Universidade de Aveiro Departamento de Biologia
Ano 2009

Susana Patrícia Pinto Pereira

Avaliação dos efeitos tóxicos de alguns desinfectantes nos trópicos

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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 do Professor Doutor António Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação de Doutora Paula Inês Borralho Domingues, Bolseiro Pós-Doutoramento, Departamento de Biologia da Universidade de Aveiro

This work was partially supported through the ASIALINK project, "Linking the post-graduate education in aquaculture and aquatic resources management with its industries through internship programme" (Contact TH/Asia-Link/016 (141-287) financed through the ASIALINK-EuropeAid Programme"

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agradecimentos Este trabalho teve várias pessoas envolvidas, que directa e indirectamente, contribuíram para a sua realização e sem as quais tal seria impossível.

À minha família, por me ter apoiado sempre nas minhas decisões, mesmo que às vezes estas parecessem demasiado tresloucadas.

Ao meu orientador Prof. Dr. António Nogueira pela oportunidade de desenvolver este trabalho, que foi muito gratificante em termos pessoais e profissionais, pelos seus ensinamentos que foram mais que muitos e pela sua paciência nas alturas mais difíceis.

À minha co-orientadora Dr. Paula Inês Domingues, pela ajuda numa fase crítica e principalmente, por me incitar ao desenvolvimento de uma visão mais abrangente e global.

À Sónia, companheira de aventuras científicas e não só, que foi o meu apoio no outro lado do mundo, partilhando todos os momentos fossem eles bons ou maus.

Na Tailândia, especialmente no AIT, ao Prof. Dr. Ram. Bujhel e Prof. Dr Amrit Bart pela recepção que nos conceder e pela orientação, Miss Lucia Sukhanenya, pela sua simpatia e disponibilidade, aos técnicos, especialmente ao Pond, pelo auxílio prestado nos laboratórios e na descoberta da cultura tailandesa; ao Warren Turner que se disponibilizou para nos dar a conhecer as instalações da sua quinta de aquacultura, Nam Sai Farm e prestar qualquer esclarecimento relevante do ponto científico; aos amigos que fiz neste país e o tornaram num cantinho especial.

Aos meus colegas de laboratório que foram sempre prestáveis, em especial à Fátima, que nunca deixou de atender às minhas dúvidas electrónicas e de partilhar os seus conhecimentos de forma paciente, ao Rhaul e Carolina, pelos ensinamentos e companhia no laboratório, ns horas mais improváveis pelo que se tornaram mais alegres .

"Last but not least"…. aos meus amigos do coração pelo seu apoio incondicional.

De modo geral, a todas as pessoas com quem me cruzei ao longo da minha vida e cuja partilha de vivências, de certo modo contribuíram para a minha formação pessoal.

palavras-chave aquacultura, Sudeste asiático, *Daphnia magna*, *Danio rerio,* químicos,cloreto de benzalcônio, glutaraldeído

resumo

A aquacultura é uma actividade generalizada por todo o mundo, no entanto o Sudeste Asiático é um dos principais centros de aquacultura graças à sua localização geográfica e clima que lhe confere vantagem para o aumento da productividade. Nos países asiáticos, a aquacultura tornou-se uma das principais fontes de proteína, emprego, rendimento para as famílias e uma importante fonte de receitas nas trocas comerciais contribuindo directamente para a sustentabilidade das populações rurais e desenvolvimento económico destes países.

O crescimento da aquacultura resultou num aumento das preocupações ambientais, visto que a aquacultura promove a contaminação por nutrientes orgânicos (fezes dos peixes, alimento e outros detritos orgânicos) que são despejados dos tanques directamente para os corpos de água próximos (costeiros ou fluviais) e contaminação química (produtos terapêuticos e biocidas que são geralmente usados no controlo de doenças e pestes). A gestão e controlo de doenças que afectam os stocks dependem de boas práticas de cultura combinadas com agentes quimioterapêuticos como pesticidas, herbicidas, bactericidas, desinfectantes entre outros, o Cloreto de benzalcônio (BKC) e o Glutaraldeído (GA), usados especificamente na desinfecção das águas dos tanques de aquacultura.

As espécies *Daphnia magna* Straus e *Danio rerio* Hamilton-Buchanan, organismos modelo em ecotoxicologia, foram usados na avaliação dos efeitos de BKC e GA em sistemas aquáticos. Testes de imobilização aguda (*D. magna*) e Testes de toxicidade embrionária em peixes (*D. rerio*) foram aplicados na tentativa de avaliar a toxicidade do BKC e GA nestas espécies e determinar os respectivos LC_{50}, 48 h em *D. magna* e 96h em *D. rerio*.

Nos resultados do teste de imobilização aguda, *D. magna* foi mais sensível ao BKC (48 h EC_{50}= 0.052 mg/l) do que ao GA (48 h EC_{50}= 8.81 mg/l). Por seu lado, os testes de toxicidade embrionária em peixes, *D. rerio* também demonstrou mais sensibilidade ao BKC (96 h LC_{50}= 3.9 mg/l) do que ao GA (96 h LC_{50}= 27.64 mg/l).

No teste com BKC observaram-se alterações significativas no estádio embrionário (alterações do fluído amniótico) e no estádio larval (distúrbios na postura) de *D. rerio*. Durante a exposição do *D. rerio* ao GA, efeitos significantes foram notados durante o desenvolvimento do embrião e larva, nomeadamente no saco embrionário (deformação e opacidade), na espinha dorsal (cauda torta), no coração (edemas pericardiais) e tamanho da larva (cauda curta). Em ambos os químicos o sucesso global de eclosão de *D. rerio* não foi afectado.

Este estudo aborda a problemática do uso inadequado de químicos em aquaculturas tropicais, contribuindo para preencher uma lacuna em termos de informação toxicológica necessária a avaliações de risco ambiental de químicos nos trópicos.

keywords aquaculture, Southeast Asia, *Daphnia magna*, *Danio rerio,* chemicals, benzalkonium chloride, glutaraldehyde

abstract

The aquaculture is an activity widespread through the world, although Southeast-Asia is a major center for aquaculture due to the geographical location and climate that are advantageous to increase productivity. In Asian countries, aquaculture has become a major source of protein, employment, income and of foreign exchange, contributing directly to food security, rural livelihoods and economic growth.

The growth of aquaculture results in increasing environmental concerns since aquaculture promotes organic nutrients contamination (fish feces, food waste and other organic debris that are flushed from the ponds into surrounding coastal or river waters) and chemical contamination (therapeutants and biocides are commonly used to control diseases and pests). The management and control of diseases that affect the stocks depends upon good culture practice combined with chemotherapeutic agents like pesticides, herbicides, bactericides, among others such Benzalkonium chloride (BKC) and Glutaraldehyde (GA) used specifically to disinfect the aquaculture ponds.

The species *Daphnia magna* Straus and and *Danio rerio* Hamilton-Buchanan, model organisms in ecotoxicology, were used to assess the effects of BKC and GA in aquatic systems. Acute immobilization tests (*D. magna*) and fish embryo toxicity Test (*D. rerio*) were performed in attempt to evaluate the toxicity of BKC and GA in those species and to determine the respective LC_{50}, 48 h in *D. magna* and 96h in *D. rerio*.

The results of acute immobilization test revealed that D. magna is much more sensitive to BKC (48 h EC_{50}= 0.052 mg/l) than to GA (48 h EC_{50}= 8.81 mg/l). The early life stages assay with *D. rerio* also shown to be more sensitive to BKC (96 h LC_{50}= 3.9 mg/l) than to GA (96 h LC_{50}= 27.64 mg/l). Furthermore, BKC significant effects occurred at embryonic stage (alterations on the consistence of amniotic fluid) and at larvae stage (posture disturbances) of *D. rerio*. During GA exposure, significant effects were noted in embryo and larvae development namely in the yolk sac (deformities and opacity), in the spine (curved tail), in the heart (pericardial oedemas) and size of the larvae (short tail) in the last days of exposure. In both chemicals, the global hatching success was not affected.

This study drive attention to the problem of inappropriate use of chemicals in tropical aquaculture systems, contributing to fulfil the data gap on ecotoxicological information necessary for ecological risk assessments of chemicals in the tropics.

“Science, in the very act of solving problems, creates more of them.”

(Abraham Flexner, 1930)

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Introduction

1.1.Aquaculture in South-East Asia

The aquaculture production of several aquatic organisms has increased in the last years. This activity is widespread throughout the world mainly since the sharp and continuous decline of many species targeted in capture fisheries (Johnston 2002).

Southeast Asia is a major center for aquaculture due to the geographical location and climate that are advantageous to increase productivity(Domingues 2007). There is a long historic connection of this region to aquaculture, but rapid expansion only started after 1975 (Hishamunda 2009), becoming one of the areas on the planet with more productivity in the field of aquaculture. Some of these countries, with its productive inland fisheries, are ranked among the top 25 countries in terms of aquaculture volume according FAO statistics (FAO 2008). In 1995, the contribution of Southeast Asia to global aquaculture production was 8.8% (weight) and 15.3% (value) (FAO 1997).

COUNTRY	SHARE OF GLOBAL PRODUCTION
China	70.2%
India	4.8%
Japan	3.1%
Philippines	2.2%
Korea, Republic of	1.8%
Indonesia	1.6%
Bangladesh	1.4%
Viet Nam	1.4%
Thailand	1.4%
Other countries	12.0%

Figure 0.1 - Top of Nine Countries for Global Aquaculture Production (FAO 2001)

As a group of countries with environmental suitable conditions, technical capacity, low costs, markets for farmed species and governments with the ambition to promote aquaculture (Hishamunda 2009), several measures were taken to improve production in aquaculture as animal breeding, broodstock development, health

management, pathogen-free, disease resistant broodstock and intensification (FAO 2006-2009).

The review of (Gräslund 2001) reported the aspects of the shrimp exploitation in Southeast Asia. The culture of these animals has a big contribution to the economy of these countries, since these species have a high value and their production is mainly for exportation. However, the importance of aquaculture as a source of domestic food is highlighted by the role of fish as animal protein. The region relies heavily on fish for protein, (exception of shrimp), since the major fish native species in the region (milkfish, the rohu, the common carp and tilapia) production is primarily for local consumption, and lately to commercial purposes (Hishamunda 2009).

Attending to previous aspects in these countries, aquaculture has become a source of protein, employment, income and of foreign exchange, contributing directly to food security, rural livelihoods and economic growth (Hishamunda 2009).

1.2. Chemicals used in Aquaculture

Fish husbandry at very high stock densities can result in several types of diseases. There are two types of diseases that can occur both in natural and cultured animals: infectious diseases caused by either a bacterial, viral or parasitic agent; non-infectious diseases caused by toxic substances, improper nutrition, poor water quality, physical damage or genetics. Total prevention of diseases in aquaculture systems is likely to be unattainable in practice, thus the good management of a stock depends upon good culture practice in combination with chemotherapeutic agents (Johnston 2002).

In attempt to manage farming conditions and to control diseases in farmed species, antibiotics, parasiticides, anaesthetics, spawning hormones, oxidants, disinfectants and herbicides are routinely used in aquaculture (Kümmerer 2001; Johnston 2002).

A disinfectant is an agent that through heat, radiation or chemical action disinfects destroying, neutralizing, or inhibiting the growth of the pathologic agents in inanimate surfaces. Frequently, formulations of disinfectants contains highly complex products or mixtures of active substances (Kümmerer 2001). Disinfectants in aquaculture are used to prevent and minimize the spread of pathogens and diseases within a system, although usually their effectiveness is hindered by the presence of organic matter (Liao 1996).

Bactericides are substances used to eliminate bacteria and include either disinfectants, antiseptics or antibiotics. Bactericides such as Benzalkonium chloride, Providone iodine, Glutaraldehyde and Formalin are usually added to pond water at concentrations of 1-10 mg/l in attempts to prevent excessive degraded by bacteria, but little information is know about the these compounds, their reaction products, or if their degradation products are bioaccumulative (Boyd 1999).

