effects of PD on the number of micronuclei (MN), nuclear abnormalities (NA) and DNA integrity are presented in Fig 4. The number of MN found on fish erythrocytes was affected by PD concentration (one-way ANOVA: F2, 17= 7.92;



P= 0.04) with differences towards control being observed at highest concentration tested (135 mg/L); the number of total nuclear abnormalities seemed to be higher in all treatments compared to control however differences were not statistically significant (one-way ANOVA: F2, 17= 1.37; P= 0.284), see Fig 4A. DNA integrity was significantly affected by PD exposure (one-way ANOVA: F 3, 17= 29.06; P< 0.001) with all concentrations exhibiting higher DNA damage than the control (see Fig 4B). The incidence of the different “comet” classes is most of the times affected by PD concentration (Fig 4C). Class 1 comets were affected by PD concentration (one-way ANOVA: F3, 17= 7.99; P= 0.002) being more abundant in the control; class 2 comets were not affected by PD concentration (one-way ANOVA: F3, 17= 2.49; P= 0.102); class 3 and 4 were affected by PD (one-way ANOVA: F3, 17= 9.78; P< 0.001 and one-way ANOVA: F3, 17= 8.83; P= 0.002 respectively) being more abundant in the highest concentration.

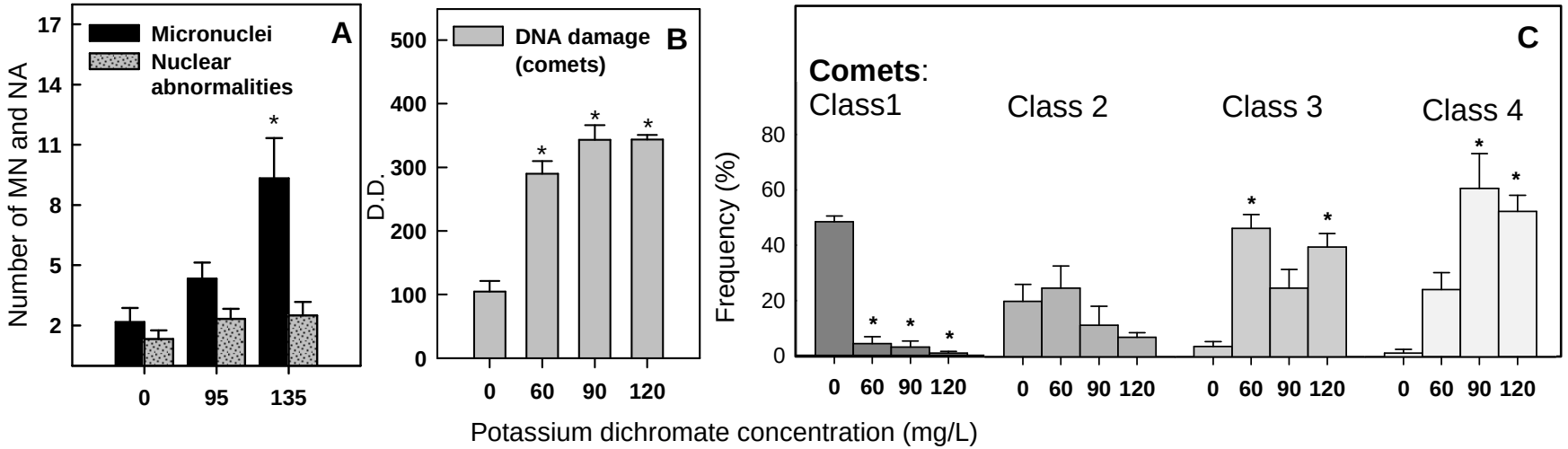
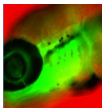


Figure 4. Endpoints of Genotoxicity. Graph A: Total number of micronuclei and nuclear abnormalities found per treatment group. Graph B: DNA Damage. Graph C: Comets of each category found per treatment group. Comet-like formations were visually graded into five progressive classes (0, undamaged; 4, extreme damage), depending on DNA damage level. Results are expressed as mean ± Standard error. An asterisk (*) represents significant differences from control (Dunnet method following One-way ANOVA).



4. Discussion

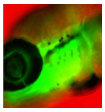
Low embryotoxicity of potassium dichromate to fish eggs comparatively to post-hatched embryos (larvae) has already been reported by several authors (see Table 2): Nguyen and Janssen (2001, 2002) worked with Clarias gariepinus and Danio rerio and calculated separately LOEC values for embryos and larvae finding in both cases a greater sensitivity of larvae; Krejci and Palikova (2006) worked with the common carp Cyprinus carpio and found no differences between the 120h-LC50 and the 48h-post hatching LC50 contributing to the idea that embryos while inside the egg are well protected against PD effects. These last authors suggested that the previtelline membrane of fish eggs may constitute a barrier preventing PD to accumulate in the embryos before hatching. Similarly no hatching delay was observed in the above mentioned works and others such as Dave et al (1987) in accordance with our results. In what concerns development abnormalities, works performed with Clarias gariepinus and Cyprinus carp describe anomalies in the post-hatched larvae including mainly problems related to the vertebral column (abnormal body axis, bowing of vertebral column) but also with head, fins, yolk sac and heart (Nguyen and Janssen, 2002; Krejci and Palikova, 2006). In our work 72h larvae also presented spine deformities agreeing with the previous data. No data on literature was found to support the observations of equilibrium disturbances verified at 96h, but the alterations of the biomarkers levels observed (Fig 3 d, e and f) might explain this behavior.

The three biochemical parameters showed to be very sensitive to chromium exposure and to our knowledge, the biomarkers analyzed have not been measured before in zebrafish larvae exposed to PD.

ChEs are a family of enzymes important for neurotransmission, being responsible for the degradation of the neurotransmitter acetylcholine in the cholinergic synapses. In our work, ChE was inhibited in a dose dependent way

suggesting disruption of the nervous system which may cause adverse effects on several functions including respiration, feeding and behavior (Cunha et al., 2007). Thus, the behavioral disturbances observed at 96h are probably related to the inhibition of ChE. Although ChEs are known for being inhibited by neurotoxic compounds like organophosphates and carbamate pesticides (Olsen et al., 2001) many authors such as Frasco et al (2005) suggest that ChEs can be inhibited by metals as well. Moreover we found several examples on literature (although none dealing with fish larvae) of ChEs being inhibited by hexavalent chromium, supporting our own findings, as it is the case of the work of Jemec et al (2008) with Daphnia magna, the work of Guilhermino et al (1998) with Mytilus galloprovincialis and the work of Elumalai et al (2002) with the marine crab Carcinus maenas.

Biotransformation mechanisms in organisms are divided into two phases: in the phase I is involved the enzyme system P450-MO catalyzing a variety of oxidative reactions and converting the initial substance into more water-soluble compounds. GSTs are a family of enzymes that play a central role in phase II being involved in the detoxification of both xenobiotics and endogenic compounds that conjugate with glutathione. Therefore, induction of GST activity has been used as a biomarker of exposure to xenobiotics with electrophilic centers. However, as Kostarapoulos et al (2005) described, contradictory results have been obtained after exposure to heavy metals, with increases or decreases in the activity being observed according to the test organism and tissue analyzed. In literature, works performed with hexavalent chromium indicated an increase in the activity measured in Daphnia magna (Jemec et al., 2008); a decrease in Carcinus maenas (Elumalai et al., 2002), no effect in the GST measured on liver or kidney of Carassius auratus (Lushchak et al., 2008) or according with the tissue analyzed a decrease (liver and kidney) or absence of response (gut) in Rana ridibunda (Kostaropoulos et al., 2005). In our work a clear inhibition of GST was observed, but since no data is available on literature regarding GST on zebrafish exposed to PD and since works with other organisms remain inconclusive regarding behavior of GST, interpretation of data becomes difficult. However, GST seemed to be a very sensitive endpoint and responded in a dose-dependent manner.



LDH is a key enzyme in the anaerobic pathway of energy production (it catalyses the interconversion of pyruvate to lactate in glycolysis). Several works indicate that many types of organisms including fish suffer a decrease on LDH levels after exposure to heavy metals. Some examples are the crab Carcinus maenas after exposure to Cr(VI) (Elumalai et al., 2002), the (post-hatching) larvae of the African catfish (Clarias gariepinus) exposed to lead nitrate (Osman et al., 2007), the fish Channa punctatus after exposure to mercuric chloride (Sastry and Rao, 1984) and the fish Oreochromis niloticus exposed to cadmium (Almeida et al., 2001). In this work we also observed a significant inhibition of LDH at last tested concentration of PD. However, in the lowest concentrations tested a significant increase of LDH was observed, which is neither a surprising result since many examples in literature refer to increases of LDH activities in organisms subjected to a situation of stress, as a way of getting additional energy from the anaerobic pathway to undergo detoxification mechanisms (Vieira et al., 2008). Some illustrative examples are Daphnia magna exposed to sodium bromide and 3,4-dichloroaniline (Guilhermino et al., 1994), Carcinus maenas exposed to zinc and mercury (Elumalai et al., 2007), Pomatoschistus microps exposed to 3,4-dichloroaniline (Monteiro et al., 2006) and even a case study with workers of electroplating industries revealed increases in LDH levels in blood samples (Saraswathy and Usharani, 2007). According with our results, the type of effect on LDH activity seems to depend on the PD concentration, suggesting that at low doses there is an increase on the anaerobic pathway of energy production probably to overcome energetic costs of detoxification processes, but at high doses cellular metabolic activity is inhibited (at 96h, the highest concentration tested caused between 30 and 40% mortality). Patterns of different LDH response over time or toxic concentration have been already observed in works such as the prawn Macrobrachium malcolmsonii exposed to endosulfan (Bhavan and Geraldine, 2001) and D. magna after in vitro exposure to zinc (Diamantino et al., 2001) and in vivo exposure to 3,4-dichloroaniline (Guilhermino et al., 1994).

All the biomarkers testes were responsive to PD exposure, LDH seemed to be the most sensitive with statistically significant differences detected at the lowest concentration tested (50 mg/L).

In the adult test, a 96h-LC50 value of 112.8 mg/L was calculated (see Table 3). In literature a wide range of 96h-LC50 values can be found for several species of fish (see Table 1) but no comparative value for zebrafish could be found. Comparing adult and early life-stages 96h-LC50 values (see Table 3) adults seem to be much more sensitive than early stages; however it should be taken into account that the 96h exposure of the early stages includes approximately 48h of embryo development inside an egg where embryo is protected from PD effects. For this reason, a more realistic comparison would be between the 96h-LC50 for adults and the 144h-LC50 for early stages and doing so, the sensitivities of the two stages are not so dissimilar.

Biomarkers seemed to be much less sensitive in adults where only GST was responsive to PD exposure (showing the same type of behavior observed in the larvae: dose dependent inhibition). In the work of Oliveira et al. (in press) in which Zebrafish was exposed to Triclosan, biomarkers measured on larvae also showed much higher sensitivity then adults.

Fish have been used as models for genotoxicity monitoring in aquatic environments and MN and NA tests have been showing to be sensitive indicators of chromosome damage (Hooftman and Raat, 1982; Carrasco et al., 1990; Al-Sabti and Metcalfe, 1995; Cavas and Ergene-Gozukara, 2005). Several authors have studied the effect of hexavalent chromium in several fish species such as Hypostomus plecotomus, Cyprinus carpio, Oryzias latipes and Pimephales promelas (De Lemos et al., 2001; Zhu et al., 2002; Goodale et al., 2008; Normann et al., 2008), observing an induction in the number of MN and confirming in this way Cr (VI) clastogenic potential, which was also observed in our work. Two studies where Oreochromis niloticus was exposed to chromium contaminated effluents showed increased frequencies of MN and NA (Cavas and Ergene-Gozukara, 2005; Matsumoto et al., 2006). DNA damage, analyzed through the comet assay, was observed in all concentrations tested in agreement with works performed with other fish species (Leuciscus cephalus and Oreochromis niloticus) exposed to chromium contaminated effluents (Matsumoto et al., 2006; Ergene et al., 2007).

