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Assessing mixture toxicity of disinfectants in zebrafish

Avaliação da toxicidade de misturas de desinfetantes em peixe zebra

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – ramo Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Paula Inês Borralho Domingues, Bolseira de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e co-orientação do Professor Doutor António Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

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palavras-chave   *Danio rerio*, cloreto de benzalcónio, formaldeído, glutaraldeído, ortoftaldeído, mistura, toxicidade

resumo   Grandes quantidades de produtos químicos (por exemplo, detergentes e desinfetantes) são usados em hospitais para limpeza e desinfeção. Os seus efluentes consistem em misturas que podem causar sérios problemas ambientais.

Neste trabalho foram estudados os efeitos de três misturas entre desinfetantes hospitalares: glutaraldeído (GA), formaldeído (FA) ou ortoftaldeído (OPA), com o surfatante cloreto de benzalcónio (BKC), nos primeiros estádios de vida do peixe zebra. Os ensaios foram baseados no protocolo OCDE do Teste de Toxicidade em Embriões de Peixe (FET). Durante 96 horas, os organismos foram observados diariamente com um estereomicroscópio, registando-se a mortalidade. BKC, FA, GA, e OPA mostraram alta toxicidade para os embriões de peixe zebra apresentando valores de CL_{50} para as 96 h de 3.9 mg/l, 546.8 mg/l, 27.97 mg/l e 64.9 μg/l respetivamente. Para os dados das misturas foram usados os modelos de ação independente e adição de concentração, a fim de determinar o modelo mais adequado para prever a sua toxicidade e analisar possíveis efeitos interativos.

Às 96 horas, os resultados mostraram que a toxicidade da mistura de BKC e FA é melhor previsível pela adição de concentração com dependência da dose (antagonismo em dose baixa e sinergismo em doses elevadas), enquanto que a adição de concentração com uma função de desvio antagonista descreveu a mistura de BKC e GA. Para BKC e OPA, é a acção independente que melhor descreve a mistura, com uma função de desvio sinergístico do modelo de referência.

Este estudo dirige a atenção para a problemática dos efluentes hospitalares devido à libertação de suas misturas complexas e sua toxicidade, que pode representar um real problema ambiental.

Abstract

Large quantities of chemicals (e.g. detergents and disinfectants) are used in hospitals for cleaning and disinfection. Their effluents consist of a mixture which can cause serious environmental problems.

In this work, the effects of binary mixtures between three hospital disinfectants: glutaraldehyde (GA), formaldehyde (FA) or ortho-phthalaldehyde (OPA) and the surfactant benzalkonium chloride (BKC) on zebrafish early life-stages were studied. The assays were based on the OECD guideline on Fish Embryo Toxicity (FET) Test. Over 96 hours the organisms were daily inspected with stereomicroscopy, registering the mortality. The BKC, FA, GA and OPA showed high toxicity for zebrafish embryos presenting LC_{50} values at 96 h of 3.9 mg/l, 546.8 mg/l, 23.97 mg/l and 64.9 µg/l, respectively. Mixtures data was fitted to the independent action and concentration addiction models in order to verify which model best described the obtained results and to analyze possible interactive responses.

At 96 hours, the results showed that the mixture toxicity of BKC and FA is best predictable by concentration addition with a dose level dependency deviation (antagonism at low dose and synergism at high dose), whereas concentration addition with an antagonist deviation function described the mixture of BKC and GA. For BKC and OPA, Independent action best described the mixture, with a synergism deviation function from the reference model.

This study drives attention to the problem of the hospital effluents due the release of its complex mixtures and its toxicity that may represent a real environmental problem.

"Science is an imaginative adventure of the mind seeking truth in a world of mystery."

(Cyril Herman Hinshelwood)

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1. Introduction

1.1. Hospital wastewater on aquatic ecosystems

Due to the medical activities, disinfection and research in medicine, hospitals represent an indisputable source of many toxic substances to the aquatic environment due to discharge of wastewaters (Ballantyne and Jordan 2001; Jolibois, Guerbet et al. 2002). Hospital effluent is referred to as wastewater from hospitals or health care centers, biological or non-biological that is discarded and not intended for further use.

From the qualitative point of view, the hospital effluents can be classified into two categories: domestic wastewater type (kitchens, laundries and toilets), and specific hospital effluents resulting of patient care and laboratory medicines (Boillot, Bazin et al. 2008)

Hospitals drain into the aquatic ecosystems an important volume of water a day that carries hazardous substances. In quantitative terms, hospitals consume 400 to 1200 l of water per day per bed. And, generally, the load of disinfectants can vary from 2 to 200 mg/l according to the size of the hospital and its consumption of disinfectants (Emmanuel, Perrodin et al. 2005). For instance, Kümmerer et al. (1997) measured concentrations of Benzalkonium chloride (BKC) in effluents from different European hospitals detecting levels from 0.05 to 6.03 mg/l. Glutaraldehyde (GA) has been detected at levels between 0.50 and 3.72 mg/l and formaldehyde (FA) at levels of 0.07 mg/l in hospital wastewaters (Jolibois, Guerbet et al. 2002; Boillot, Bazin et al. 2008) .