1.2.1. Environmental Impacts

Since governmental incentive measures were taken, these countries had successful rates of aquaculture production and a quick expansion without order. This occurrence was associated to problems of disease and other sustainable affairs, creating serious concerns about environmental impacts of aquaculture, although is an activity with high rates of income. After this events, those governments developed policies to reduce the environmental risks involving aquaculture pratices (Hishamunda 2009).

Most of aquaculture operations are still based on extensive and semi-intensive methods of aquaculture to enhance yields and to improve the efficiency of production and growth of these activities some serious constraints are involved especially in fish farming, requiring both land and water — two resources already in short supply in many areas (Holmes 1996).

Among the major environmental effects of aquaculture activities is genetic pollution, spread of parasites and diseases, misuse of chemicals, toxicity of non-target populations, release of wastes and degradation of habitats (Primavera 2006).

Mainly, in trends of intensification if appropriate planning and management of farming systems, is not followed, especially regarding the excessive or incorrect use of resources and inputs as chemicals, it can induce toxicological effects into non-target populations (cultured species, human consumers and wild biota) and accumulation of residues (Holmstrom 2003; FAO 2005-2009). However, closed and semi-closed water systems recycle water by the use of reservoirs, treatment ponds and canals back to production ponds, reducing this way the amount of discharged wastes (Primavera 2006).

This review focuses on the use of some disinfectants in aquaculture systems, as Benzalkonium chloride (BKC) and Glutaraldehyde (GA), widely used in aquaculture

operations in Southeast Asia and two distinct toxicological model organisms, *Daphnia magna* and *Danio rerio*.

1.2.2. Benzalkonium Chloride

Chemical and Physical Properties

Benzalkonium Chloride (BKC; $C_{21}H_{38}NCl$; CAS no. 8001-54-5) is a cationic surfactant, also known as quaternary ammonium compound. This substance is a mixture of alkylbenzyldimethylammonium chlorides [C6H5CH2N(CH3)2C*n*H2*n*+1·Cl] in which the alkyl groups have a chain length from C8 to C18, creating homologues (BACs) (O'Neil et al., 2001; Pavlostathis 2009).

BKC occurs in different physical forms, being hygroscopic, soapy to the touch and has a moldy aromatic odour and very bitter taste. Practically insoluble in ether, but very soluble in acetone, ethanol (95%), methanol, propanol and water (Wade 1994). Aqueous solutions of BKC foam when shaken, with a low surface tension, possess detergent and emulsifying properties (Wade 1994).

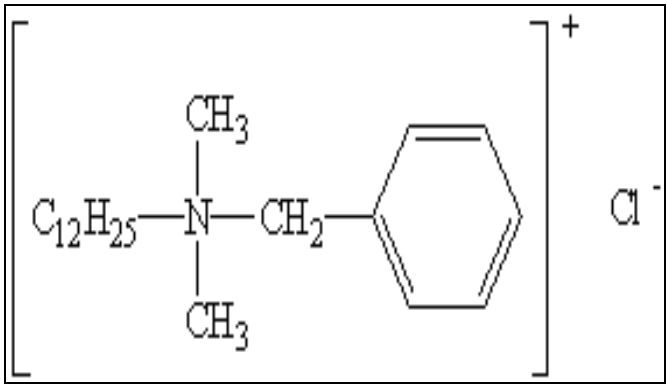

Figure 0.2 Structural formula of Benzalkonium chloride (THWATER 2002-2006)

Applications in Industry

BKC is a substance widely used industrially as a disinfectant, preservative and stabilizer. In the pharmaceutical industry is used in low concentrations (0.007 – 0.01%) as a preservative or stabilizer (Fan 1993), while disinfectants often contain higher concentrations of benzalkonium chloride (0.01 – 80%) (ACT 1989). In aquaculture, BKC is broadly used as a disinfectant and as a very effective herbicide and bactericide (Cheng 2003). The target for the antibacterial activity of BKC is the membrane; the

lipophilic alkyl chain penetrates into the membrane with the polar quaternary ammonium group on the membrane surface. The mechanism of toxicity is based on the alteration of the phospholipid bilayer that leads to the impairment of the membrane (Pérez 2009).

Environmental Fate

The knowledge about the biodegradation pathways of alkyldimethylbenzylammonium salts, as cloride (ADMBAC), is very scarce (Madsen 2001). Biodegradation of aqueousphase (bioavailable) BAC in aerobic biological systems has been demonstrated (Patrauchan 2003). However, in anaerobic biological systems BAC exhibit toxicity and resistance to biodegradation, which results in its environmental persistence (Pavlostathis 2009). In the work developed by Fenger (1973) with alkyldimethylbenzylammoniumchloride (ADMBAC), the identified metabolites (benzoate, acetate, and tetradecyldimethyl amine) formed under degradation of C14 ADMBAC in activated sludge pilot plants indicates that ADMBAC is degraded via a cleavage of the bond linking the benzene group to the alkyldimethylammonium.

Since, BAC soil/water sorption coefficient is faster than biodegradation in aerobic systems, it is transfered to anoxic/anaerobic compartments, such as anaerobic digesters and aquatic sediments (Tezel 2006). Based on the value of *n*-octanol/water partition coefficient (log $P_{o/w}$ <1), benzalkonium chloride is not be expected to bioaccumulate.

Effects on Aquatic Organisms

BKC is a substance highly toxic for aquatic organisms. The effects include mortality in crustaceans; behavior and histological alterations, and mortality in fish; biochemical effects, growth inhibition, physiological injuries and population level effects in phytoplankton; behavioral changes and mortality in zooplankton (PAN 2009). *Oncorhynchus mykiss*, *Danio rerio* (fish), *Daphnia magna* (zooplankton), *Elodea canadensis, Pseudokirchneriella subcapitata* (algae) (FEF 2008; Vervliet-Scheebaum 2008; PAN 2009) are among the species tested.

Table 0.I - Toxicity of BKC in aquatic species

Species	Value (mg/l)	Endpoint	Time (days)	Reference
Algae				
Chaetoceros gracilis	87.3	EC_{50}	4	(Pérez 2009)
Isochrysis galbana	66.4	EC_{50}	4	(Pérez 2009)
Pseudokirchneriella subcapitata	0.07	IC_{50}	3	(FEF 2008)
Crustacea				
Daphnia magna	0.02	EC_{50}	2	(FEF 2008)
Fish				
Danio rerio	0.31	LC_{50}	4	(FEF 2008)
Oncorhynchus mykiss	11.5	LC_{50}	4	(Mayer

1.2.3. Glutaraldehyde

Chemical and Physical Properties

Glutaraldehyde (GA; $C_5H_8O_2$; CAS no. 111-30-8) is an aliphatic dialdehyde frequently used as disinfectant and sterilizing agent against bacteria and viruses. Such a large number of applications since industrial, scientific and biomedical fields are a consequence of the rapid reaction of glutaraldehyde with proteins as a cross-linking agent, which is very important for biocidal activity (HSDB 1996).

Figure 0.3 - Structural Formula of Glutaraldehyde (Chemblink 2009)

Table 0.II - Physical and chemical properties of Glutaraldehyde (HSDB 1996)

Characteristic	Value
Molecular formula	C5H8O2
Molecular weight (g mol−1)	100.11
Density (kgm−3)	0.72
Melting point (C)	−14
Boiling point (◦C, at 1002 hPa)	188
Solubility	Miscible
log*K*ow	−0.11
Vapor pressure (Torr, at 25 ◦C)	0.6
Henry's constant (atmm−3 mol)	1.1×10−7
Aquatic biodegradation (aerobic) (h)	*t*1/2 = 10.6

Applications in Industry

GA has assorted applications in the industry: Cold disinfectant in the health care industry; hardener in x-ray film processing; water treatment, biocide in the pulp and paper, and petroleum industries; cleaning agent in animal health industry; tanning; embalming agent; microscope/ histology; sterilization of heat sensitive dental and medical equipment; cosmetics and aquaculture (SIDS 2005). Usually this chemical is sold commercially as a 45% or 50% aqueous solution. GA is a fish egg surface disinfectant for aquaculture (Katharios 2007), although the concentration and exposition times should be evaluated according the species fish.

Half Life and Decomposition

The applications of Glutaraldehyde entail exposure of aquatic and atmospheric compartments. However, does not persist in the atmosphere since it suffers a photochemically-induced degradation. Ideally, wastes of glutaraldehyde solutions are discharged and treated into sewer, after the respective effluents are discharged to receiving waters. Any glutaraldehyde that may enter into receiving waters is likely to be rapidly diluted and undergo further biodegradation (SIDS 2005).

GA is completely soluble showing relatively good hydrolytic and photolytic stability in water. Biodegradable in fresh and sea-water with complete mineralization,

this substance is neither persistent nor does it produce problematical metabolites (SIDS 2005).

Metabolism of GA was studied under aerobic and anaerobic conditions in the presence of river water-sediment system (Leung 2001 a; Bioshare 2002). The results showed that glutaraldehyde degradation was quite rapid under aerobic conditions (half life of 10.6 h), based on the disappearance of the parent compound from the water phase and in anaerobic conditions was also rapid (half life of of 7.7 h). In constrast, extrapolated half-life of GA in abiotic degradation was 508 days at pH 5, 102 days at pH 7, and 46 days at pH 9 (Leung 2001 a).

The proposed degradation pathway of GA in aerobic systems takes first to glutaric acid and finally to carbon dioxide, without any intermediate metabolite. After 48 hours, there were no traces of either GA or glutaric acid (Leung 2001 a; Bioshare 2002).

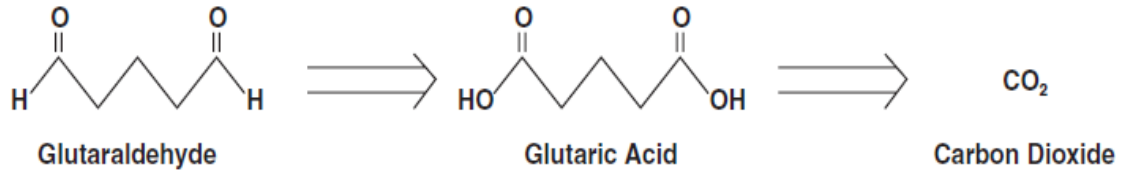

Figure 0.4 - Decomposition path of Glutaraldehyde in aerobic systems (Bioshare 2002)

Meanwhile in anaerobic systems, the results indicate a pathway where glutaraldehyde is first metabolized to 5-hydroxypentanal as an intermediate, which then undergoes transformation into 1,5-pentanediol, without significant biocidal properties (Leung 2001 a; Bioshare 2002).

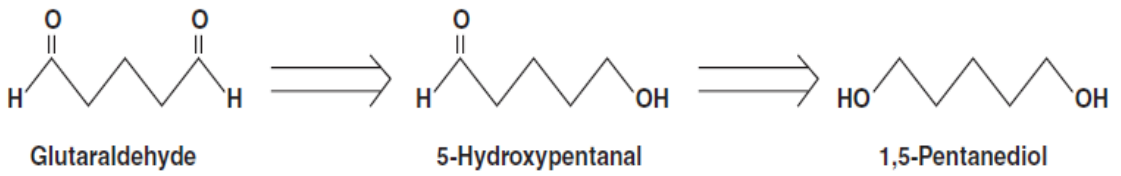

Figure 0.5 - Decomposition path of Glutaraldehyde in anaerobic systems (Bioshare 2002)

Interaction with Air, Water and Sediment

According to environmental partitioning studies, it can be concluded that GA has low tendency to enter the atmosphere from the aqueous environment due to its low air/water partition coeficient. In relation to terrestrial environment, GA displays a moderate to high potential to leach from soil, since soil/water sorption coefficient is low. Thus, the principal ecosystem of relevance for glutaraldehyde is the aquatic environment (Leung 2001 b).The tendency of GA to bioaccumulate in aquatic organisms is low, based on its high water solubility and low *n*-octanol/ water partition coeficient.