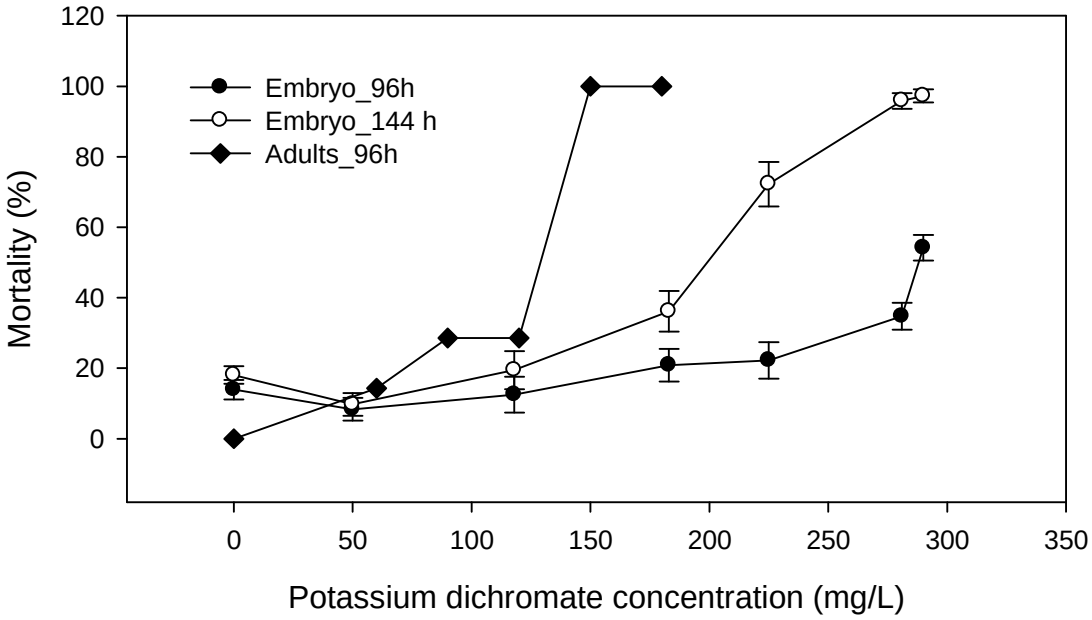


Figure 5. Comparison between mortality curves of adults at 96h and larvae at 96 and 144h**.**

From the biomarkers analyzed, only GST was responsive to PD exposure showing the same type of behavior observed in the larvae (dose dependent inhibition).

5. Conclusion

Zebrafish eggs showed to have low sensitivity to PD probably because this chemical does not easily cross the egg shell. Thus, no anomalies or delays were observed on embryo development including hatching. 96h-LC50 was much higher for larvae (362.42 mg/L) compared to adults (112.76 mg/L) which might be explained by the fact that larvae spent approximately half of these 96 hours inside the egg and protected from PD action. Moreover, biochemical parameters were much more sensitive in larvae and based on our results, PD seems to be neurotoxic (inhibited ChE), seems to interfere with cellular metabolic activity (changes in LDH activity) and inhibit GST activity. Early life-stages assay also

contributed with relevant information regarding anomalies in larvae development and behavior, especially at 96 h where PD seemed to cause swimming and equilibrium disturbances (probably related to the neurotoxic effects suggested by the inhibition of ChE). In this way, embryo/larvae assay was very informative and sensitive. In the future, genotoxic parameters should be included in the test (as the comet assay) which, in the adult assay, confirmed the genotoxicity of PD. Oxidative stress enzymes (such as superoxide dismutase and catalase) would be also useful to complement information as PD is known to induce oxidative stress at molecular level.

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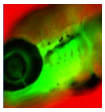

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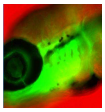

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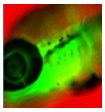

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CHAPTER III – GENOTOXIC EVALUATION OF DIFFERENT δ-ENDOTOXINS FROM BACILLUS THURINGIENSIS ON ZEBRAFISH ADULTS AND DEVELOPMENT IN EARLY-LIFE STAGES

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Abstract

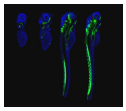
The use of toxins from *Bacillus thuringiensis* as biopesticides in the biological control of insects and in transgenic plants has increased their availability in the environment. Ecotoxicological evaluations of four δ-endotoxins cry 1Aa, cry 1Ab, cry 1Ac, and cry 2A from *Bacillus thuringiensis* were carried out on zebrafish *Danio rerio* to explore the adverse effects on their genome and embryos. Cry 1Aa increased the micronucleus (MN) frequency in peripheral erythrocytes of adult *D. rerio,* while cry 1Ab, cry 1Ac and cry 2A did not show genotoxicity, after 96-h of exposure at concentration of 100 mg/L. Exposures to binary mixtures (cry 1Aa + cry 1Ac, 50:50 mg/L) and (cry 1Aa + cry 2A, 50:50 mg/L) for 96-h showed also significant increased MN frequency. Other evaluated binary mixtures did not show genotoxicity. Activity of glutathione S-transferase measured in the gills did not seem to be altered after exposure to all tested toxins as well as to the six combinations of binary mixtures. In the embryo-larval study, all tested δ-endotoxins showed embryo toxicity and developmental delay after exposure to the concentrations of 25, 50, 100 and 150 mg/L for 96-h. However, each toxin presented a different pattern of toxic response suggesting that a specific approach should be taken into account to its toxicological evaluations.

Keywords: micronucleus, glutathione S-transferase, embryo toxicity, zebrafish, *Bacillus thuringiensis,* binary mixtures.

1. Introduction

Advances in genetic engineering in recent years have lead to the development of plants that present resistance to some insects through incorporation and expression of genes encoding δ-endotoxins from the bacterium *Bacillus thuringiensis.* A number of plant species, particularly crops such as cotton, corn, potatoes, tobacco, tomato, and sugarcane have been modified to produce δ-endotoxin proteins from *B. thuringiensis* (Mendelsohn et al., 2003; OECD, 2007; PrietoSamsonov et al., 1997; Romeis et al., 2006). Moreover, the insecticidal properties of toxins from *Bacillus* have been used to insect control for a long time as commercial spray formulations. Thus, the concern about availability of microbial toxins in plants with incorporated protection as well as in biopesticides has been raised, due to its potential adverse effects on non-target species in the environment, including aquatic ecosystems. The δ-endotoxin is a protein that is toxic to insects of the Coleoptera, Diptera and Lepdoptera families. After ingestion by insects, in the alkaline environment of the gut, proteases dissolve the crystal to yield the toxic form. The active crystal-toxins bind to specific receptor sites and produce pores in the insect gut which results in loss of homeostasis and septicemia, which are lethal to the insect (Broderick et al., 2006; Schnepf et al., 1998). There are no known equivalent receptor sites for binding of the δ-endotoxins in mammals (Broderick et al., 2006; Gill and Ellar, 2002; Noteborn et al., 1995). The toxicological database on *B. thuringiensis* shows no mammalian health effects attributable to δ-endotoxins (McClintock et al., 1995a; McClintock et al., 1995b).

The development of insect resistance to transgenic crops producing Bt toxins poses a major threat to their sustainable use in agriculture. One strategy to avoid resistance is the introduction of different Bt genes into the same cultivar, which means gene pyramiding. Cao *et al.* (2002), reported the increased lethality of a broccoli carrying both cry 1Ac gene and cry 1C gene, compared to both parental lines with a single Bt resistance gene. In this case, pyramid of both Bt



genes was obtained through sexual crosses. The principle of gene pyramiding is that a species can not easily develop resistance to both toxins, because that would require two simultaneous independent mutations in genes encoding receptors. Dual Bt cottons (cry 1Ac + cry 2Ab) and (cry 1Ac + cry 1F), expressing two Bt endotoxins were much more effective in the control of *Helicoverpa zea* than Bt cotton expressing cry 1Ac alone (Jackson et al., 2003). As referred above, the increased use of modified crops has raised environmental concerns since no conclusive studies on non-target organisms are available. Especially in the case of aquatic environments, different entry paths for Bt toxins are to be considered: addition of pollen and crop dust during harvest to surface water, runoff of crop residues or leaching of decomposing material and root exudates (Prihoda and Coats, 2008; Saxena et al., 2004). This study aims to contribute to the understanding of the risks for aquatic environments of Bt toxins. The main objectives were (i) to evaluate the effects of the δ-endotoxins cry 1Aa, cry 1Ab, cry 1Ac, and cry 2A on *Danio rerio* (zebrafish) early stages and adults and (ii) to explore the toxicological consequences of gene pyramiding in transgenic Bt-crops. To achieve the first objective a toxicity test was developed for zebrafish embryo and larvae with mortality and embryo development as endpoints and another test was performed with adult zebrafish where mortality, GST activity and genotoxic effects were chosen as endpoints. To achieve the second objective a set of Bt toxins binary mixtures was included in the test performed with adult zebrafish.

2. Materials and Methods

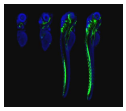
2.1 Bacillus thuringiensis - esporo-crystal (strains tested)

Four Brazilian entomopathogenic strains were used in this assay. Each one represented a different serotype: *B. thuringiensis* serotype *kurstaki*, encoding cry 1Aa, cry 1Ab, cry 1Ac and cry 2A proteins, toxic to lepidopteran larvae (Monnerat et al., 2007). These strains were isolated from Brazilian soils and are stored at a

Collection of Entomopathogenic *Bacillus* spp. of Embrapa Genetic Resources and Biotechnology, Brazil. The bacterial strains were grown to sporulation on NYSM medium in a rotative stirrer (200 rpm for 72 h) at 30 ºC, harvest in water and centrifuged (10.000Xg, 10 min, 4 ºC). Cry proteins are water soluble at neutral pH. The pellet was washed two times in water to remove cell debris, lyophilized and stored at 4 ºC. Prior to use in bioassays, the strains of *B. thuringiensis* were resuspended in water and the volume adjusted to desired concentration.

2.2 Adult zebrafish toxicity test

Zebrafish (*Danio rerio*), sexually mature, from a colony established at the department of Biology, University of Aveiro (Portugal) are maintained in carbon-filtered water system at standardized conditions of temperature (27.0 ± 1 ºC), conductivity (550 ± 50 µS), pH (7.5 ± 0.5), dissolved oxygen (95% saturation) and photoperiod (16:8-h light:dark). Fish are fed twice a day with commercially available artificial diet (ZM 400 Granular). Adult zebrafish test followed the OECD guideline 203 (OECD, 1992)using adult fish of similar length and age Based on preliminary tests the treatments described in Table 1 were chosen including a negative control (dechlorinated tap water) and a positive control (cyclophosphamide monohydrate (Sigma) at 50 mg/L. In each treatment, two aquaria were used with four organisms each. The assays were carried out in aquaria of 1L during which fish were not fed. The ammonium level in water was constantly monitored; water conductivity was kept at 500 µS and pH at 7.2. Mortality and abnormal behaviour were recorded daily. Test ended at 96h when fish were scarified and samples taken for micronucleus assay and GST activity determinations.



2.3 Zebrafish micronucleus assay

Peripheral blood was obtained by cardiac puncture with a heparinized syringe and immediately smeared. After fixation in ethanol for 15 min, slides were left to air-dry and then were stained Acridine Orange at a concentration of 0.003%. The stained slides were viewed under an epi-fluorescent microscope at a magnification of 1000x and evaluated for the presence of micronuclei exhibiting yellow-green fluorescence in the peripheral blood erythrocytes. For each treatment, all 8 fish were sampled and three thousand erythrocyte cells with complete cytoplasm were scored per fish (total of 24000 cells per treatment). 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 must not touch the main nuclei, (c) MN must be of the same color and intensity as the main nuclei. This data were statistically analyzed by non-parametric Mann-Withney *U* test, considering $\alpha = 5\%$.

2.4 Glutathione S-Transferase (GST) activity

Gills were collected and immediately frozen at -80 ºC in 1 ml potassium phosphate buffer (0.1 M, pH 6.5) until analysis. On the day of enzymatic analysis; samples were defrosted on ice and homogenized with an Ystral GmbH D-7801 tissue homogenizer. Enzymatic determinations were performed in the supernatants obtained by centrifugation of the homogenates (4 ºC, 8367 rpm, 30 min). GST activity was determined at 340nm following the general methodology described by Habig and Jakoby (1981) and adapted to the microplate reader (Frasco and Guilhermino, 2002), using 0.1mL of homogenate and 0.200 mL of the reaction mixture (reduced 10mM glutathione (GSH) and 60mM 1-chloro- 2.4-dinitrobenzene (CDNB)). Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute per mg of protein. Protein concentration in the samples was determined in quadruplicate by the Bradford method (Bradford, 1976a), at 595 nm, using γ-globulin as standard. A

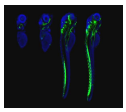
Labsystem Multiskan EX microplate reader was used for all biochemical determinations.

2.5 Embryo-larval toxicity test

This test was based on OECD Guideline for (OECD, 1998). Zebrafish fertilized eggs were individually exposed in 24-well microtiter plates to nominal concentrations of 0, 25, 50, 100 and 150 mg/L of each of the four δ-endotoxins: cry 1Aa, cry 1Ab, cry 1Ac and cry 2A. Different concentrations were achieved by dilution of a stock solution in the water used for fish maintenance. The test was initiated immediately after egg fertilization and was continued for 4 days. Each δ-endotoxin was tested in parallel in two independent replicates plus a control. In the embryo phase the following parameters were evaluated: (i) egg coagulation (ii) lack of otolith formation, (iii) lack of eye pigmentation, (iv) lack of somite formation, (v) lack of detachment of the tail-bud from the yolk sac, (vi) lack of heart-beat, and (vii) lack of body pigmentation. After hatching, in the larval phase the following parameters were evaluated: (i) heart-beat, (ii) oedemas, (iii) tail malformation, (iv) larval pigmentation and (v) larval behaviour. The test was carried out at temperature of 28.0 ± 2 ºC and the observations were done under stereomicroscope.

2.6 Statistical analysis

Genotoxicity (MN) data was analyzed by the non-parametric test Mann Withney-*U* (α = 5%). GST and embryo development data did not pass the Kolmogorov Smirnov normality test and so Kruskal-Wallis tests were performed. If significant results were found, the Dunn's test was used to verify differences between tested concentrations and control. Sigma Stat 3.1 statistical package was used for all statistical analyses (SPSS, 2004). For embryo/larvae mortality, lethal concentrations at 50% (LC_{50}) were calculated with probit analysis using MINITAB 13 statistical package (Minitab, 2000). All statistical analyses were performed with a significance level of 0.05.



3. Results

Neither mortality nor visible signals of toxicity were observed in the adult fish exposed to tested-concentration of all studied strains of *Bacillus thuringiensis* esporo-crystal during the experiments. From the four δ-endotoxins, only cry 1Aa increased the MN frequency in *D. rerio* (Table 1, p = 0.0009). When evaluated in binary mixtures, cry 1Aa + cry 1Ac and 1Aa + cry 2A showed a significant increase of MN frequency (p = 0.0074 and p = 0.0046, respectively). Other binary mixtures did not increase the MN frequencies. Table 1 shows all possible binary combinations among the four tested δ-endotoxins and the results of MN evaluation. GST activities measured in fish exposed to δ-endotoxins alone and in binary mixtures (Figure 1) did not show differences when compared to control (Kruskal-Wallis; H = 8.309, p = 0.685).

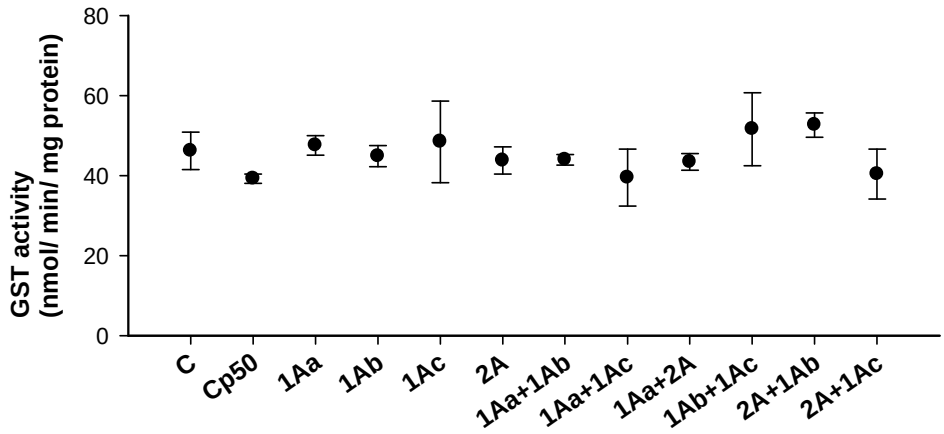


Figure 1. GST activity (mean values ± SE) of *D. rerio* gills after 96-h exposure to four Bt-toxins and its binary mixtures.

Mortality of zebrafish embryo and larvae is shown on Figure 2. In the first 48 hours δ-endotoxins do not seem to elicit high levels of mortality on embryos, however at 72 and 96 hours mortality highly increases at concentrations higher than 50 mg/L. A 96h-LC_{50} was calculated for all δ-endotoxin (Table 2), showing that larvae are much more resistant to cry 1Ac since the LC_{50} value for this toxin is about 2.2 times higher than LC_{50} values for the other toxins.

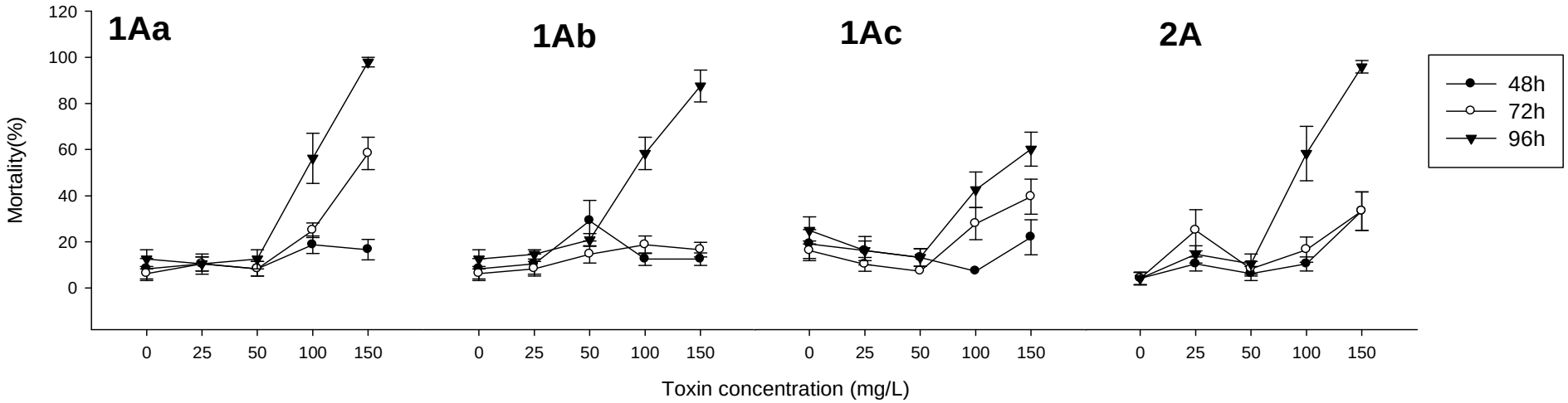


Figure 2. Effect of time and δ-endotoxin concentration on zebrafish embryo/larvae mortality (mean value ± SE).

Table 1. Micronucleus evaluation in peripheral erythrocytes of *Danio rerio* exposed to four δ-endotoxins and its binary mixtures.

Treatments	Total of MN found per treatment group	Mean ± SD	Mann-Withney P
Control	3	0.37 ± 0.51	
Cyclophosphamide (50 mg/L)	60	8.62 ± 2.44	**0,0009***
cry 1Aa (100 mg/L)	22	2.71 ± 0.70	**0,0009***
cry 1Ab (100 mg/L)	2	0.25 ± 0.46	0,7132
cry 1Ac (100 mg/L)	2	0.25 ± 0.46	0,7132
cry 2A (100 mg/L)	7	0.87 ± 0.83	0,2701
cry 1Aa + cry 1Ab (50 mg/L + 50 mg/L)	5	0.62 ± 0.51	0,4309
cry 1Aa + cry 1Ac (50 mg/L + 50 mg/L)	17	2.12 ± 1.35	**0,0074***
cry 1Aa + cry 2A (50 mg/L + 50 mg/L)	18	2.25 ± 1.48	**0,0046***
cry 1Ab + cry 2A (50 mg/L + 50 mg/L)	8	1.00 ± 0.92	0,2076
cry 1Ac + cry 2A (50 mg/L + 50 mg/L)	7	0.87 ± 0.83	0,2701
cry 1Ab + cry 1Ac (50 mg/L + 50 mg/L)	8	1.00 ± 0.75	0,1278

* statistically significant, $p < 0.05$

Some parameters of embryo development were highly affected by exposure to δ-endotoxins. At 24 hours a dose dependent response was observed for the lack of otolith formation, eye pigmentation and tail detachment. As the anomalies in these three parameters were always associated, they were treated as a single endpoint. Figure 3 (A) (grey bars) shows that for all δ-endotoxin tested except for cry 2A; 100% of the embryos exposed at the highest concentration did not present otolith, eye pigmentation or tail detachment at 24 hours. Body pigmentation was also verified but no embryos with abnormal responses were found (data not shown). At 48 h the number of embryos with abnormal development (otolith, eye pigmentation and tail detachment) decreased, especially in cry 1Ab and 2A where recovery was almost complete (Figure 3 (A), black bars). Lack of somite formation was observed in the embryos exposed to cry 1Aa and 1Ac (96.9 and 81.3 % of embryos respectively in the highest concentration tested). For all toxins except cry 2A, heart beat and tail circulation were absent in embryos exposed to some concentrations (see Table 2)

At 96 hours δ-endotoxins cry 1Aa, 1Ab and 2A caused 100% mortality on larvae exposed to highest concentration (150 mg/L). Remaining concentrations elicited serious behavioral disturbs on larvae characterized by a lack of reaction to mechanical stimulus (as a soft pinch with Pasteur pipette) and difficulties in keeping the equilibrium (Figure 3 (B)). Spine deformations were observed for all δ-endotoxins particularly in cry 1Aa and 1Ab with 32.1 and 36.9 % respectively of embryos affected at concentration of 100 mg/L. Table 2 to shows a summary of developmental endpoints analyzed, including EC50 values for dose responsive parameters.

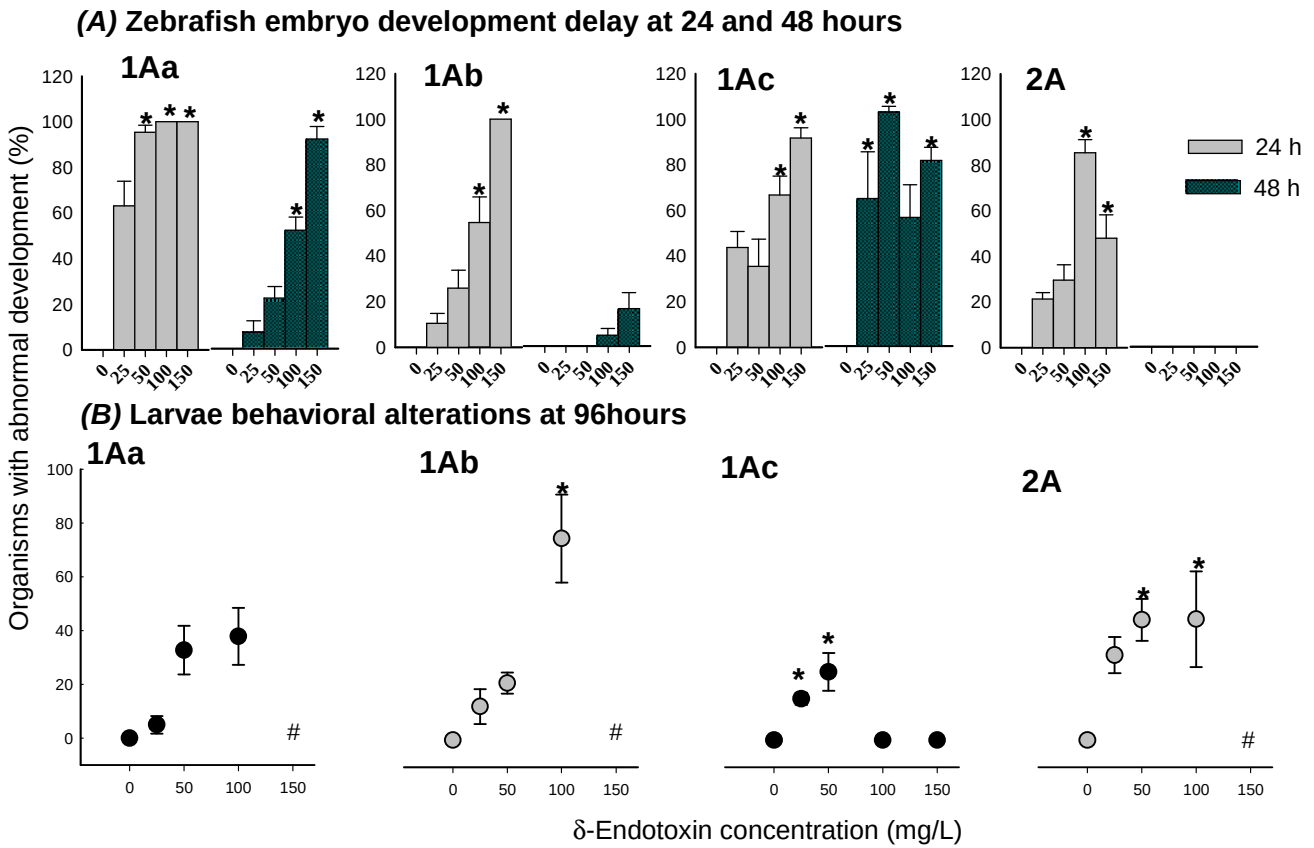


Figure 3. Effects of different δ-endotoxin concentrations on zebrafish embryo and larval development (mean value ± SE); **(A)**: lack of otolith formation, eye pigmentation and tail detachment at 24 and 48 hours; **(B)**; lack of reaction to mechanical stimulus and equilibrium difficulties at 96 hours. "#" means all embryos died at 96h. Asterisks above the plots mean significantly different from control (Dunn's test after Kruskal-Wallis, $p<0.05$)

Table 2- Effects of different δ-endotoxins on developmental parameters of zebrafish embryos and larvae.