In summary, Glutaraldehyde is hydrophilic, biodegradable and non-bioaccumulative.

Effects on Aquatic Organisms

Some bioassays with toxic compunds showed that glutaraldehyde is slightly toxic to crabs, shrimp and sewage micro-organisms, slightly to moderately toxic to fish and daphnids, moderately toxic to oyster larvae, and moderately to highly toxic to algae. This substance loses its biological activity below about 10 mg/L (SIDS 2005; PAN 2009).

Table 0.III - Toxicity of Glutaraldehyde in aquatic species

Species	Value (mg/l)	Endpoint	Time (days)	Reference
Algae				
Pseudokirchneriella subcapitata	1.8	LC_{50}	4	(Landrum 2005)
Scenedesmus supspicatus	2.1	EC_{50} (Biomass)	4	(RCC 1990)
Crustacea				
Americamysis bahia	0.0071	LC_{50}	4	(EPA 2000)
Carcinus maenas	465	LC_{50}	4	(UCC 1975)
Cerodaphnia dubia	0.0049	LOEC	4	(Landrum 2005)
Daphnia magna	5	LC_{50}	2	(UCC 1981)
Mollusca				
Crassostrea virginica	0.55	LC_{50}	4	(UCC 1975)
Fish				
Lepomis macrochirus	11	LC_{50}	4	(UCC 1978)
Oncorhynchus mykiss	0.0239	LC_{50}	4	(EPA 2000)
Oncorhynchus kisutch	0.011	LC_{50}	4	(UCC 1977)
Pimephales promelas	0.0054	LC_{50}	4	(UCC 1996)
Oncorhynchu mykiss	1.82	IC_{50} (Hatch rate)	35	(Landrum 2005)

1.3. Tested Species

1.3.1. Daphnia magna Straus

Life Cycle

Daphnia magna is a small planktonic invertebrate crustacean (0.5-5 mm) with a short life cycle (Ren 2007) and the capacity to reproduce by two methods: sexually (ephipial eggs or amphigonic) and asexually (ameiotic parthenogenesis) (Peters 1987). Usually, a adult female can produce more than 100 parthenogenic eggs per brood, but

when adverse conditions are triggered the production of ephipial start the sexual reproduction.

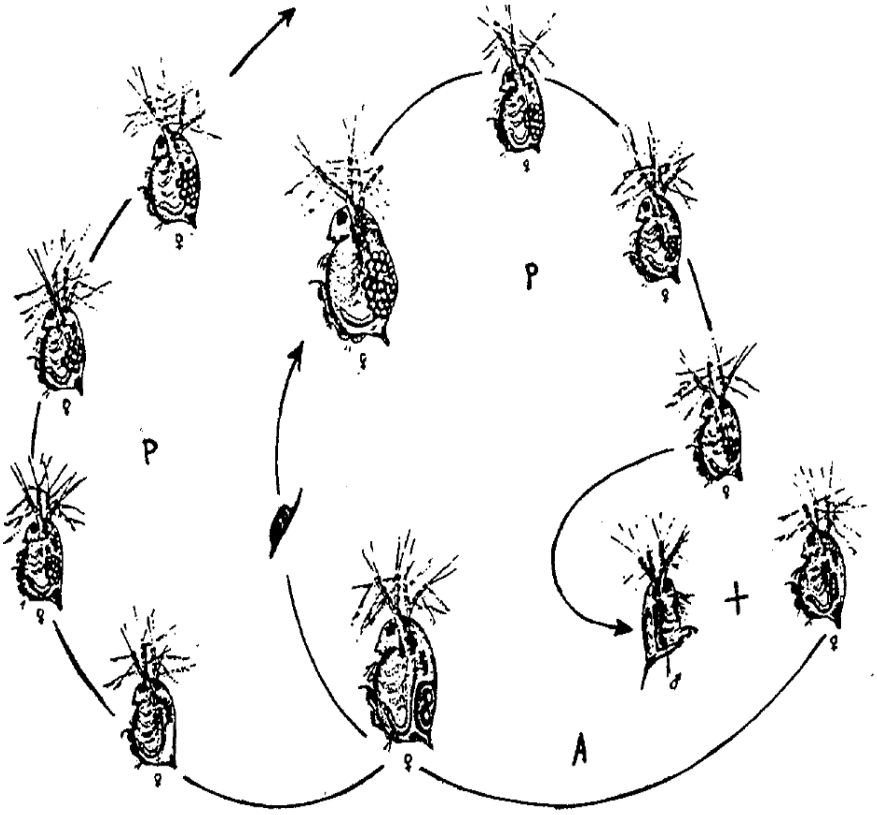

Figure 0.6 - Reproductive cycle of *Daphnia magna*. P Partenogenetic reproduction: many summer eggs which usually develop into females but under adverse environmental conditions may develop into males. A Amphigonic: reproduction two winter eggs that after being fertilized are extruded into the brood chamber which is modified to form the ephippium (Peters 1987).

Ecological Relevance

D.magna belong to important group with a crucial role in aquatic ecosystem, being highly sensitive to changes of the chemical composition in aquatic environments. This cladoceran possesses some requirements for a good model organism in a bioassay: intermediate size that enable a good management in culture and also allow the representation of a range of forms, physiologies and ecologies but still enough to keep some homogeneity (Peters 1987); a short life cycle, with high reproductive rate, and

available at different times of the year, in sufficient number and size required (Peters 1987).

Daphnia magna display an important role in aquatic food webs (Baird 1989), between primary producers and fish, thus their life-history changes can trigger community or ecosystem-level responses (Barbosa 2008).

Some parameters, like food availability, can influence the daphnid populations in the field (Lampert 1993) and passively limit reproduction. Therefore, daphnids adapt their reproductive strategies according this highly variable environmental factor (Threlkeld 1987).

Ecotoxicological Relevance

At present, biotesting plays an important role in the system of water quality control. Currently used methods of bioassay provide only the integral evaluation of the pollutants effect, but not the determination of the xenobiotics origin (Flerov 1989; Tonkopii 2007). In the ecotoxicological research, the interaction between the chemicals and their effects at different levels of biological organization has a crucial role (Malty 1989).

Daphnia magna is a standard organism routinely used to assess the health of aquatic ecosystems through the identification of main classes of toxic xenobiotics (organophosphates, carbamates, heavy metals, organochlorines, pyrethroids) (Tonkopii 2007). Characteristics as sensitivity to alterations of chemical expression in aquatic ecosystems (Ren 2007), rapid reproduction and critical role in freshwater ecosystems (Baird 1989) corroborate their assessment ability.

In toxic assessments is essential obtain uniform results with minimum interference of genetic variability influence, and hence the repeatability, reproducibility and robustness of toxicity test increase (Baird 1989). Thus, *D. magna* is a reliable organism, since in good conditions reproduces asexually by parthenogenesis, causing low genetic variability (Barata 2006).

The interference of xenobiotics in the endocrine regulation of *D. magna* egg production (Zaffagnini 1987) may disturb the natural shift of biomass allocation and consequently hinder the adaptation to environmental stress (Coors 2004).

1.3.2. Zebrafish (*Danio rerio* Hamilton-Buchanan)

Life Cycle

The zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) is a small freshwater benthopelagic cyprinid, which is original from Ganges River system, Burma, Malakka Peninsula and Sumatra (Eaton 1974).

In contrast to many other fish species, in adult phase zebrafish only reach approximately 3-5 cm long, allowing an easy management in laboratory in large numbers (Kishi 2003). At 26ºC, their growth is significant and, respective life cycle is complete within three months. Males are easily distinct from females, under spawning conditions, since their body shape is more slender and females get swollen bellies. Another advantage of this species is its high fecundity, daily a female can spawn between fifty and two-hundred eggs (Nagel 2002).

Zebrafish embryonic development has been well characterized (Kimmel 1995). *Danio rerio* egg is telolecithal, with a cleavage meroblastic and discoidal. Fertilization triggers cytoplasmatic movements, as accumulation of nonyolky cytoplasm in the animal pole. Only this portion of the clearer yolk granule-rich vegetal cytoplasm, identified as blastocist, undergoes to cleavage (Kimmel 1995; Nagel 2002). Organogenesis occurs rapidly, basic body plan is defined after 24 hours post-fertilization (hpf) and hatching goes on at 2-3 days post-fertilization (dpf) and major organs are present in larvae by 5 to 6 dpf (Rubinstein 2003; Scholz 2008).

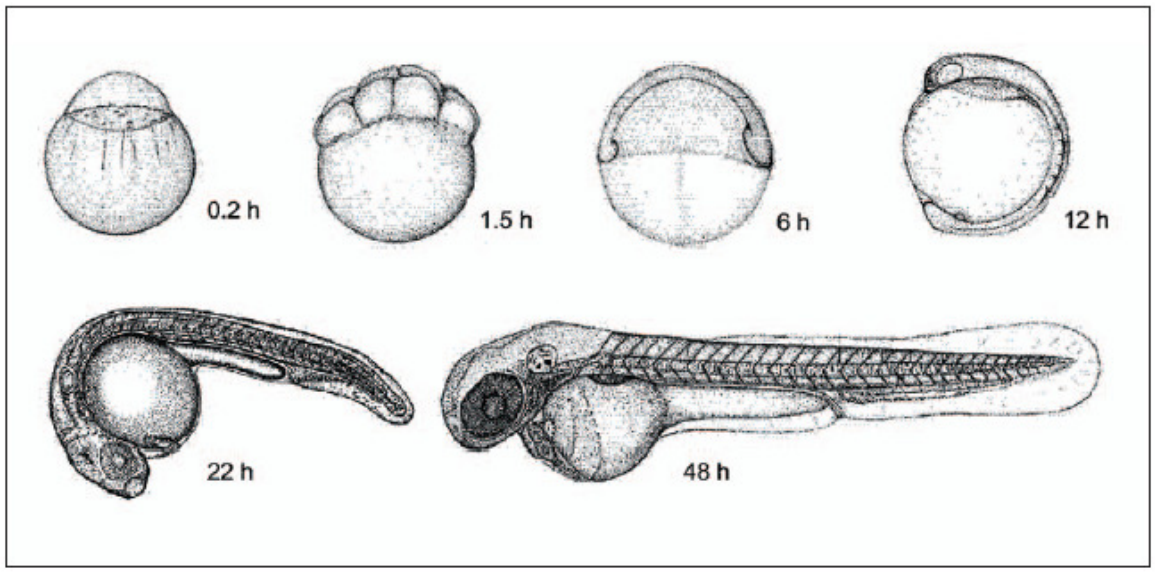

Figure 0.7 - Stages of zebrafish (*Danio rerio*) development. For ease of differentiation, the 22 h embryo has been dechorionated. Note: variable scaling of different stages. Top row stages from Kimmel (Kimmel 1995; Braunbeck 2004)

Ecotoxicological Relevance

Zebrafish is frequently used as model in development toxicological assays, since has a quick development with transparency allowing easy visualization of tissues and organs, tests can start immediately after fertilisation including more information than lethal and sublethal effects (Rubinstein 2003; Haendel 2004).

D. rerio is a fish with suitable features to evaluate possible hazardous effects of water-soluble compounds to wild vertebrates, since it has many organs and cell types similar to different classes of aquatic vertebrates (Rubinstein 2003).

In toxicology and pharmacology, quantities of chemicals to deploy and volumes of potentially hazardous waste can be minimized by the introduction of assays performed in a miniaturized format, as multi-well plates (Spitsbergen 2003; Hill 2005). This method is supported by the small size of eggs and juveniles, which enable an increase of replicate samples, thus the increase of database, improving statistical evaluation and at last the validation of results (Bopp 2006).