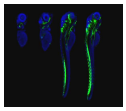
	1Aa	1Ab	1Ac	2[a]
At 24 h				
Development delay [(1)]	**23.5** (19.5-27.2)	**83.1** (73.8-94.0)	**68.0** (56.9-79.9)	**97.1** (81.6-117.9)
Body pigmentation	n.e.	n.e.	n.e.	n.e.
At 48 h				
Development delay [(1)]	**91.9** (81.5-103.9)	n.e.	* (25,0)	n.e.
Somite formation	**81.2** (72.4-90.9)	n.e.	* (25,0)	n.e.
Heart beat	**74.1** (63.0-86.9)	* (100,0)	* (25,0)	n.e.
Tail circulation	**71.6** (60.8-83.9)	* (100,0)	* (25,0)	n.e.
At 96 h				
Spine deformation	**120.9** (101.0-172.0)	* (50,0)	* (50,0)	* (25,0)
Reaction stimulus	**88.8** (73.9-115.4)	**75.0** (64.3-90.7)	* (25,0)	**91.5** (68.6-151.8)
Mortality (LC_{50})	**85.9** (75.8-97.1)	**87.0** (75.4-100.5)	**188.4** (151.5-277.3)	**87.6** (78.1-98.4)

Bold values are EC_{50} (in mg/L) of dose responsive endpoints followed by 95% CI between brackets.

"n.e." means no effect on the endpoint analysed.

"*****" means not dose responsive; in these cases LOEC value (lowest observed effect concentration) is presented.

[(1)] This endpoint takes into account the otolith development, eye pigmentation and tail detachment.



4. Discussion

As no data was found on toxicity and genotoxicity of these δ-endotoxins to adult D. rerio, the concentration chosen in this work was based on the embryo toxicity study. At 100 mg/L the four δ-endotoxins caused severe embryo toxicity (Figure 3) which was taken as an indicator that some adverse effect would be also expectable in adults. The absence of mortality of adult fish was not surprising since no acute effects were expected based on information provided in several EPA documents (eg: U.S. Environmental Protection Agency, 2001). In this work the number of micronucleus in fish exposed to cry 1Aa indicated genotoxicity of this toxin; moreover, the presence of cry 1Aa determined the genotoxicity of the mixtures cry 1Aa + cry 1Ac and cry 1Aa + cry 2A, because cry 1Ac and cry 2A, when evaluated alone, did not cause genotoxicity and nor when tested as binary mixture cry 1Ac + cry 2A (Table 1). On the other hand, the binary mixture cry 1Aa + cry 1Ab, did not induced MN in zebrafish, even in the presence of δ-endotoxin cry 1Aa. However, some data in literature report low sensitivity of the MN assay in fish erythrocytes (Jha, 2008) which can lead to an underestimation of genotoxicity. Future works should include complementary tests such as the comet assay so that a more precise assessment of genotoxic effects can be obtained.

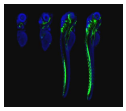
Bacillus thuringiensis serotype H-1 and H-14 were tested on Salmonella typhimurium strains TA 98 or TA100 (Ames test). No increases in revertent colony were observed. Even with preparations tenfold concentrated there were considered non-mutagenic (Carlberg et al., 1995). Ren et al (2002), reported chromosome aberration induced by B. thuringiensis (HD-1) emulsion (2750 IU/ml) in the grasshoppers' testicular cells (O. chinensis). They showed that Bt-toxin may induce chromosome aberrations, mainly chromatid and chromosome breaks in spermatogonia. By statistical analysis, Bt-toxin showed significant dose–effect relationships and it may be mutagenic in this species. The mode of action of Bt-toxins occurs through cytolytic activity against cell membrane receptor of the epithelial cells, causing membrane disruption which is essential for its toxicity. Thus, δ-endotoxins within the cell can restrain the action of RNA polymerases

which acts also as DNA repair enzyme during DNA transcription (Zhang, 2000). Moreover, cell membrane disruption increases cytoplasm vacuolization releasing microsomal enzymes, which cause DNA hydrolysis. These would be considered indirect mechanisms of genotoxicity.

Strains of B. thuringiensis that express multiple toxins can exhibit an unexpected toxicity due to interactions with each other (Liu et al., 1998). Many experiments, both in vitro and in vivo, suggest synergistic interactions between two or more δ-endotoxins. However, antagonism was already observed between Cry1Aa and Cry1Ab in the gypsy moth (Schnepf et al., 1998; Tabashnik, 1992). This antagonism could explain the non-genotoxic results observed in our study with binary combination of Cry1Aa + Cry1Ab on zebrafish. Two toxins, with affinity to different cell membrane receptors have independent actions. Thus, cry 1Ac and cry 2A did not interfere with the binding site of cry 1Aa and this is the reason that the genotoxicity found by the binary mixtures was similar to that observed by cry 1Aa.

No significant alteration on GST activity was observed in fish exposed to δ-endotoxin or cyclophosphamide, indicating that this biomarker is not sensitive to these chemicals. No data on literature was found to compare with the obtained results. GST activity was used because it has shown to be a useful biomarker of exposure to organic pollutants in a series of aquatic organisms including microalgaes, macrophytes, mussels and zebrafish (Wiegand et al., 1999). Gill plays an important endpoint for monitoring contaminants in water because they are in direct and permanent contact with the toxicant during the extent of the experiments. Many studies have reported GST activity evaluation on gills of different fish species, such as rainbow trout exposed to caffeic acid, Anguilla anguilla exposed to polluted water, Channa punctata exposed to fly ach leacheate (Ahmad et al., 2006; Ali et al., 2004; Chung et al., 2006).

Toxicity test with zebrafih embryo and larvae indicated a much higher sensitivity of these life stages when compared to adults. This is especially concerning since risk assessment of substances are usually performed based on adult fish tests, underestimation risk evaluation. Mortality observed on this test



was very low in the first two days when embryos had not hatched yet, being more pronounced on the last 2 days (larvae). 96h-LC50 values were very similar between δ-endotoxins (± 87 mg/L) except for cry 1Ac (188 mg/L) that seems to be much less toxic. This agrees with the work of Tounou et al (2005) where cry 1Aa shows lower toxicity compared to other δ-endotoxins. Despite the low mortality at embryo phase, effects of δ-endotoxins were already visible at this stage, with development delay verified with all endotoxins tested. Effects were especially marked on organisms exposed to cry 1Aa where a 24h-EC50 for development delay of 23.5 mg/L was calculated. Cry 1Aa elicited response in almost all endpoints tested and after hatching larvae presented high rates of spine deformations. Comparatively, cry 1Ab and 2A presented similar levels of acute toxicity (similar 96-LC50) but lower sublethal effects. Development delay observed at 24 hours was not verified at 48 hours and many parameters analyzed were not affected. Finally, cry 1Ac elicited comparatively lower mortality at 96 hours and irregular sublethal effects. No studies were found in literature to support results obtained for effects on embryo development. All δ-endotoxins tested seem to be embryo toxic but with different toxic properties since different patterns of response on the endpoints analyzed were observed. This idea is also supported by micronucleus evaluation where genotoxicity was only elicited by δ-endotoxin cry 1Aa.

In conclusion, genotoxicity on adult zebrafish as well as embryo toxic effects were observed. Moreover, each δ-endotoxins tested showed to have their specific toxic properties and for this reason risk evaluation should not be performed generally for Bt toxins but their specificities might be taken into account. Interactions between δ-endotoxin and with other chemicals are likely to occur, for instance in this work antagonism was verified between cry 1Aa and cry 1Ab in the genotoxic response. Published works also suggest that Bt toxins may interact synergistically with chemicals like pesticides (eg: Kaur, 2000).

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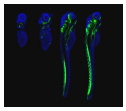

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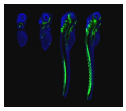

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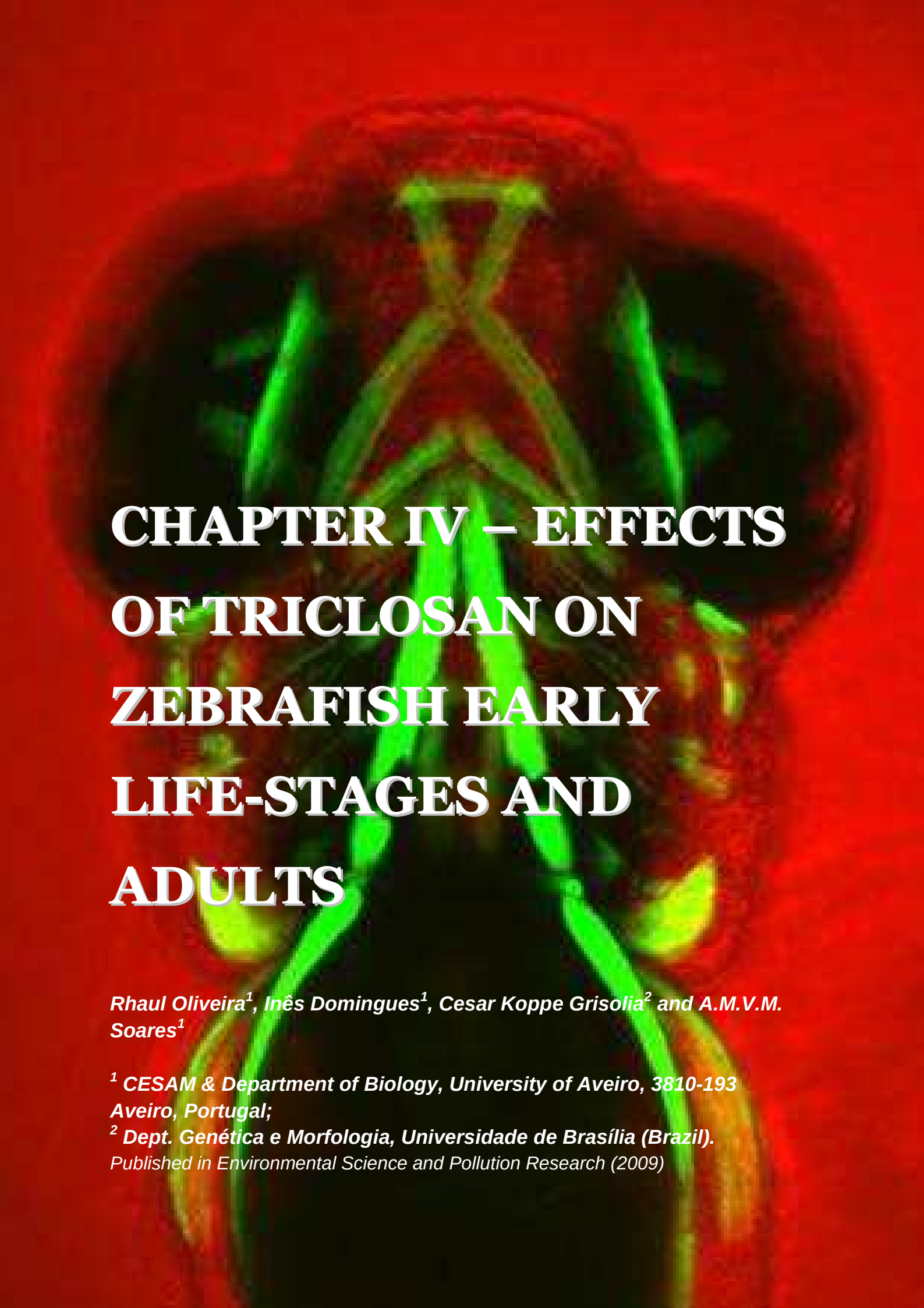

CHAPTER IV – EFFECTS OF TRICLOSAN ON ZEBRAFISH EARLY LIFE-STAGES AND ADULTS

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Abstract

The biocide triclosan (TCS) is commonly used in personal care, acrylic, plastic, and textiles products. TCS has been detected in surface water in several countries, and its ecological impact is largely unknown. In this work, the toxicity of TCS in zebrafish (Danio rerio), embryos and adults, was studied. Several lethal and sub-lethal endpoints were analysed in organisms exposed to TCS such as mortality, embryo development and behaviour, hatching, micronuclei and biochemical markers (cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH)).