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Toxicity of two chemicals (Benzalkonium chloride and Glutaraldehyde) used in aquaculture ponds in the tropics to Daphnia magna and Danio rerio

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Abstract

The aquaculture is an activity widespread through the world, but in Southeast Asia it is an economically important activity that continues to grow every year. The intensification of aquaculture can promote diseases outbreaks that are controlled by the use of chemicals for the different kind of threats. This work focuses on two disinfectants, Benzalkonium chloride (BKC) and Glutaraldehyde (GA), used specifically to sterilize the aquaculture ponds. The species *Daphnia magna* Straus and *Danio rerio* were the selected model organisms, since are important organisms to assess the effects of chemicals in the health of the aquatic ecosystem. In the results of acute immobilization test, D. magna revealed to be much more sensitive to BKC (48 h EC50= 0.052 mg/l) than to GA (48 h EC50= 8.81 mg/l). Regarding the early life stages assay, *D.rerio* also shown to be more sensitive to BKC (96 h LC50= 3.9 mg/l) than to GA (96 h LC50= 27.64 mg/l). In BKC assay two significant effects occurred: alterations on the consistence of amniotic fluid (bubbles effect), in embryos and posture disturbances, in larvae. In GA exposure, significant hatching delays was observed during the test, although at the end did not affect global hatching success; significant deformities and opacity in yolk sac, spine malformations (curved tail) and pericardial oedemas occurred during embryo and larvae development; and significant number of undersize larvae (low length of tail) in the last days of exposure.

Measured acute toxicity values for BKC and GA on *D. Magna*, under tropical conditions, are different and similar (respectively for each chemical) to same values under standard (temperate) conditions.Moreover, since BKC is a cationic surfactant it changes the chorionic membrane permeability of zebrafish, the consistence of amniotic fluid, and probably interferes with vital physiological processes associated with embryonic development.

Keywords: aquaculture, Southeast Asia, *Daphnia magna*, *Danio rerio,* benzalkonium chloride, glutaraldehyde, acute immobilization test, early-life stages assay

1.5. Introduction

Southeast Asia is a major center for aquaculture due to the geographical location and climate that are advantageous to increase productivity (Domingues 2007). In Asian countries, aquaculture has become a major source of protein, employment, income and of foreign exchange, contributing directly to food security, rural livelihoods and economic growth.

Meanwhile, the growing and intensification of aquaculture increases drastically the subsequent use of organic compounds, which can promote outbreaks of diseases in farmed species (Naylor 2000). To control these threats is essential to combine good culture practices with chemotherapeutic agents like pesticides, herbicides, bactericides (Johnston 2002).

Excessive and incorrect use of chemicals can result in toxicity to non target populations (cultured species, human consumers and wild biota) and accumulation of residues (Holmstrom 2003).

When the water from the ponds is flushed into surrounding coastal or river waters it drags high amounts of mixed organic debris, nutrients and chemical residues (Mock 2001), increasing the environmental impacts. However, closed and semi-closed water systems recycle water by using reservoirs, treatment ponds and canals back to production ponds, thus reducing the amount of discharged wastes (Primavera 2006).

Benzalkonium chloride (BKC) and Glutaraldehyde (GA) are disinfectants frequently used in aquaculture pond systems, because of their biocidal activity (Boyd 1999; Gräslund 2001).

Benzalkonium chloride is a cationic surfactant, also known as quaternary ammonium compound. Aqueous solutions of benzalkonium chloride foam when shaken, have a low surface tension and possess detergent and emulsifying properties (Wade 1994). The target for the antibacterial activity of BKC is the membrane. The lipophilic alkyl chain breaks into the membrane with the polar quaternary ammonium group on the membrane surface. The mechanism of toxicity acts provoking alterations into phospholipid bilayer, thus leading to the injury of the membrane (Perez, 2008). Information concerning the biodegradation pathways of alkyldimethylbenzylammonium salts, as cloride (BKC), is very scarce (Madsen 2001), although biodegradation of aqueousphase (bioavailable) of Benzalkonium chloride homologues (BACs) in aerobic biological systems has already been demonstrated (Patrauchan 2003). BKC is highly toxic for aquatic organisms, however toxicity values are scarce and could be found only

for the algae *Pseudokirchneriella subcapitata* (72h IC50= 0,07 mg/l) (FEF 2008), for the crustacean *Daphnia magna* (48h LC50= 0,02 mg/l) (FEF 2008) and for the fish *Oncorhynchus mykiss* (96h LC50= 11.5 mg/l) (Mayer 1986) and *Danio rerio* (96h LC50 = 0,31 mg/l) (FEF 2008).

Glutaraldehyde (GA) is an aliphatic dialdehyde frequently used as disinfectant and sterilizing agent against bacteria and viruses. It is also used as fish egg surface disinfectant for aquaculture (Katharios 2007), although the concentration and exposure times should be evaluated according the fish species. GA is hydrophilic, biodegradable and non-bioaccumulative (SIDS 2005). In aerobic conditions, glutaraldehyde is quickly degraded (half lifeof 10.6 h) into glutaric acid and finally into carbon dioxide. Traces of either glutaraldehyde or glutaric acid were not found after 48 hours (Leung 2001 a; Bioshare 2002). Several studies report the adverse effects of Glutaraldehyde on aquatic organisms, namely on algae: Pseudokirchneriella subcapitata (96h LC50 = 1.8 mg/l) (Landrum 2005), *Scenedesmus supspicatus* (96h EC50 = 2.1 mg/L) (RCC 1990); molluscs: *Crassostrea virginica* (96h EC50 = 780 µg/l) (EPA 2000); crustaceans: *Daphnia magna* (48h LC50 = 5 mg/l) (UCC 1981), *Americamysis bahia* (96h LC50= 7,1 mg/l) (EPA 2000); and fish: *Lepomis macrochirus* (96h LC50 = 11 mg/l) (UCC 1978); *Oncorhynchus mykiss* (96h LC50 = 0.0239 mg/l) (EPA 2000); *Oncorhynchus kisutch* (96h LC50 = 0.011 mg/l) (UCC 1977) and *Pimephales promelas* (96h LC50= 5.4 mg/l) (UCC 1996).

The main purposes of the present study were: (1) to evaluate acute responses of Daphnia magna to BKC and GA exposure and determine both 48h LC50; (2) to assess possible influence of BKC and GA in the developing zebrafish embryos, and respective 96h LC50. Both studies were developed in tropical conditions.

1.6. Materials and Methods

1.6.1. Preliminary Acute Toxicity Assay with *Daphnia magna*

Chemicals

High purity (≥ 95%) Benzalkonium chloride and Glutaraldehyde (≈ 50% solution in water) were purchased from Fluka (St. Louis, MO, USA).

Test Organisms

D. magna (clone A *sensu*, (Baird 1989) cultured under laboratory conditions was used in the experiments. Cultures were kept in beakers with 100 ml of ASTM hard water (ASTM 1998), enriched with the organic additive Marinure ‘‘25” (Pann Britannica Industries Ltd, Waltham Abbey, UK), an extract from the algae *Ascophyllum nodosum* (Baird 1989). The culture media had a total hardness of 175.41 ± 5.53 mg/l CaCO3, a pH range of 8.15 ± 0.27 and a conductivity of 577.63 ± 9.01 µS/cm.

The culture medium was renewed three times a week. Daphnids were fed daily with algae (*Chlorella vulgaris*) with a density of 5.5 µg dry weight/ml, maintained with a photoperiod of 16 h light: 8 h dark and temperature of 24 ± 1 ºC. Adults were distributed individually per beaker, while in juveniles density was below 3 per 100 ml of culture medium. These culture conditions proved to be suitable since they led us to a consistent healthy parental stock with exclusive female offspring. Only newly released neonates from the third to sixth clutch were used in the experiments (≤ 24 h). Experimental conditions were similar to culture conditions.

Acute Immobilization Assay

The acute test was performed in accordance with the OECD guideline (OECD 2000) for Daphnia magna acute immobilization test. Neonates were statically exposed to control medium, BKC and GA at nominal concentrations of 125-0.5 µg/L and 20-0.625 mg/L, respectively. Each treatment concentration had 3 replicates, containing 100ml of chemical solution and 5 juveniles of *D. magna*. During the exposure experiments, no food was added. Incubation conditions were set at 24 ± 1ºC in a 16:8 h photoperiod. The endpoint examined was immobilization, defined as inability to swim (actively move) after 15 s of gentle agitation.

1.6.2. Bioassay with *Danio rerio*

Test Organisms

Zebrafish (D. rerio) from a culture system established at the Department of Biology, University of Aveiro, kept in aquaria with carbon-filtered water at 28.0±2ºC under a 16h light/ 8 h dark photoperiod cycle were used. Conductivity was kept at 750±50 µS, pH at 7.5±0.5 and dissolved oxygen at 95% saturation. Adult zebrafish were

fed twice daily with a combination of freshwater aquarium flakefood (ZM 400 Granular) and brine shrimp.

Early-life stages assay

This assay was based on OECD Guideline (OECD 2006) of Fish Embryo Toxicity Test. After collection and cleaning with system water, eggs were examined under the a stereomicroscope (Stereoscopic Zoom Microscope- SMZ 1500, Nikon). Only fertilized eggs between the 4- and 128-cell stages were used. Unfertilized eggs (non-transparent) or eggs with overt anomalies were discarded. Seven nominal concentrations of BKC (1.7, 2.1, 2.5, 3.0, 3.6, 4.3 and 5.2 mg/L) and GA (2.5, 5, 10, 25, 50, 75 and 100 mg/L) were tested, plus the respective controls. Each treatment contains forty-eight eggs that were set individually with 2 ml of the test solution in each well of 24-well microplates. Both disinfectants are soluble in water, since BKC is a soap and GA in liquid form. Test solutions were prepared by dilution of stock solution in water, with controlled pH (7.5±0.5) and conductivity (750±50 µS/cm), except in the lower concentrations where successive dilutions were used. The temperature during the test was 26.0±1ºC and the photoperiod had 16 h light and 8 h dark.

The examination of the eggs was carried out with the aid of a stereomicroscope at 24, 48, 72, 96, 120 and 144 hours post fertilization (hpf). The resolution to observe the eggs was between x30 and x50. The parameters evaluated were: egg coagulation, otolith formation, eye and body pigmentation, somite formation, heart beat, tail blood circulation, detachment of the tail-bud from yolk sac, alterations onamniotic fluid and hatching. The parameters post-hatching were: pericardial oedema, posture, reducted body size, spine malformations and mortality.

1.6.3. Statistical Analysis

Daphnia magna

Statistical treatment of acute toxicity assay was performed. Determination of median effect concentration (EC_{50}) at 48 h with 95% confidence limit was made plotting the number of immobilized organisms from acute immobilization test of BKC and GA against the test concentrations, using standard Probit method (Finney 1971).

Danio rerio

Sigma Stat 3.5 statistical package was used for statistical analyses (SPSS 2004). Values are expressed as mean ± SE. The EC_{50} values were estimated by SPSS probit at a level of p <0.05. ANOVA statistical analysis was performed, except when data did not pass the Kolmogorov Smirnov normality test, in which case a Kruskal–Wallis test was performed. In case of significant results, the Dunett or Dunn's test was used to check differences between tested concentrations and control. The estimation of the value of Lethal Concentration at 50% (LC_{50}) at 96 h, values of Effect Concentration at 50 % (EC_{50}) of parameters and values of Lowest Observed Concentration Effect (LOEC) was made through Sigma Stat 3.1. All statistical analyses were performed with a significance level of 0.05.

1.7. Results

1.7.1. Acute Toxicity in *Daphnia magna*

The acute toxicity test for both chemicals was valid, since both control survival was 100%. For Benzalkonium chloride, 48 h EC_{50} was determined as 0.052 µg/L (95% Confidence Interval = 0.050 - 0.053 µg/L) (**Figure 0.1**), while Glutaraldehyde 48h EC_{50} value was 8.81mg/l (95% Confidence Interval = 8.10 - 9.58mg/l) (**Figure 0.2**).

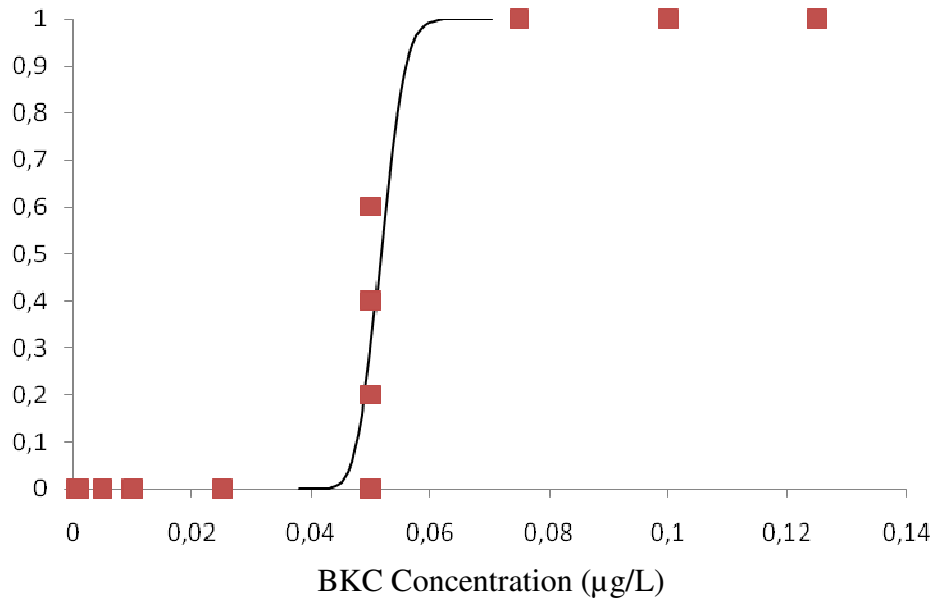

Figure 0.1 - Sigmoid Curve of BKC effect at D. magna in acute immobilization test

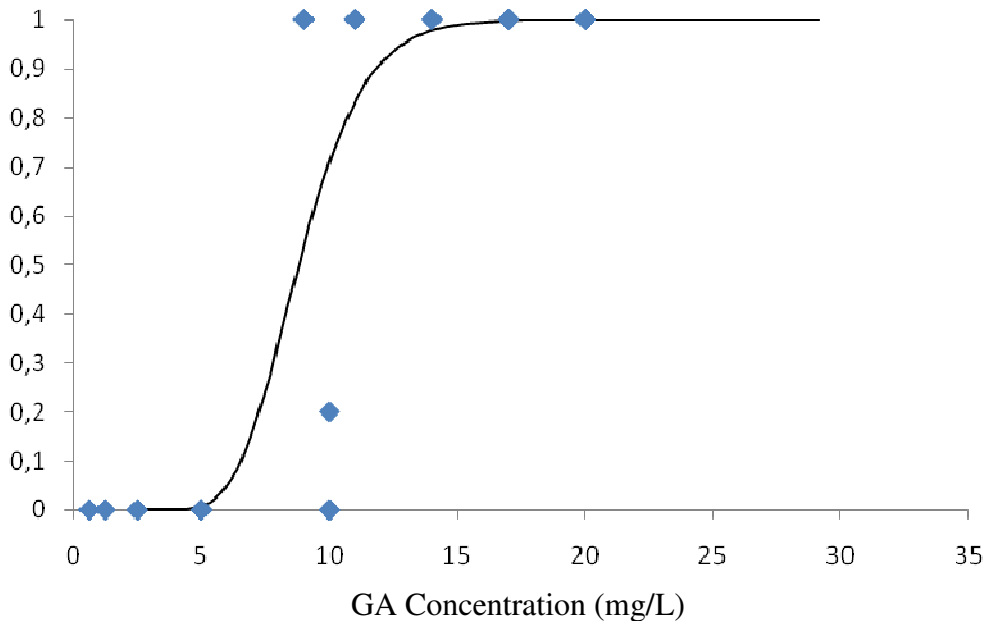

Figure 0.2 - Sigmoid Curve of GA effect at D. magna in acute immobilization test

1.7.2. Early-life stages assay with *Danio rerio*

Embriotoxicity of BKC

The exposure of fertilized zebrafish eggs to different concentrations of BKC was prolonged for 144 h.

The distribution of embryos that died along the experiment (black bars), alive embryos (grey bars), hatched embryos (white bars) and larvae that died (spotted dark grey bars) is presented as stacked bars **(Figure 0.3)**

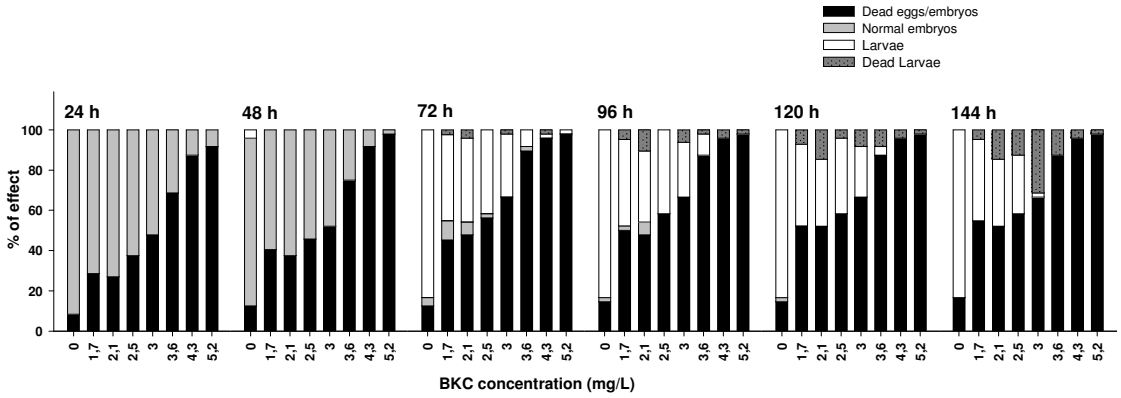

Figure 0.3 - General overview of BKC effects on *D. rerio* embryo and larvae during 144 h (6 days) of exposure

In the first 48 h, the concentrations 5.2 and 4.3 mg/l had respectively, 97.917% and 91.67% of embryo mortality, and at 72 h, 87.5 % of embryos exposed to 3.6 mg/l had died as well, (**Figure 0.4**). Mortality of both larvae and embryo has reached 100% only at 96 h (4.3 and 5.2 mg/l) and 144 h (3.6 mg/l). Embryos mortality was correlated with BKC concentration, and a 96h-LC_{50} of 3.9 mg/l (SE= 3.7483) was calculated.

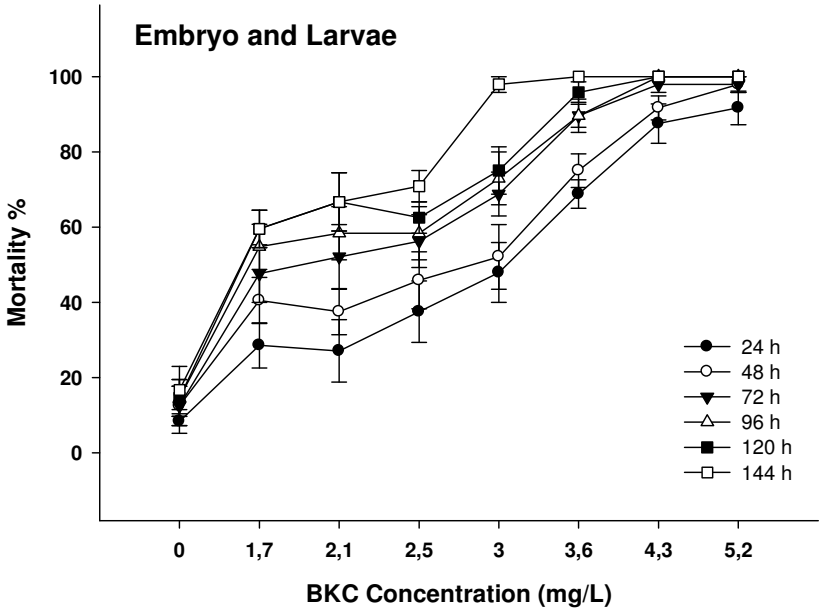

Figure 0.4 - Mortality rates of *D. rerio* embryo and larvae during 144 h (6 days) of BKC exposure

At 48 h only 4.167% of the control eggs had hatched, while at 72 h hatching occurred in all concentrations (83.3% in the control) without any significant difference observed between concentrations (Kruskal-Wallis $H = 7.654$ $P > 0.001$).

The development of embryos of the control group was normal, according to the descriptions made by (Kimmel 1995) (**Figure 0.12 b** and **Figure 0.13 d**). During the first 24 h, development of control embryos was normal, as described by (Kimmel 1995).

Table 0.I - Effects of BKC on developmental parameters of zebrafish early-life stages

Embryotoxicology Parameters	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf
Eye Pigmentation (**LOEC**)	2.1	n.e.	_	_	_	_
Spine malformations ($\mathbf{EC_{50}}$)	_	n.d.r.	n.d.r.	n.d.r.	**2.84** (0.13)	n.d.r.
Pericardial oedema (**LOEC**)	1.7	n.d.r.	n.e.	n.d.r.	n.e.	n.e.
Bubble effect ($\mathbf{EC_{50}}$)	**2.58** (0.14)	**2.49** (0.15)	_	_	_	_
Posture ($\mathbf{EC_{50}}$)	_	_	_	_	n.d.r.	**2.47** (0.085)

n.e.− no effect on the endpoint analyzed, n.d.r. − no dose response on the endpoint analyzed, *hpf* − hours post-fertilization

EC_{50} values are in mg/l followed by standard error

.Along six days of exposure to BKC, anomalies were shown in some parameters of development: deformation and opacity on yolk sac, delay on tail detachment, spine malformations as curved tail, weak eye pigmentation, irregular or weak tail blood circulation, occurence of pericardial oedemas, alterations of amniotic fluid and posture disturbance (lack of equilibrium). However, statistically significant differences among treatment groups and control were low (**Table 0.I**).

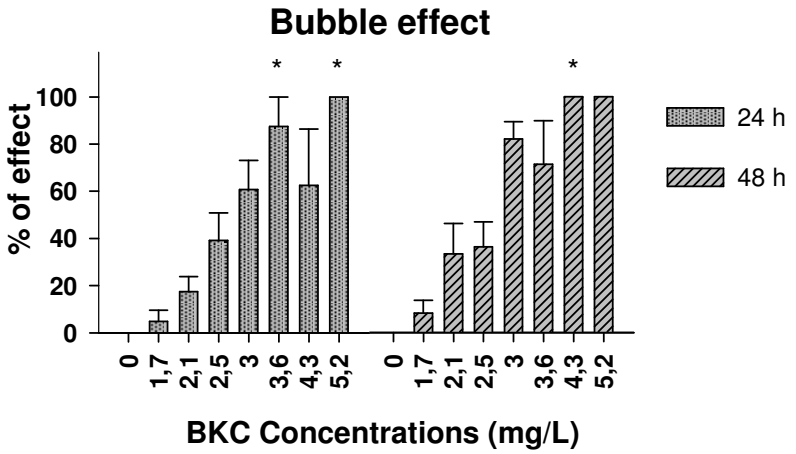

Figure 0.5 - Effect of BKC in the endpoint bubble effect in the first 48h of exposure. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryos

By 24 h, embryos exposed to 3.6 and 5.2 mg/l (Kruskal-Wallis H =32.621, P <0.001); and at 48 h, 4.3 mg/l exposed embryos (Kruskal-Wallis H = 30.267, P <0.001), presented alterations in the amniotic fluid in significantly higher frequency than control group where no organisms with such anomalies were found (**Figure 0.5** and **Figure 0.12 a**). Although most of embryos at 4.3 and 5.2 mg/l with amniotic fluid alterations died until 48h, the survival ones hatched at 72 h, and died until 96h. The alterations of amniotic fluid were like a bubble effect, since the consistence of amniotic fluid became as gelatinous foam.