Embryo/larvae assay followed the OECD guideline on Fish Embryo Toxicity (FET) Test. Embryos were exposed at nominal concentrations of 0.1, 0.3, 0.5, 0.7 and 0.9 mg/L of TCS for 6 days and were daily inspected with the help of a stereomicroscopy for mortality, developmental parameters (otolith formation, eye and body pigmentation, somite formation, heart-beat, tail circulation, detachment of the tail-bud from the yolk sac) and hatching. A similar test was run to obtain larvae for ChE, GST and LDH analysis. The adult test followed the OECD Guideline TG 203 in semi static conditions. Adult zebrafish of similar length and age were exposed to nominal concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l of TCS for 96h and were inspected daily for mortality and behaviour alterations. A second test was run to obtain organs for biomarkers analysis: heads, muscles and gills were isolated and snap-frozen in eppendorfs and used for ChE, LDH and GST determinations, respectively. Adult zebrafish testing also comprised a third test for micronucleus analysis in which the nominal concentrations of 0, 0.175 and 0.350 mg/l were used. Peripheral blood was obtained by cardiac puncture and used for the analysis.

TCS showed acute toxicity for embryo/larvae (96h-LC_{50}=0.42 mg/L) and delayed hatching. Moreover, embryo toxicity was evident: delay on the otolith formation, eye and body pigmentation was found and malformations were also evident including spine malformations, pericardial oedema and undersize. Biomarkers

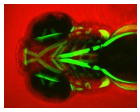
levels were affected: ChE and LDH activity were increased in larvae exposed to 0.25 mg/L and GST activity was increased in larvae exposed to 0.25 and 0.35 mg/L. TCS also demonstrated acute toxicity to adult zebrafish (96h-LC_{50}=0.34 mg/L). However, TCS did not change biomarkers levels and did not elicit a micronucleus in adults.

Despite the fact that similar 96h-LC50 values have been found for D. rerio embryos and adults (0.42 and 0.34 mg/L, respectively), the embryo assay was much more informative, showing important effects at several levels including teratogenic response, hatching delay and alteration of biomarker levels. TCS does not seem to be genotoxic for adult fish or to interfere with biomarkers levels at the concentrations tested.

TCS has deleterious effects on zebrafish adults and during early stages, (including embryotoxicity, hatching delay and alterations of biomarkers levels). The range of endpoints used on the embryo test allows an integrated analysis that contributes to a better understanding of the toxicity and mode of action of TCS.

Future works should focus on a deeper investigation of TCS modes of action on zebrafish early-life stages. As embryo testing was revealed to be so informative, a refinement of the test could be made including other endpoints such as different biochemical markers as well as DNA microarrays to assess a gene expression level for the effect of exposure to TCS. In the perspective of risk assessment, these endpoints should be explored in order to assess their usefulness as early warning signs and links should be sought between these short-term tests and effects of long-term exposures as it is observed in more realistic scenarios.

Keywords: Acute toxicity • Biomarkers • Danio rerio • Embryotoxicity • Triclosan • Micronucleus • Zebrafish



1. Introduction

Personal care products (PCPs) such as shampoos, fragrances, cosmetics, toothpaste and soap, are increasingly being used. Under typical use conditions, PCPs are discarded down the drain, become components of domestic wastewater and are treated in wastewater treatment plants. However, several chemicals used in their composition are frequently detected in aquatic ecosystems and, although present at low concentrations, processes of bioaccumulation, transfer through the food chain and interaction with other chemicals might increase their risk for aquatic ecosystems.

According to Kolplin et al. (2002) Triclosan (TCS) is widely used in PCPs and is one of the most frequently detected chemicals in wastewater in the USA. In Europe, 350 tons of TCS are utilized per year and approximately 1,500 tons are produced annually worldwide (Singer et al. 2002). TCS (5-Chloro-2-(2,4-dichlorophenoxy) phenol) is a common antibacterial agent that has been used extensively for more than 30 years. TCS was developed in the 1960s by JR Geigy AG; it is a relatively small compound with a molecular weight of 289.5 g/mol, sparingly soluble in water (10 mg/L in distilled water at 20ºC), hydrolytically stable and relatively non-volatile (vapour pressure [Pvp] = 4 × 10–6 mm Hg at 20ºC) (McAvoy et al. 2002). This compound is currently used in oral care products (Irgacare MP), acrylic products (Biofresh©), and incorporated into plastic materials (Microban©), as well as distributed as Irgasan DP 300 for skin applications (Glaser 2004). These uses have been evaluated and approved by the regulatory authorities responsible for the safety of personal hygiene products, food contact materials and chemicals in Britain, Canada, European Union, USA and many other countries (Dayan 2007). Several studies report, however, the adverse effects of TCS on aquatic organisms, namely on algae, molluscs, crustaceans and fish (Table 1). Reiss et al. (2002) drew attention to the case of TCS discharged from low technology plants to waters with low dilution volumes. The risk of TCS might not be negligible, especially for algae communities located downstream of the wastewater treatment plants, which are continuously exposed to chemicals

contained in the effluent plume and might thus show evidence of adaptation or species selection due to prolonged exposure (Wilson et al. 2003). Pernicious effects of TCS on algae are of special concern since, as primary producers, they are prone to influence the entire aquatic system (Tatarazako et al. 2003). A study of Coogan and La Point (2008) on TCS bioaccumulation at the outfall of Pecan Creek (Tx, USA) (using the caged aquatic snail Helisoma trivolis and the algae Cladophora spp.) showed a rapid bioaccumulation among algae and adult snails. The concentration of TCS in the water was 0.112 ppb, very close to the PNEC of 0.07 □g/L based on the toxicity of the most sensitive aquatic species Scenedesmus subspicatus (Capdevielle et al. 2008). Moreover, TCS levels reached 162 ppb in algae and 58.7 ppb in snail tissue. Other studies report the bioaccumulation of TCS and methyl-triclosan in fish, with high levels being found in bile samples (Adolfsson-Erici et al. 2002, Balmer et al. 2004). In this way, although TCS concentrations in water rarely exceed the PNEC of 1.55 □g/L derived from a species sensitivity distribution (SSD) (Capdevielle et al. 2008), the transference and bioaccumulation of TCS within the aquatic environment may result in higher exposure concentrations among the organisms of the different trophic levels.

Recent studies have also indicated that TCS may act as an endocrine disruptor. Veldhoen et al. (2006) showed that the exposure of premetamorphic Rana catesbeiana to TCS induced metamorphosis in the tadpole, although these animals were not naturally ready to undergo this process at that life stage. Others studies also indicate that TCS is a potential endocrine disruptor in fish, namely in Oryzias latipes where it proved to be weakly androgenic (Foran et al. 2000) or oestrogenic (Ishibashi et al. 2004).

Table 1. Effects of TCS on several aquatic organisms

Species	Value (µg/L)	Endpoint	Time (days)	Reference
Algae				
Anabaena	1.6	LC_{50}	4	Orvos et al. 2002
Closterium ehrenbergii	0.62	[1]EC_{50}	4	Ciniglia et al. 2005
Navicula	19.1	LC_{50}	4	Orvos et al. 2002
Scenedesmus subspicatus	1.4	LC_{50}	4	Orvos et al. 2002
Selenastrum Capricornutum	4.46	LC_{50}	4	Orvos et al. 2002
Skeletonema	66	LC_{50}	4	Orvos et al. 2002
Pseudokirchnesiella subcapitata	0.53	[2]IC50	3	Yang *et al.* 2008
Macrophyte				
Lemna gibba	62.5	LC_{50}	10	Orvos et al. 2002
Crustacea				
Ceriodaphnia dubia	184.7	LC_{50}	2	Orvos et al. 2002
Daphnia magna	343.8	LC_{50}	2	Orvos et al. 2002
Hyalella azteca	1.0	LC_{50}	10	Dussalt et al. 2008
Artrophoda				
Chironomus tetans	3.0	LC_{50}	10	Dussalt et al. 2008
Mollusca				
Mytilus galloprovincialis	10 µM	[3]EC_{50}	30 mim	Canesi et al. 2007
Amphibia				

Continuation table 1. Effects of TCS on several aquatic organisms

Species	Value (µg/L)	Endpoint	Time (days)	Reference
Bufo americanus	2.3	[4]LOEC	14	Smith and Burgett 2005
Rana pipiens	0.23	[5]LOEC	14	Fraker and Smith 2004
Xenopus laevis	23	[6]LOEC	14	Fraker and Smith 2005
Fish				
Lepomis macrochirus	370	LC_{50}	4	Orvos et al. 2002
Oryzias latipes	399	LC_{50}	4	Ishibashi et al. 2004
Oncorhynchus mykiss	34.1	[7]NOEC	61	Orvos et al. 2002
Pimephales promelas	260	LC_{50}	4	Orvos et al. 2002

[1] Effect observed: Lysosomal membrane destabilization (LMS)

[2] Effect observed: Growth inhibition

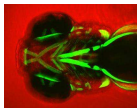
[3] Effect observed: Inhibition of asexual reproduction

[4] Effect observed: Mortality

[5] Effect observed: Behaviour alteration

[6] Effect observed: Mortality

[7] Effect observed: Hatchability and larvae survival



The zebrafish, Danio rerio (Hamilton-Buchanan 1822), is a small cyprinid widely used as a model organism in studies of developmental biology, physiology, molecular genetics, and toxicology. To acquire a deeper knowledge of TCS effects and mechanism of action on non-target organisms, several tests were performed in this work involving different life stages of zebrafish (Danio rerio) exposed to TCS. Effects analysed were lethality, development (embryo and larvae), biochemical parameters (biomarkers) and genetic (micronuclei). Furthermore, to elucidate the mechanism of toxicity of TCS in zebrafish and also to highlight parameters that work as early warning signals, a comparative analysis of the different endpoints was performed.

2. Materials and methods

2.1 Chemical

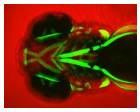
TCS (Irgasan, 5-Chloro-2-(2,4-dichlorophenoxy)phenol, ≥97.0% purity (HPLC)) was purchased from Sigma – Aldrich.

2.2 Test organisms

Zebrafish (Danio rerio) from a culture established at the Department of Biology, University of Aveiro are maintained in carbon-filtered water at 27.0±1ºC; under a 16:8h light:dark photoperiod cycle. Conductivity is kept at 550±50 µS, pH at 7.5±0.5 and dissolved oxygen at 95% saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

2.3 Early-life stages assay

The assay was based on the OECD guideline on Fish Embryo Toxicity (FET) Test (OECD 2006) and on the embryo test described by Fraysse et al. (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 eggs with irregularities during cleavage or injured were discarded. 72 eggs per treatment were used and distributed in three 24-wells microplates (replicates). Eggs were placed in each well individually with 2 ml of test solution. Seven treatments were used: control, solvent control and TCS treatments at nominal concentrations of 0.1, 0.3, 0.5, 0.7 and 0.9 mg /L. Test solutions were prepared by dilution of stock solution in water (pH 7.00±0.5 and conductivity 500±50 µS). TCS stock solution was prepared by dissolving TCS on acetone. Solvent (acetone) control contains 250 µl/L of acetone which is the highest concentration of solvent in the treatments. This procedure was adopted for all assays realized. The temperature during the test was 28.0±2ºC. Embryos and larvae were observed daily with the help of stereomicroscopy. Magnification used for observations of eggs was 70 x and was 40 x for larvae. Test run for 6 days. In the embryo phase, the following parameters were evaluated: egg coagulation, otolith formation, eye and body pigmentation, somite formation, heart-beat, tail circulation, detachment of the tail-bud from the yolk sac and hatching. After hatching, the following parameters were evaluated: oedemas, spine malformation and mortality. A second test using a similar design was performed for collection of larvae for biomarkers analyses (cholinesterase (ChE), glutathione S-transferase (GST) and lactate dehydrogenase (LDH)). Test ended at day 4 when clusters of eight larvae were snap-frozen in eppendorfs with 0.4 ml of the adequate buffer (described ahead). Samples were stored at –20ºC until enzymatic analysis.