At 144 h, posture disturbances as impossibility of keeping an upright posture (**Figure 0.6**) occurred in larvae at the concentration of 2.5 mg/l (Kruskal-Wallis H =14.018, P <0.001) with statistical significance. Dose-dependent effect was observed at amniotic fluid and posture endpoints, while remaining effects tested did not have consistent results, most respective percentages were below 20%.

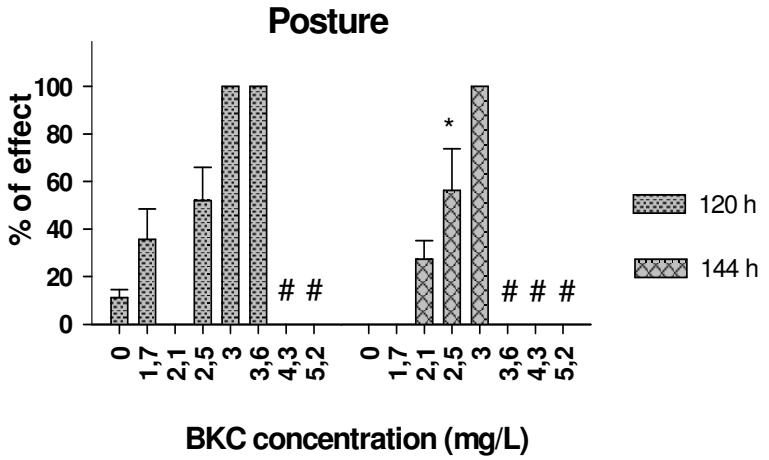

Figure 0.6 - Effect of BKC in the endpoint posture in the last 48h of exposure. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryos

Embriotoxicty of Glutaraldehyde

In the fish embryotocixity test using GA as contaminant, fertilized zebrafish embryos were just exposed for a period of 120 h. The results are presented as stacked bars, each proportion of dead (black bars) and alive (grey bars) embryos, hatched larvae (white bars) and larvae that died (spotted dark grey bars) (**Figure 0.7**).

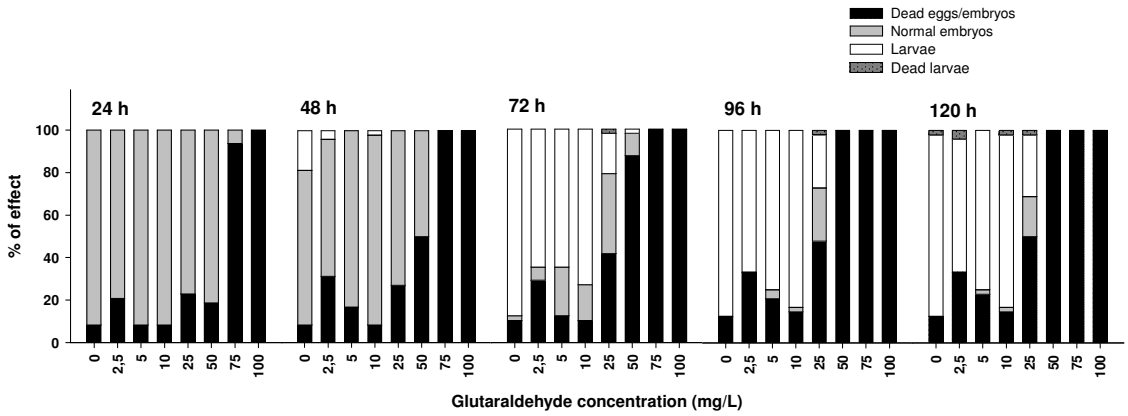

Figure 0.7 - General overview of GA effects on *D. rerio* embryo and larvae during during 120 h (5 days) of exposure

Within the first 48 h, all embryos exposed to 100 and 75 mg/l had died, and by the 72 h, 50 % of embryos exposed to 50 mg/l also had died. (spotted dark grey bars) (**Figure 0.8**). In the zebrafish early life stage test with GA was quite informative. Embryos mortality was correlated with GA concentration and 96h LC_{50} of 23.97 mg/l (SE= 2.50).

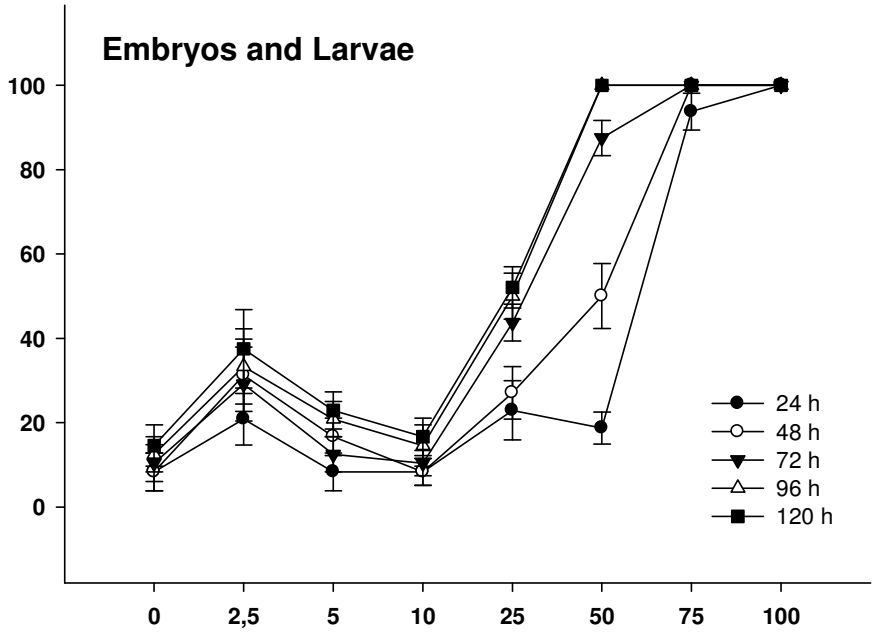

Figure 0.8 - Mortality rates of *D. rerio* embryo and larvae during 120 h (5 days) of GA exposure

Hatching rates are shown along the test (**Figure 0.9**). Embryos started to hatch at 48 h (21.41% in control) and differences between concentrations were observed (Kruskal-Wallis H = 22.369, P <0.001) especially at the concentrations of 5, 25 and 50 mg/l where no embryos hatched. At 72h, almost 100% of the control embryos hatched; at concentrations between 2.5 and 10 the majority of the larvae hatched but a delay was still observed in the concentrations 25 (32.29%) and 50 mg/l where none of the surviving embryos had hatched. These differences were statistically significant: Kruskal-Wallis H = 25.531, P <0.001. By the fourth day (96h) the control group showed 100% of hatching, with similar results in 2.5, 5 and 10 mg/l, except at 25 mg/l where was observed a delay on the hatching (59.375 %, 120 h).

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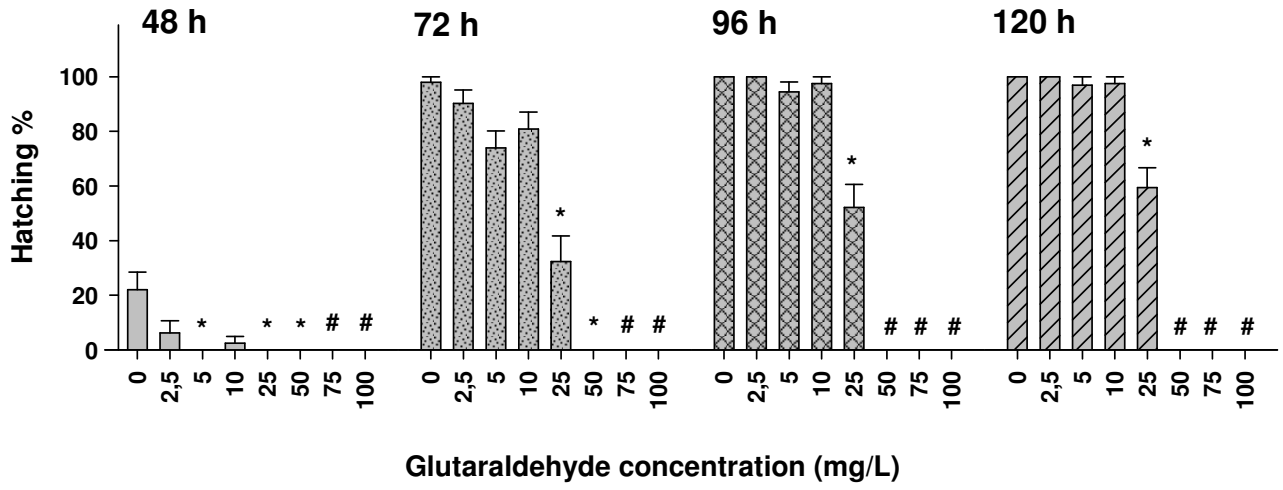

Figure 0.9 - Effects of GA in the Hatching rates of *D.rerio* embryo during 120 h (5 days) of exposure. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryos that could hatch

The development of embryos present in control group was regular, according to the descriptions made by (Kimmel 1995). The effects of GA in the development parameters analyzed are summarized in the Table 3. On the first day of study (24h), embryos in the control and 2.5 mg/l had a well-developed head, body and tail, without significant deformities (**Figure 0.14** b). LOEC of 5 mg/l was noted at somite malformation.

The effects of GA in the developmental parameters are resumed in **Table 0.II**.

Table 0.II - Effects of Glutaraldehyde on developmental parameters of zebrafish early stages

Embryotoxicology parameters	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf
Eye Deformation	_	n.e	n.e.	n.e.	n.e.
Somite formation (**LOEC**)	5	n.e.	_	_	_
Alterations in amniotic fluid	n.e.	n.e.	_	_	_
Yolk Sac deformity (**EC_{50}**)	n.d.r	n.d.r	n.d.r	**18.38** (21.55)	**9.978** (2.94)
Tail detachment (**EC_{50}**)	**11.22** (4.22)	_	_	_	_
Spine malformation (**EC_{50}**)	_	n.d.r	n.d.r	n.d.r	**15.26** (13.13)
Pericardial oedema (**EC_{50}**)	n.e.	n.d.r	**58.37** (7.37)	**14.50** (11.57)	n.d.r
Pigmentation Eye	n.e	**24.28** (n.c.)	_	_	_
Pigmentation Body (**LOEC**)	n.e	50	_	_	_
Tail blood circulation	_	n.e.	n.e.	n.e.	n.e.
Undersize	_	_	_	**6.53** (n.c.)	**6.53** (n.c.)
Heart beat	_	n.e	n.e.	n.e.	n.e.
Posture (**LOEC**)	_	_	_	5	n.e.

EC_{50} values are in mg/l followed by standard error
n.e.−no effect on the endpoint analyzed, n.d.r. − no dose response on the endpoint analyzed, n.c.- not calculated value; *hpf* hours post-fertilization

By the next days of study, effects on the development could be noticed especially in the deformation and opacity of yolk sac, delay on tail detachment and occurrence of pericardial oedema at several test concentrations, although statistical significant differences were only observed at concentration of 25 mg/l.

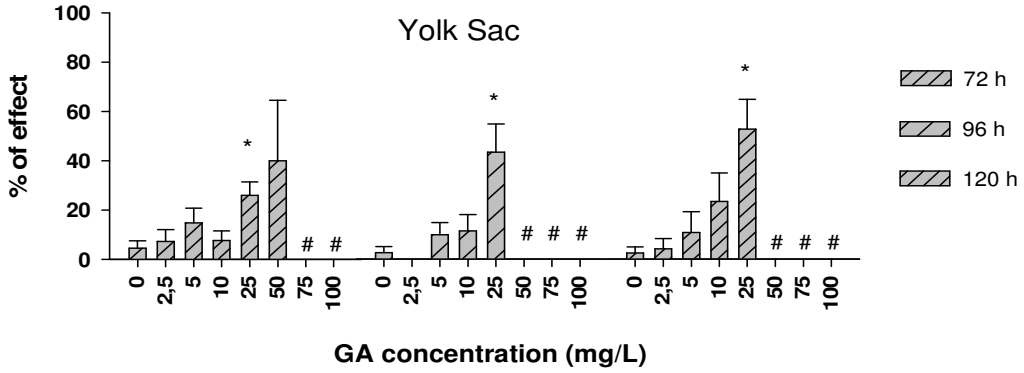

Figure 0.10 - Effect of GA in the endpoint Yolk sac in the last 72h of exposure. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryos

Thus, considerable deformation and opacity of yolk sac at 72 h (Kruskal-Wallis H = 13.773, P <0.001) – as described in **Figure 0.15 c** – 96 h (Kruskal-Wallis H = 18.039, P <0.001) and 120 h (Kruskal-Wallis H = 19.322, P <0.001) was observed (**Figure 0.10**); pericardial oedemas (Kruskal-Wallis H = 27.709; P <0.001) occurred at 48 h in higher frequency than in control group, although some control embryos presented this effect as well (Figure 0.14 a); Significant spine malformations, as curved tail (Kruskal-Wallis H = 16.672, P <0.001) were observed at 120 h.

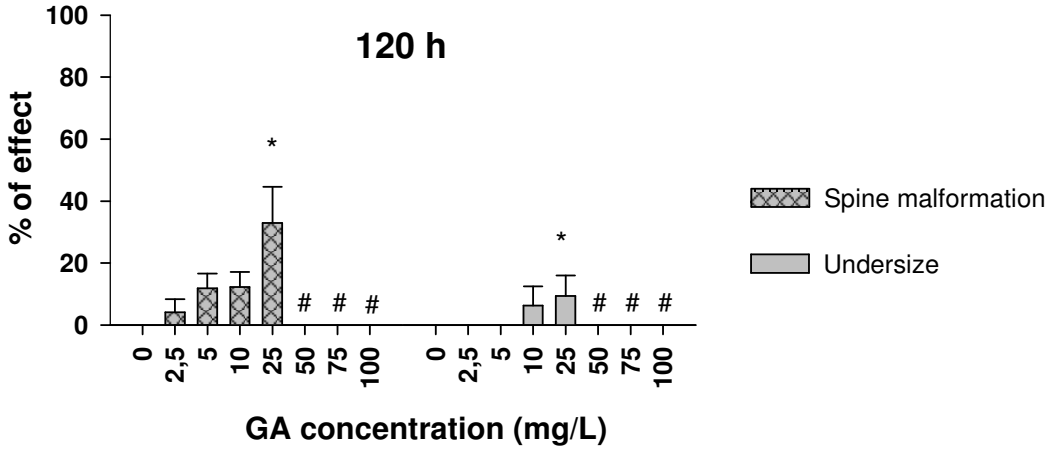

Figure 0.11 - Effect of GA in the endpoints Spine malformation and undersize at 120 h of exposure. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryos

1.8.Discussion

1.8.1. Acute Toxicity in *Daphnia magna*

D. magna was much more sensitive to BKC (48h-EC50= 0.052 µg/l= 0.0000516 mg/l) than to GA (48h-EC50= 8.8145 mg/l). For GA a similar LC50 value (48h LC50= 5 mg/l) has been reported (UCC 1981). Values reported for BKC by FEF (2008), correspond to an 48 h LC50 of 0.02 mg/l that is quite higher than the values determined in this study.

Although in previous studies the information about the test temperature is not provided, it is assumed that temperature is in the range specified in the standard protocols (18 - 22 ± 1º C) (OECD 2000). The objective of this test was to determine EC50 values for both chemicals under tropical conditions (24± 1º C). Therefore, we can conclude that *D. magna* acute toxicity values of GA under temperature standard and tropical conditions are similar, where BKC show some significant differences. Furthermore, in the GA test mortality was similar at 24 h and 48 h, which is in concordance with rapid degradation (half life of 10.6 h) into CO2 (final metabolite) under aerobic conditions (Leung 2001 a; Bioshare 2002).

1.8.2. .Early-life stages assay with *Danio rerio*

Embryotoxicity of BKC

Embryos mortality was correlated with BKC concentration, and a 96h-LC_{50} of 3.9 mg/l ± 3.7483 (SE) was calculated (**Figure 0.4**). BKC showed higher toxicity to *D. rerio* embryos than to *Oncorhynchus mykiss* embryos (96 h-LC_{50} = 11.5 mg/l; Mayer (1986); however in another work with *D.rerio* (FEF 2008) BKC toxicity (96 h-LC50 = 0.31 mg/l) was much higher compared to the previous study with *O. mykiss*. No significant hatching delays were verified at early life stage assay with BKC.

No studies were found in literature relating toxicity of BKC with embryo development in *D. rerio*.

The chorion of the egg, considered as a barrier, protect embryo from the surrounding environment but might allow different pollutants to penetrate embryo at specific stages of embryogenesis (Manner 1976). In D. rerio exposure to BKC, the main effect was the alteration of chorion integrity, which was noted through visible changes

on amniotic fluid, which became a gelatinous foam (**Figure 0.12 a**). Since BKC is a soap with detergent and emulsifying properties, the alterations in the consistence of amniotic fluid might be explained by a saponification reaction, which probably brought modifications to essential physiological activities, however there are no studies supporting this explanation.

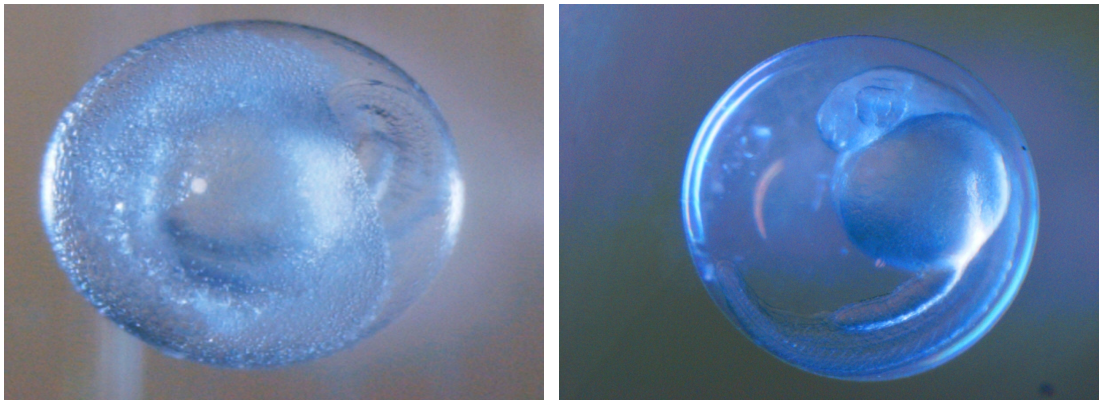

Figure 0.12 - Zebrafish embryos abnormalities during exposure to BKC: a 24-h-old embryo exposed to 4.3 mg/l with bubble effect on amniotic fluid; b normal embryo 24 h after fertilization with normal amniotic fluid

In higher toxic concentrations some adverse effects as spine deformations (curved tail) were induced and eventually led to embryos death. While in low concentrations, BKC induced a bubble effect although it consistence/density was lower in comparison to higher concentrations. However if embryo survived in this phase, low quality effects and no significant hatching delays were noted on embryo development.

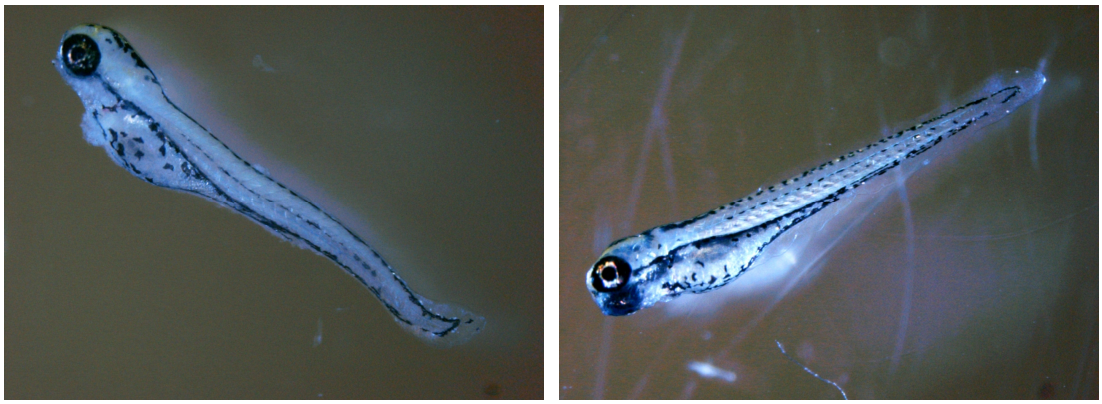

Figure 0.13 - Zebrafish larvae abnormalities during exposure to BKC: c 96 h larvae exposed to 2.1 mg/l of BKC with deformity and opacity of yolk sac, pericardial oedema and spine malformations although without significance; d normal larvae (96h) with normal body structure

The mechanisms of embryotoxicity of BKC in aquatic organisms are not yet described. BKC, as LAS (linear alkyl benzene sulfonate) is a surface acting agent. Studies with LAS (Manner 1976), concluded that this property may induce chemical or morphological alterations in the chorionic membrane, decreasing the rates of diffusion or carrier transport through the membrane.

In the last days of exposure significant posture disturbances (**Figure 0.6**) occurred in larvae in a dose-dependent manner. This abnormality is characterized by an impossibility of larvae in keeping an upright posture, either swimming or stopped. The reason for this phenomenon remains unknown.

Embryotoxicity of GA

In the zebrafish early life stage test with GA was quite informative. Embryos mortality was correlated with GA concentration, and a 96h LC_{50} of 27.64 (SE= 48.77) mg/l was calculated (**Figure 0.8**). This was a high LC50 value compared with other embryotoxicity studies performed with fish species: 96 h LC50= 11 mg/l for *Lepomis macrochirus* (UCC 1978), 96 h LC50= 0.0239 mg/l for *Oncorhynchus mykiss* (EPA 2000) and 96 h LC50= 0.011 mg/l for *Oncorhynchus kisutch* (UCC 1977).

In GA embryotoxicity, significant delays in hatching occurred at 48 and 72h on embryos exposed to 25 and 50 mg/l; and at 96 and 120h, only in embryos exposed to 25 mg/. In the work of (Landrum 2005), hatching delay was observed on embryos of

Oncorhynchus mykiss exposed to 2.5 mg/l of GA. The delay in hatch did not appear to affect global hatching success at the end of the test (**Figure 0.9**). Different toxicity processes can explain this hatching delay: inability of embryo to break the chorion; induction of abnormal embryo activity and function of hatching enzyme (Hallare 2005).

In the literature, studies dealing with GA teratogenic effects on embryo development could not be found.