2.4 Adults assay

The assay with adult fish followed the OECD Guideline TG 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. All test solutions were prepared by dilution of stock solutions in water (pH 7.00±0.5 and conductivity 500±50 µS). The values of dissolved oxygen were maintained above 80% of air saturation, a 16:8-h light:dark photoperiod cycle was used and the temperature was set to 21.0±2ºC. During the 96-h acute toxicity experiment, test solutions were exchanged daily to maintain water quality and TCS concentration. Seven treatments were used: control, acetone control (250 µl/L) and TCS treatments at nominal concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l. Groups of eight Danio rerio were exposed per treatment. The fish were not fed during the test period. Mortality was recorded each 24 h.

A second test using a similar design was performed for collection of organs for biomarker analysis. Five treatments were used: control, acetone control and TCS treatments at nominal concentrations of 0.15, 0.25 or 0.35 mg/l. Groups of 12 Danio rerio were used per treatment. At the end of the test, the number of dead fish was recorded and the living fish were sacrificed. Heads, muscles and gills were isolated and snap-frozen in eppendorfs with 0.5 ml of the adequate buffer and used for ChE, LDH and GST determinations, respectively. Samples were stored at –20ºC until enzymatic analysis.

2.5 Biomarkers assay for adults and larvae

Assays were performed to analyse ChE, GST and LDH activities on larvae and adults of Danio rerio. On the day of enzymatic analysis, samples were defrosted on ice and the homogeniser Ystral GmbH D-7801 was used for homogenisation.

For ChE analyses, individual heads of adults or clusters of eight larvae per concentration were homogenised in 0.5 and 0.4 ml of potassium phosphate buffer (0.1 M, pH 7.2) respectively. The supernatant obtained after the centrifugation of the homogenate (4ºC, 6000 rpm, and 4 min) was removed and used as enzyme extract for ChE activity determination. Total ChE activity was performed at 414 nm according to Ellman's method (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996).

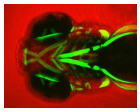
For the LDH determinations, the dorsal muscle of adults or clusters of eight larvae were homogenised in 0.5 and 0.4 ml of Tris–NaCl buffer (0.1 M, pH 7.2), respectively, on ice. Samples were centrifuged (4ºC, 6,000 rpm, 3 min) and the supernatant was removed and used to determine LDH activity. LDH activity was measured following the methodology described by Vassault et al. (1983) with the modifications introduced by Diamantino et al. (2001).

For the GST determinations, a pair of gills or clusters of eight larvae were homogenised in 0.5 and 0.4 ml of potassium phosphate buffer (0.1 M, pH 6.5), respectively. After centrifugation (4ºC, 8,367 rpm, 30 min) the supernatant was used to determine GST activity. GST activity was measured following the general methodology described by Habig and Jakoby (1981) and adapted to the microplate by Frasco and Guilhermino (2002).

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute per mg of protein. Protein concentration in the samples was determined in quadruplicate by the Bradford method (Bradford 1976), at 595 nm, using γ-globulin as standard. A Labsystem Multiskan EX microplate reader was used for all biochemical determinations.

2.6 Zebrafish micronucleus (MN) assay

The zebrafish micronucleus assay followed the same procedure of the adults assay, but with fewer treatments: 0, 0.175 and 0.350 mg/l of TCS.



Peripheral blood was obtained by cardiac puncture with a heparinised syringe and immediately smeared on a microscope slide. After fixation in ethanol for 15 min, slides were left to air-dry and then were stained with Acridine Orange at a concentration of 0.003%. The stained slides were viewed under an epi-fluorescent microscope at a magnification of 1000x and evaluated for the presence of micronuclei exhibiting yellow-green fluorescence in the peripheral blood erythrocytes. 3000 erythrocyte cells were scored per fish. 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 ovoid chromatin bodies showing the same staining pattern as the main nuclei (c); MN must not touch the main nuclei.

2.7 Statistical analysis

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS 2004). One-way ANOVA was performed except when data did not pass the Kolmogorov Smirnov normality test, in which case a Kruskal-Wallis test was performed. If significant results were found, the Dunett or Dunn's test was used to verify differences between tested concentrations and control. Lethal concentrations at 50% (LC50) for early-life stages and adults were calculated with probit analysis using the MINITAB 13 statistical package (Minitab 2000). Genotoxicity data was analysed with the non-parametric test Mann Withney-U. All statistical analyses were performed with a significance level of 0.05.

3. Results

3.1 Embryotoxicity of TCS

In the present study fertilized zebrafish eggs were exposed for 144 hours to several concentrations of TCS. Fig. 1 shows the proportion of eggs and embryos that died during the experiment (black bars), the proportion of embryos that stayed

alive (grey bars), that hatched (white bars) and finally proportion of larvae that died (spotted dark grey bars). The control group presented a normal embryo development as described by Kimmel et al. (1995). Embryo mortality in the control was always below 10% (8.3% at 96 hours) as is required for test validity.

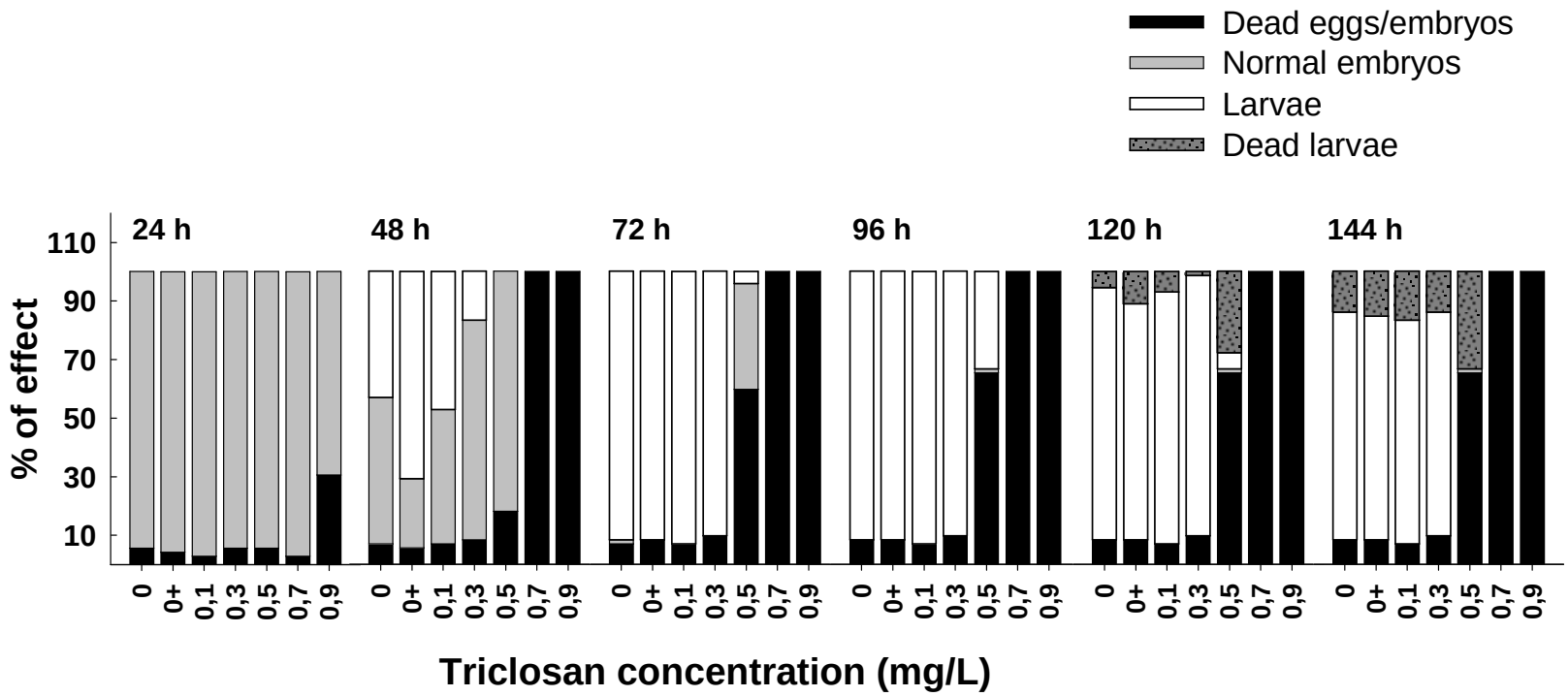


Figure 1. General overview of TCS effects on *Danio rerio* embryo and larvae mortality and hatching during the 144 h (6 days) of exposure.

TCS showed acute toxicity for embryos and larvae with a 96h-LC50 of 0.42 mg/L (95% confidence interval: 0,38–0.45) (Table 3, Fig. 2). Within the first 48 hours, all embryos exposed to 0.7 and 0.9 mg/L had died and at 72 hours 60% of embryos exposed to 0.5 mg/L had died as well. Embryos started to hatch at 48 hours (48% of hatching in the control) (Table 2 and see Fig. 1) and differences between concentrations were observed (Kruskal-Wallis H=38.5, P<0.001), especially at a concentration of 0.5 mg/L where no embryos hatched. At 72 hours, 100% of embryos had hatched in the control, 0.1 and 0.3 mg/L concentration but a delay was still observed in the 0.5 mg/L treatment (Kruskal-Wallis H= 55.12, P<0.001) where only 9% of the surviving embryos had hatched. At 96 hours, the remaining embryos of the 0.5 mg/L treatment group finally hatched but died the next day (120 hours).

Table 2. Percentage of eggs hatched (standard error between brackets)

TCS (mg/L)	48 h	72 h	96 h
0	45.8 (7.2)	98.6 (1.4)	100.0 (0)
0.1	49.9 (7.7)	100.0 (0)	100.0 (0)
0.3	19.4 (5.9)	100.0 (0)	100.0 (0)
0.5	0.0 * (0)	9.0 * (4.9)	97.0 (3.0)
0+	74.7 (7.7)	100.0 (0)	100.0 (0)

Asterisks mean significantly different from control (Dunn's test, p<0.05)

Developmental parameters proved to be excellent indicators of TCS toxicity. Table 3 summarizes effects on the parameters analysed. At 24 hours embryo development in the control and 0.1 mg/L treatments was normal, as described by Kimmel et al. (1995): embryos presented a well-developed head, body, and tail. In all other concentrations, effects could be noticed, although statistical significance was only observed at a concentration of 0.9 mg/L where a considerable delay on the otolith formation, eye and body pigmentation was found (Kruskal-Wallis: H= 83.00; P<0.001) (Fig. 3A and B). After 96 hours, larvae

exposed to 0.5 mg/L presented several developmental effects including spine malformations (Kruskal-Wallis: H= 29.962; P<0.001), pericardial oedema (Kruskal-Wallis: H=45.56; P<0.001) and were undersized (Kruskal-Wallis: H= 29.96; P<0.001) to a higher frequency than in the control group where no organisms with such anomalies were found (Fig. 3 C and D).

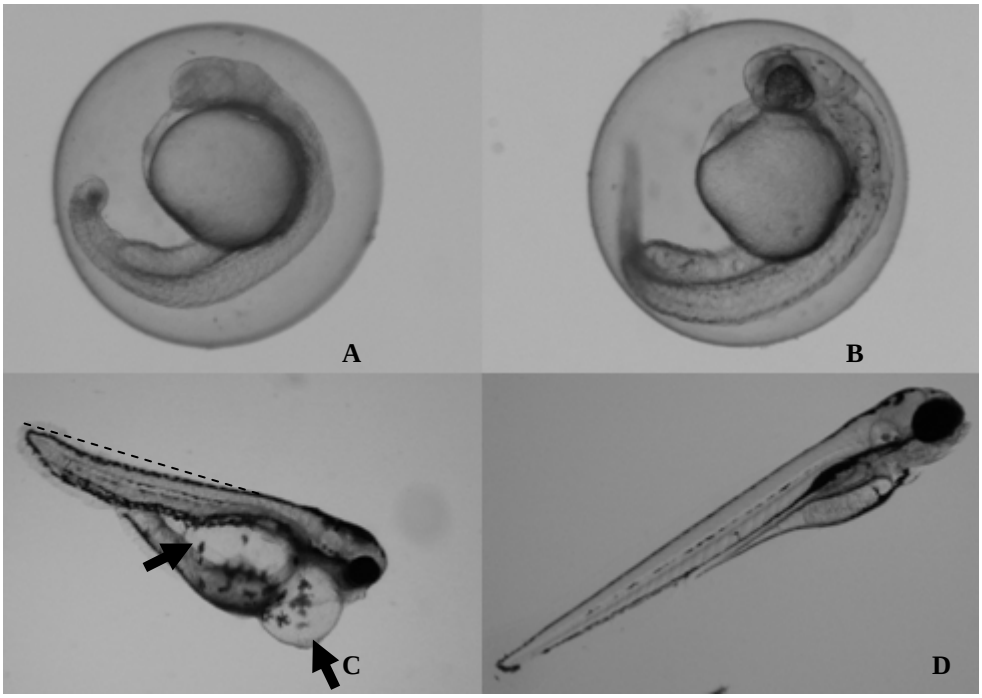


Figure 2. Zebrafish embryos and larvae abnormalities during exposure to TCS: (A) 24h old embryo exposed to 0.9 mg/L TCS with weakly pigmented retina and body; (B) Normal embryo 24h after fertilization, body and eyes with adequate pigmentation, (C) 96h larvae exposed to 0.5 mg/L of TCS with abnormal length, spine deformity and cardiac and yolk-sac edema; (D) Normal larvae (96h) with well developed tail and normal body structure.

The effects of TCS on the activities of the biomarkers ChE, LDH and GST of larvae are presented in Fig. 4: ChE activity was increased in larvae exposed to 0.25 mg/L (one-way ANOVA: F3, 37 = 7.09; P< 0.001) (Fig. 4A); GST activity was increased in larvae exposed to 0.25 and 0.35 mg/L (one-way ANOVA: F4, 48 = 10.42; p< 0.001) (Fig. 4B) and LDH was increased in larvae exposed to 0.25 mg/L (one-way ANOVA: F4, 48= 10.29; p <0.001) (Fig. 4C).

Table 3. Effects of TCS on developmental parameters of zebrafish early stages

Endpoints	24hpf	48hpf	72hpf	96hpf	120hpf	144hpf
Otolith formation	**0.7**	–	–	–	–	–
Eye pigmentation	**0.7**	–	–	–	–	–
Body pigmentation	**0.7**	–	–	–	–	–
Somite formation	n.e.	–	–	–	–	–
Heart beat	n.e.	n.e.	n.e.	n.e.	n.e.	–
Tail blood circulation	n.e.	n.e.	n.e.	n.e.	n.e.	–
Tail detachment	n.e.	n.e.	n.e.	–	–	–
Spine malformation	n.e.	n.e.	n.e.	**0.3**	n.e.	n.e.
Pericardial oedema	n.e.	n.e.	n.e.	**0.3**	–	–
Undersize	–	–	–	**0.3**	–	–
Mortality (LC_{50}) *	**1.57** (1.12–2.03)	**0.52** (0.49–0.56)	**0.43** (0.40–0.47)	**0.42** (0.38–0.45)	**0.34** (0.31–0.37)	**0.27** (0.24–0.30)

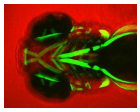
Bold values are NOEC (mg/L)

* LC_{50} values are in mg/L followed by 95% CI between brackets.

n.e.—means no effect on the endpoint analysed.

– —endpoint not analysed.

Hpf—Hours post fertilization



3.2 TCS toxicity for adults

The 96 h-LC50 value for adult Danio rerio was 0.34 mg/L (95% CI: 0.29–0.38) (see Fig. 2). Alteration on the behaviour of fish exposed to TCS was observed during the assay including erratic swimming, equilibrium loss, and abnormal operculum movement, especially at the highest concentration; however, no detailed record of this information is available.

In vivo effects of TCS on ChE, LDH and GST activities are presented in Figure 4. No alterations on the enzymatic levels were observed for any of the enzymes (ChE: Kruskal-Wallis, H=11.52; p=0.021, see Fig. 4A; GST: one-way ANOVA: F 4, 51=0.09; p=0.985, see Fig 4 B and LDH: Kruskal-Wallis, H=7.80; p= 0.099, see Fig. 4C).

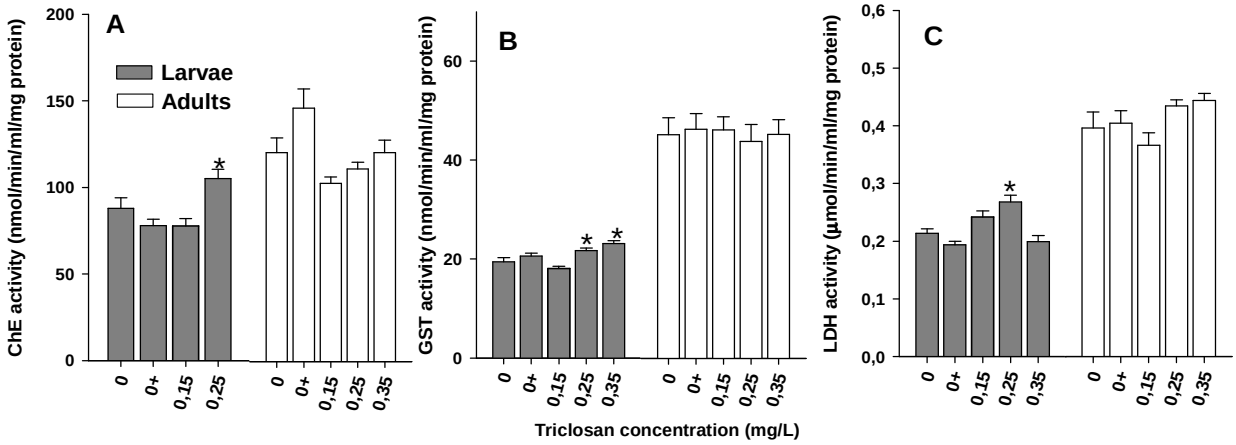


Figure 3. Variation of biomarkers activities (mean value ± standard error) on zebrafish larvae and adults after 96 h exposure to TCS: (A) ChE activity, (B) GST and (C) LDH. B. "*" mean significantly different from control treatment (Dunnet test p<0.05).

The Genotoxicity assay with adult zebrafish showed no induction of MN at concentrations of 0.175 or 0.35 mg/L compared to the control group (Table 4).

Table 4. Micronucleus frequencies in erythrocytes of adult zebrafish after 96-h of exposure to TCS

Treatments	Total of MN found per treatment group	MN per 1000 cells analyzed (‰)	Mean ± SD	P, Man Withney-*U* test
Control	3	0.125	0.3±0.5175	–
TCS 0.175 mg/L	4	0.166	0.5±0.7440	0.878
TCS 0.350 mg/L	5	0.208	0.6±0.7559	0.574

4. Discussion

Personal care products have been increasingly used and incorporate chemicals with the potential of affecting various aquatic organisms through unexpected modes of action. In this work, D. rerio early-life stages test were found to be much more informative than adult fish test. Embryos mortality was dependent on the TCS concentration and a 96h-LC50 of 0.42 mg/L was calculated. In the study of Ishibashi et al. (2004) with TCS, Oryzias latipes early stages demonstrated similar sensitivity (96h-LC50=0.40 mg/L), while in the study of Orvos et al. (2002) Oncorhynchus mykiss early stages exhibited greater sensitivity to TCS, with significant effects on survival under the 0.071 mg/L treatment. For some toxicants, the chorion of the egg may act as a barrier of embryo protection, although the relatively high mortality of embryos reported here and in other works (see above) suggests an incorporation of TCS into the egg, which can be explained by its high lipophilicity (Hallare et al. 2006).

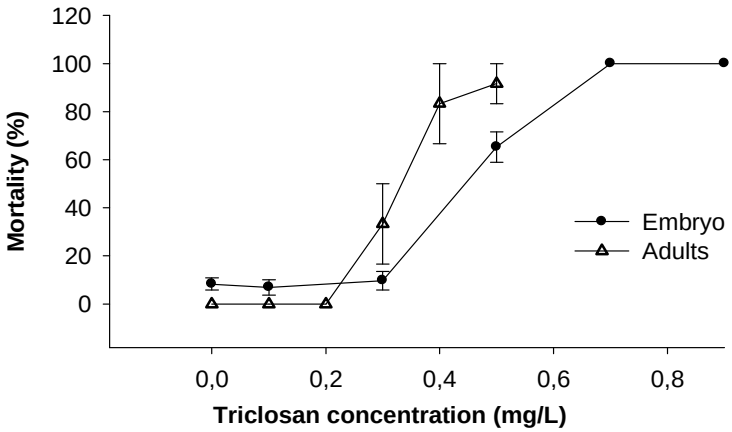


Figure 4. Embryo (dots) and adults (triangles) mortality at 96 hours (mean value ± standard error)

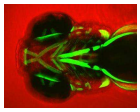
Hatching time has been widely used as an endpoint in fish early life stage tests. In this study a significant delay in hatching was observed, at 48 and 72 hours, on embryos exposed to 0.5 mg/L. This is in concordance with the work of Ishibashi et al. (2004) where a hatching delay was found on embryos of O. latipes exposed to 0.31mg/L of TCS. Different toxic mechanisms could justify hatching delay or failure like the induction of abnormal function of the enzyme chorionase and/or inability of the emerging larvae to break the egg shell (Hallare et al. 2005).

No studies were found in literature to support teratogenic responses to TCS verified during embryo development in D. rerio. Embryos exposed to 0.7 and 0.9 mg/L presenting abnormal pigmentation of eyes and body and lack of otolith at 24 hours died at 48 hours. Embryos exposed to 0.5 mg/L suffered a significant delay in hatching (see above) and presented high incidence of pericardial oedemas and spine malformations, dying within the next 48 hours. The mechanisms of embryotoxicity of TCS in aquatic organisms are not yet described, but pericardial oedema is often associated with leaks in the endothelial vessels and usually results in cardio-vascular dysfunctions (Hallare et al. 2005). Spine deformations can be linked with depletion or deregulation of ions like calcium and phosphorus or

with a reduction in myosin and myotonia, both required for normal development (Cheng et al. 2000; Muramoto 1985).

The use of biomarkers in ecotoxicology is becoming a useful routine, and various endpoints have been proposed as valuable tools to assess the effects of environmental chemical contamination (Nunes et al. 2006). The ChE, LDH and GST are biomarkers much used in monitoring of the quality of aquatic ecosystems (Domingues et al. 2007; Monteiro et al. 2006; Quintaneiro et al. 2008; Vieira et al. 2008). Analyses of biomarkers levels on larvae exposed for 96 hours to Triclosan showed important differences on the activities at concentrations that had not induced any effect at survival or embryo development level (eg. 0.25 mg/L), suggesting that enzymatic levels are very sensitive parameters for embryo/larvae TCS toxicity assessment. ChE activity was increased by 19.4% in larvae exposed to 0.25 mg/L of TCS. Several studies analysing ChE activity in zebrafish embryos indicate that this enzyme is very important in neuronal and muscular development (Behra et al. 2002; Hanneman 1992) suggesting that its alteration may be linked to effects like the larvae abnormal posture and spine malformations described in this work. Glutathione S-transferases are involved in the biotransformation of several pollutants; therefore an induction of GST activity has been widely used as an environmental biomarker (Monteiro et al. 2006; Oost et al. 2003). In this study, induction of larvae GST activity was observed in the two highest concentrations tested, namely 23.87% at 0.25 mg/L and 32.26% at 0.35 mg/L, suggesting the occurrence of detoxification processes. LDH activity increased 12.9% in larvae exposed to 0.25 mg/L of TCS. However, at the highest concentration tested (0.35 mg/L) no significant differences were found relative to the control group. Increased LDH activity levels have been observed in conditions of chemical stress when high levels of energy are required; at 0.35 mg/L, effects of TCS on larvae are probably too serious and detoxification mechanisms are no longer able to overcome toxic effects, hence the lack of induction of LDH activity.

In this study, serious effects of TCS on D. rerio early stages were observed. Results suggest high toxicity of TCS with adverse effects on survival, embryonic



development, hatching, and enzyme activities. The GST, ChE, LDH activity were the most sensitive endpoints analysed.

Toxicity of TCS to adult Danio rerio was assessed using lethal, genotoxic, and biochemical parameters. The 96h-LC50 value calculated in this study (0.34 mg/L) is very similar to the values found by Orvos et al. (2002) for Lepomis macrochirus (0.37 mg/L) and Pimephales promelas (0.26 mg/L). Alteration on fish behaviour (erratic swimming, equilibrium loss, and abnormal operculum movement) observed at high concentrations of TCS (0.5 and 0.4 mg/L) were also reported in the same study by Orvos et al. (2002) with Oncorhynchus mykiss, 61 days after exposure to 0.071 mg/L of TCS. Effects of TCS on the behaviour of fish species may increase predation risk. Further studies should investigate carefully the behavioural response of adult zebrafish to TCS.

Fish are an excellent model for the study of genotoxicity of contaminants in water. The erythrocyte micronucleus test has demonstrated efficiency and sensitivity in different fish species to monitor aquatic pollutants displaying mutagenic features (Al-Sabti and Metcalfe 1995; Ali et al. 2008; Grisolia and Cordeiro 2000; Kligerman 1982). Our results did not show a statistically significant difference between control and exposed groups. These results are in line with the results of several studies carried out in Salmonella for point mutation and eukaryotic cells for chromosome aberration that did not report genotoxicity in response to TCS (Bhargava and Leonard 1996).