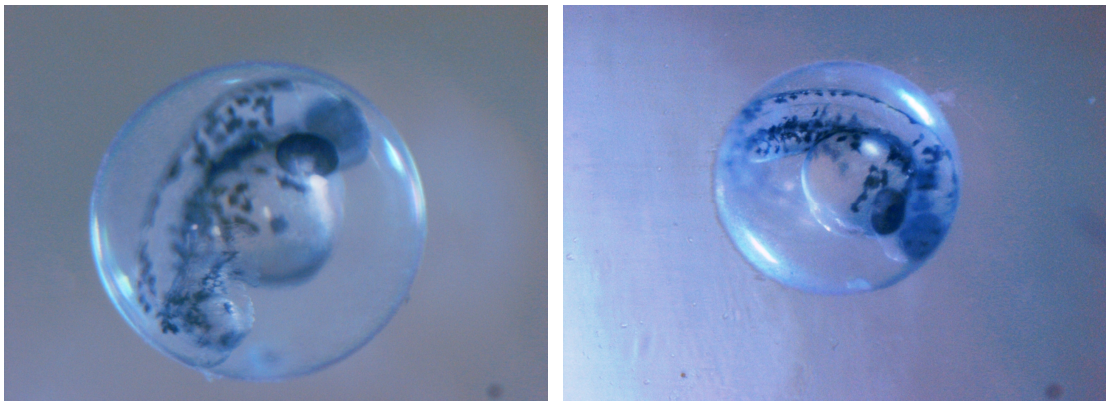

Figure 0.14 - Zebrafish embryos abnormalities during exposure to GA: a 48-h-old embryo exposed to 25 mg/l with significant ocurrance of pericardial oedema; b normal embryo 48 h after fertilization with normal heart

Embryos at highest concentration tested died in the beginning of test, while embryos at fourth highest concentration developed some significant abnormalities, as spine deformations (**Figure 0.11**), pericardial oedemas (**Figure 0.14 a**) and deformity and opacity in yolk sac (**Figure 0.15 c**) and that eventually led to embryos death, during the test. At the same time, the percentage of effect in the same abnormalities increased in lower concentrations during the assay, although it did not affect hatching. In the last days of exposure (120h), significant undersized larvae were observed, characterized by short length of tail.

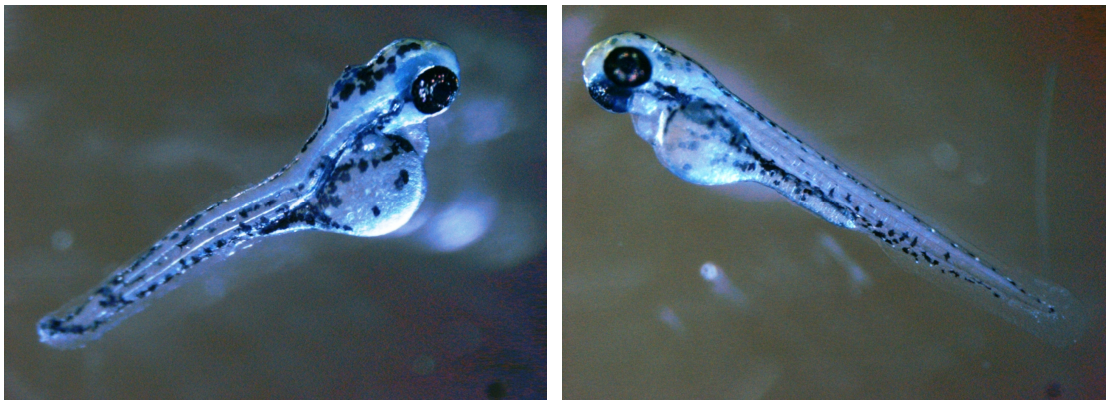

Figure 0.15 - Zebrafish larvae abnormalities during exposure to GA: c 72 h larvae exposed to 25 mg/l of GA with significant deformity and opacity of yolk sac; d normal larvae (72h) with normal body structure

The mechanisms of embryotoxicity of GA in aquatic environment are not yet described. Pericardial oedemas are frequently linked to leaks of endothelial vessels, which provide nutrients to the yolk sac and usually result in cardio-vascular dysfunction (Guiney 1990). While, yolk sac presented some deformities and opaque aspect compared with control during the whole test at most of exposure concentrations, although the reason for this phenomenon remains unknown.

Spine malformations, occurred during this test and according to (Muramoto 1985) this events are linked to the reduction of calcium and phosphorus ions; while for (Cheng 2000) are related to depletion of proteins as myosin and myotonia essential to muscle-skeletal system, although it can be proved by this study.

1.9.References

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Conclusions and final remarks

Since 1975, Southeast Asia had a rapid expansion in aquaculture becoming one of the areas on the planet with more productivity (Hishamunda, 2009).

The high demand for source of animal protein induced the development of aquaculture techniques, technology and measures in order to improve production, including health management and intensification of production of aquaculture species (Mock 2001; FAO 2006-2009). Antibiotics, parasiticides, anaesthetics, spawning hormones, oxidants, disinfectants and herbicides were introduced and are routinely used in aquaculture, in an attempt to control threats associated with aquaculture (Johnston 2002). Thus, increased concerns, about the potential harm of aquaculture effluents on receiving water bodies, the contamination of farmed organisms with bioaccumulative and potentially harmful chemicals started to grow (Boyd 1999).

This study is focus on two chemicals widely used in aquaculture, Benzalkonium chloride (BKC) and Glutaraldehyde (GA), both used to sterilize water ponds from pathologic agents (bacteria, fungus, phytoplankton), plus GA usage in fish egg surface disinfection (Katharios 2007).

According to Lee 1994, when BKC is applied as an algaecide, the ideal concentration's range into pond systems should be between 0.5 and 2.0 mg/l. The amount of chemicals added to the ponds is calculated based on the area. Most ponds have an area of one rai (1200 m^2) and the average depth of ponds is around 1.2 meters, so their average volume is equivalent to 1920 m^3.

In aquaculture, BKC and GA are usually combined as binary mixtures (Boillot 2008), as provided by the manufacturer. However, there is no guaranty that this formulation (10% of BKC and 15% of GA) is optimized to provide better results while keeping environmental impacts as low as possible and maximizing farmers' revenue. The manipulation and usage of chemicals in aquaculture is made based on empirical knowledge acquired through experience in field practice, for this reason the amounts applied, most of the times, exceed the recommended dose.

As an example, if a farmer adds 2.5 l of BKC/GA mixture (value provided by the farmer of Nam Sai Farm) per rai, the expected concentration of BKC and GA in the pond system water would be 130.2 mg/l and 195.3 mg/l, respectively.

Comparing with the values of 96 h LC50 in zebrafish (*D. rerio*), determined in this study, for each chemical separately (3.9 mg/l to BKC and 27.6 mg/l to GA), the above mentioned chemical concentrations could have extremely serious effects on this model organism. Although, zebrafish is not used in aquaculture, this species from Cyprinidea family, is native of tropical environment and widely distributed in Southeast Asia, which makes this model organism a suitable species to perform comparisons with farmed tropical freshwater fish. Thus, these results can be extrapolated to other early-life toxicity assessments already made with related farmed tropical freshwater fish species, as Tilapia (same family). Besides this study contribute to fulfill the data gap on ecotoxicological information necessary for ecological risk assessments of chemicals in the tropics.

The main effect of BKC in *D. rerio* was the alteration of chorion integrity, only noted through alterations of amniotic fluid, since it became like gelatinous foam, what might be explained as a saponification reaction. However, this theory does not have supportive data, since is based only in visual observations. A future line of work should take detailed chemical analysis to check the effects of BKC on the composition of amniotic fluid. During this study, some difficulties were found with the manipulation of the chemicals, in the future to improve these conditions, it is essential to assess the better physical state of chemicals to apply in this study.

Concerning the acute toxicity assay with Daphnia magna, the results can be useful to assess eventual environmental impacts of aquaculture operations in aquatic ecosystems, since this model species display an essential role in aquatic food webs (Baird et al., 1989). Both 48h EC50 values of BKC (0.052µg/l) and GA (8.8 mg/l) were extremely low, when compared to concentrations of each chemical in the binary mixture applied into ponds.

As an example, (Cheng 2003) reported that prawn farmers often apply excess amounts of BKC, and the potential effect of this on the disease resistance or immune functions of the prawns is of some concern.

A future line of work to pursue should be the identification of the correct proportion of BKC and GA in the binary mixture, where the same results of disinfection can be reached without production of chemical wastes, thus minimizing impacts in tropical environment.

The interaction between the elements of this binary mixture should be evaluated, in order to determine what kind BKC and GA interaction is produced and to define the

subsequent effects on aquatic organism. Since, the effects of a mixture of chemicals can be simply additive, more than additive (synergistic) or less than additive (antagonistic) (Calamari 1980; Hermens 1984). A study regarding the assessment of joint toxicity of surfactants with GA in hospital effluents, found several considerable acute toxicity effects on aquatic organisms (Emmanuel 2005).

Although, as decribed by (Dierberg 1996), pond effluents are generally not treated before discharged into receiving waters, this task is crucial to reduce adverse environmental impacts. Thus, measures as application of sustainable and cost-effective operations in effluents management should be taken. The use of treatment ponds, where a sequential use of species optimize nutrient use, can be helpful.

In these countries, there is a notorious lack of control and legislation by the government in the certification of chemicals, as commercial products. In fact, this situation allows the growth of parallel markets, and subsequent arising of counterfeit products with same trade name of original commercial products, although often do not provide essential information about ingredients.

Therefore, is essential to improve environmental education, so that aquaculturists could apply those products in correct doses in tropical pond systems. In this way, further impacts of these chemicals in tropical environment would be minimized.

1.10. References

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Annexes

Table 0.I - Values of one-way ANOVA analysis to BKC endpoints

D1		Tail Deformation	H = 8.794 (7df)
			(P = 0.268)
Tail Deformation	H = 7.591 (7df)	Yolk Sac	H = 2.036 (7df)
	(P =0.370)		(P = 0.958)
Yolk Sac	H = 10.015 (7df)	**D4**	
	(P = 0.188)		
Corion	H = 32.621 (7df)	Tail Deformation	H = 0.374 (3 df)
	(P <0.001)		(P = 0.945)
D2		Yolk Sac	H = 0.806 (3 df)
			(P = 0.848)
Tail Deformation	H = 5.671 (7df)	Oedema	H = 0.374 (3 df)
	(P = 0.579)		(P = 0.945)
Yolk Sac	H = 4.020 (7df)	**D5**	
	(P = 0.777)		
Corion	H = 30.267 (7df)	Tail Deformation	H = 8.241 (5 df)
	(P <0.001)		(P = 0.041)
Oedema	H = 8.406 (7df)	Posture	H = 12.350 (5 df)
	(P = 0.298)		(P =0.006)
Pigmentation Eye	H = 3.846 (7df)	**D6**	
	(P = 0.797)		
Tail Circulation	H = 8.406 (7df)	Tail Deformation	H = 8.399 (3 df)
	(P = 0.298)		(P = 0.038)
D3		Posture	H = 14.018 (3 df)
			(P = 0.003)

Table 0.II - Values of one-way ANOVA analysis to GA endpoints

D1	
Tail Deformation	H = 14.315 (5 df) (P = 0.014)
Yolk Sac	H = 12.518 (5 df) (P = 0.028)
D2	
Pigmentation Eye	H = 8.529 (5 df) (P = 0.129)
Tail Deformation	H = 9.488 (5 df) (P = 0.091)
Yolk Sac	H = 17.684 (5 df) (P = 0.003)
Oedema	H = 27.709 (5 df) (P = <0.001)
Tail Circulation	
D3	
Tail Deformation	H = 10.394 (5 df) (P = 0.065)
Yolk Sac	H = 13.773 (5 df) (P = 0.017)
D4	
Tail Deformation	H = 8.934 (4 df) (P = 0.063)
Yolk Sac	H = 18.039 (4 df) (P = 0.001)
Oedema	H = 14.976 (4 df) (P = 0.005)
Body Size	H = 3.080 (4 df)(P = 0.545)
D5	
Tail Deformation	H = 16.672 (4 df) (P = 0.002)
Yolk Sac	H = 19.322 (4 df) (P = <0.001)
Body Size	H = 3.080 (4 df) (P = 0.545)
Oedema	H = 32.384 (4 df) (P = <0.001)