Biomarkers analyses did not show any alteration on ChE, LDH or GST activities in adult zebrafish. No data from the literature were found to compare the results.

Despite embryo and adults, tests have been done at different temperatures, thereby invalidating a direct comparison of results, although similar 96h-LC50 values were found (0.42 and 0.34 mg/L for embryos and adults, respectively), suggesting similar sensitivities. However, the early-life stages test was much more informative in terms of sublethal effects due to the clear response of biomarkers

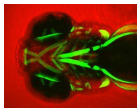
that was not observed in adults and, moreover, because monitoring of embryo development and hatching contributed with important information that has no parallel in adults. The range of endpoints used on the embryo test allows an integrated analysis that contributes to a better understanding of the toxicity and mode of action of chemicals. This is in line with the ideas of Sholz et al. (2008), who reviewed the wide range of applications of fish embryos testing in environmental sciences. Moreover, our results support the actual trend of replacing adult fish testing by embryo testing for several reasons (Nagel 2002). Several works also support the idea that fish early stages are commonly most sensitive to chemicals exposure (e.g.: Hallare et al. 2004; Luckenbach et al. 2001).

5. Conclusions

The effects of TCS in the different life stages of zebrafish were analysed. TCS seemed to be teratogenic at concentrations higher than 0.7 mg/L, leading to deep delays in embryo development and consequent death (at 48 hours). At 0.5 mg/L, TCS caused a significant decrease in embryo mortality. Surviving organisms suffered a significant hatching delay (embryos), oedemas and spine deformations (larvae), with all organisms dying at 120 hours. At 0.3 mg/L, biomarkers worked as early warning signs, being induced when none of the other parameters responded. Based on these results, concentrations higher or equal to 0.3 mg/L seem to pose a high risk for the environment. The embryo test seemed to be much more informative than the adult test, although 96h-LC50 values are similar (0.42 and 0.34 mg/L, respectively). Biomarkers levels and genotoxic parameters (MN) were not affected by TCS in the test with adult fish.

6. Recommendations and perspectives

Future works should focus on a deeper investigation of TCS modes of action on zebrafish early-life stages. As embryo testing were revealed to be so informative, a refinement of the test could be made including other endpoints such as different biochemical markers as well as DNA microarrays to assess at which



gene expression level the effect of exposure to TCS occurs. In the perspective of risk assessment, these endpoints should be explored in order to assess their usefulness as early warning signs and links should be sought between these short term tests and effects of long-term exposures as it is observed in more realistic scenarios.

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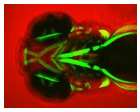

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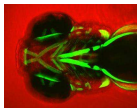

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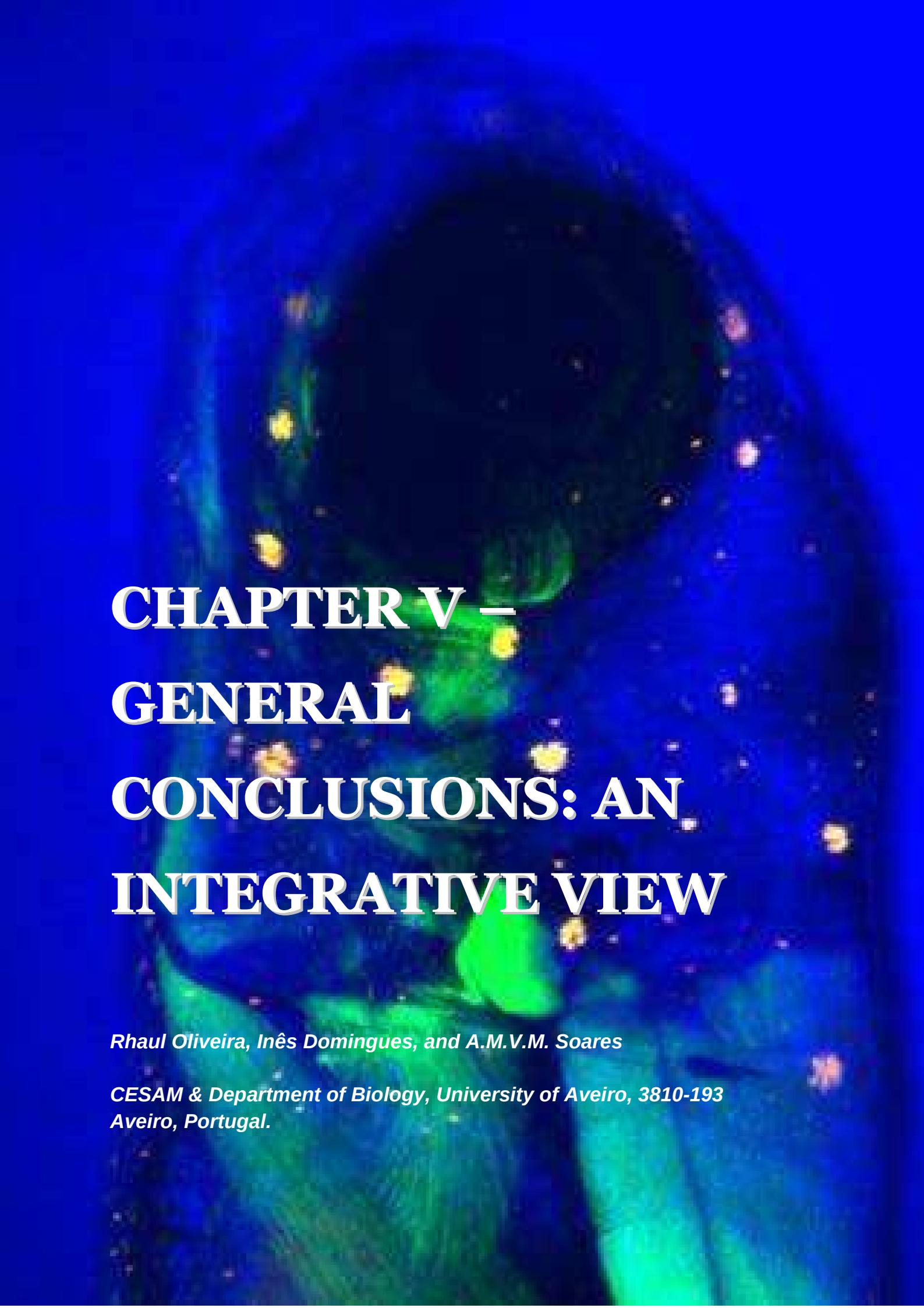

CHAPTER V – GENERAL CONCLUSIONS: AN INTEGRATIVE VIEW

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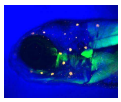


1. General conclusions: an integrative view

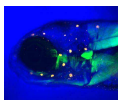
This study provides information about embryo and adult toxicity for diferent toxicants. A general overview of effects of the studied chemicals on *Danio rerio* early-life stages and adults after 96 hours of exposure is presented in Table 1. Results from the performed work reinforce the ideia of the zebrafish early-life stages and adults as model organisms in ecotoxicology (Lammer et al., 2009; Nagel, 2002; Scholz and Mayer, 2008). Eggs are easy to obtain and maintain, and, due to their transparence, effects on embryonic development are monitored with notable precision. Adults are also easy to handle and their small size allows the easy preparation of experimental set ups using lower test solution volumes when compared with other species.

When comparing embryo and adult tests in terms of lethality, our results showed that there are different behaviors among chemivals. While Bt test indicated higher sensitivity of embryos, TCS test showed equal sensitivities between early-life stages and adults and PD showed that embryo were less sensitive. Despite the apparent disagreement amog adults and embyos lethality values for each substance tested, the diference is very small with a factor minor than 3. The good correlation among $LC_{50\ embryo}$ and $LC_{50\ adults}$ is a very important question to the validation of fish embryo test as an alternative for acute assessment. Our data corroborate several interlaboratotial studies that indicate a good correlation between the fish embryo test and the acute fish test with adults (Braunbeck et al., 2005; Braunbeck and Lammer, 2006; Nagel, 2002).

Table 1. General over view of effects of the studied chemical on *Danio rerio* early-life stages and adults after 96 hours of exposure.

Toxics	**Adults LC50**	**Embryos LC50**	**Adult GST**	**Adult LDH**	**Adult ChE**	**Larvae GST**	**LarvaeLDH**	**LarvaeChE**	**Larvae Behavior**	**Adults Behavior**	**Comet**	**MN**	**NA**	**Hatch**	**Teratology**
PD	112.76	362.42	**120**	n.e	n.e	**183**	**50**	**281**	**118**	n.e	**60**	**135**	n.e	n.e	n.e
TCS	0,32	0,42	n.e	n.e	n.e	**0,25**	**0,25**	n.e	n.e	n.e		n.e		**0.5**	**0.3**
Cry1Aa	n.e	85.9	n.e						88.8	n.e		**100**		n.e	120.9
Cry1Ab	n.e	87.0	n.e						75.0	n.e		n.e		n.e	**50**
Cry1Ac	n.e	188	n.e						**25**	n.e		n.e		n.e	**50**
Cry2A	n.e	87.6	n.e						91.5	n.e		n.e		n.e	**25**

Presented values are LC50 and EC50 (in mg/L) values of dose responsive endpoints (between Brackets). When a EC50 value is not possible tocalculate, a LOEC value (lowest observed effect concentration) is presented (bold values)
"n.e." means no effect on the endpoint analyzed.
Shaded cells represent no evaluated endpoints.



With no doubt, the monitoring of embryonic development over 48 hours gives a considerable amount of information: it may show severe general effects on embryo development conducing to embryo death in the following days; it may indicate damage or malformations in a specific organ that can or not interfere with viability of embryos or it can show a delay on the development. On the other hand, evaluation of hatching and pos-hatching period (72 to 120 hours) provides important information on larvae behavior and malformations as it is the case of the PD test. The limitation of the test to 48 hours is a decision that excludes the fish embryo test from the Directive 86/609/EEC but in our opinion, the extended fish embryo test (24 to 120 hours) provides a better perspective of toxicity of subtances allowing evaluation of more sublethal endpoints with toxicological and ecotoxicological relevance.

Many studies on early life-stages of *Danio rerio* have developed tools for sublethal effects such as monitoring of behavior by MFB tecnology (Kienle et al., 2008) or diferential gene expression applied for detection of endocrine disruptors (Ankley et al., 2009; McGonnell and Fowkes, 2006). As well as the emphasis on the extension of the embryo test for evaluation of a wide range of developmental endpoints, in our work, biomarkers responses on larvae were also performed and compared with adults. Our results showed that embryos present higher sensitivity and much more responsive biomarkers than adult test. . Enzimatic activity on larvae may be an useful tool for ecotoxicity assessment in laboratorial assays and *in situ* scenarios. The development of new methotologies to identify more specific modes of action of pollutants are due to the increasing requeriment of a more acurate environmental risk evaluation.

Parameters indicative of Genotoxicity (micronuclei, nuclear abnormalities and “comets”) used in the adults tests were useful in distinguishing chemicals with this kind of toxicity (PD and Cry 1Aa). The use of Genotoxicity parameters in larvae (especially the comet assay which is a methodology already adapted for fish larvae) would be an important complement for the battery of endpoints already used in this assay.

In summary, the data presented in the present study (survivorship, embryotoxicity, genotoxicity, behavior and enzymatic activity) contribute to a better understanding of the effects of the chosen chemicals on zebrafish model with numerous applications for ecotoxicological assessment. In the future, it would be important to establish links between these effects observed at early-life stages and eventual fitness impairments in the adult stage. This could be done by performing chronic tests where growth, reproductive performance, etc could be evaluated.

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CHAPTER VI – RECOMMENDATIONS AND PERSPECTIVES

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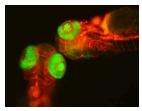

Recommendations and perspectives

The importance of zebrafish early-life stages assays in the assessment of chemicals effects is reinforced with this work. As embryo testing revealed to be very informative, it is important to refine the tests and to define the limitations of the model, including more studies about chorionic membrane interference on toxicity and ecotoxicological assessment techiniques. Future works should focus on modes of action of chemicals on zebrafish at genetic level and/or on chronic exposure. Also, a better perspective about relationships among embryotoxicological sublethal endpoints and long-term effects is required in order to perform risk assessment more corectly and accurately. The establishment of standard techniques for genotoxicity assessment for zebrafish early-life stages is important for detection of mutagenic and carcinogenic compounds and linkages with teratogenic responses. More studies with others endpoints such as different biochemical markers and DNA microarrays to assess a gene expression level are necessary. Furthermore, the development of new techniques, protocols and regulatory guidelines is necessary for standardization of zebrafish as model for ecotoxicological evaluations.