Hospital wastewater as well as urban wastewater constitutes a complex mixture of different substances generally containing hundreds of chemicals (Jolibois, Guerbet et al. 2002) U.S. EPA (1989) has detected 400 toxic and hazardous pollutants in hospital wastewater. Their presence in the environment may pose serious environmental health risk due to their toxic, genotoxic and/or

carcinogenic effect and could have potential negative effects on biological balance of natural environment. Riviére (1998) distinguishes the hazardous substances by their capacity to provoke toxic short-term effects (mortality) or long term effects (appearance of cancers, reproduction impairment, etc). The ecotoxicological studies performed with hospital effluents confirm the existence of these hazardous substances (Table 1.1)

The detected compounds include products directly related to medical activities such as disinfectants and antiseptics commonly used to ensure hygiene and avoid nosocomial infections, drugs excreted by patients, heavy metals such as silver (radiology departments) and radio-elements injected to the patients and discharged by urine. These effluents also have very high AOX contents (organohalogenic compounds absorbable on active carbon). Concentrations higher than 10 mg/L have been measured in the effluents from a German university hospital (Boillot, Bazin et al. 2008).

Hospital wastewater is not subjected to any specific pre-treatment before being discharged into urban sewage and is liable to disseminate pathogenic microbes or multi-resistant strains of bacteria (some of which are multi-resistant to antibiotics), heavy metals, radioisotopes, organohalogens, (arising in particular from the use of bleach on organic compounds present in effluents) and drug residues. Some of these pollutants, especially drug residues and organohalogens, are frequently discharged from sewage plants after having undergone little degradation. In the case of environmental conditions not favorable to the degradation of these substances, hospital pollutants risk remaining in the natural environment for a long time, thereby representing a risk in the short, medium and long terms for the species living in these ecosystems (Panouillères, Boillot et al. 2007; Emmanuel, Pierre et al. 2009).

The work of Emmanuel (2009), refers that, in certain developing countries, hospitals usually discharge their wastewater into septic tanks equipped with diffusion wells. This type of discharge can pollute the groundwater resources used intensively for drinking water by the population. Beyond the groundwater, all ecosystems may be affected by this type of pollution. In the air, the susceptible

elements to be affected are the birds and the insects. In the soil, the microorganisms, wildlife of soils (insects, earthworms, etc), and plants. In the surface water, the primary producers (phytoplankton) of which unicellular and pluricellular green algae; the primary consumers (invertebrate) in particular of the crustaceans; and secondary consumers of which fish and water birds (Emmanuel, Perrodin et al. 2005).

In general, hospital liquid effluents and domestic effluent are both collected by the sewer system and sent to the same wastewater treatment plants (Figure 1.1), which can cause ecological risks (Emmanuel E. 2002; Emmanuel, Perrodin et al. 2005).

In Portugal, the concern with hospital sewage started to receive attention only in 2005. Some recommendations for wastewater management were made that are not yet fully in vigor. Currently the exact situation of hospital wastewaters is unknown, due to lack of national legislation (Falcão 2009).

According to the study of the Quantitative and Qualitative Characterization of Wastewater Hospital, prepared by the National Laboratory Civil Engineering (LNEC, 2003):

> *"(…) were not obtained data or information indicating that in current situation, the hospital effluents should be cause for concern about its impacts on environment and public health , when their discharges are properly treated in municipal wastewater treatment plant, or other, prior to discharge into the environment ".*

In general, it is argued that infected wastewater should be fully independent and directed to a station of wastewater treatment before being sent on the municipal sewer network, and it is considered that the wastewater from rooms resulting from disinfection of beds should also be connected to this network due the use of disinfectants and detergents (Falcão 2009).

Falcão (2009) found that, in Portugal, some modern hospitals (less than 10 years) have a wastewater treatment plant (WWTP), but many of these treatment

plants are not operating regularly due to lack of maintenance or technical conditions. However, the consequences for public health may be burdensome because in this way a large set of pathogenic microorganisms, drugs and substances with ecotoxicological risk are channeled into rivers and the sea.

Even if these effluents undergo dilution after reaching the treatment plant, the possibility that certain substances produce a cumulative effect with long term detrimental effects to ecosystems should not be excluded.
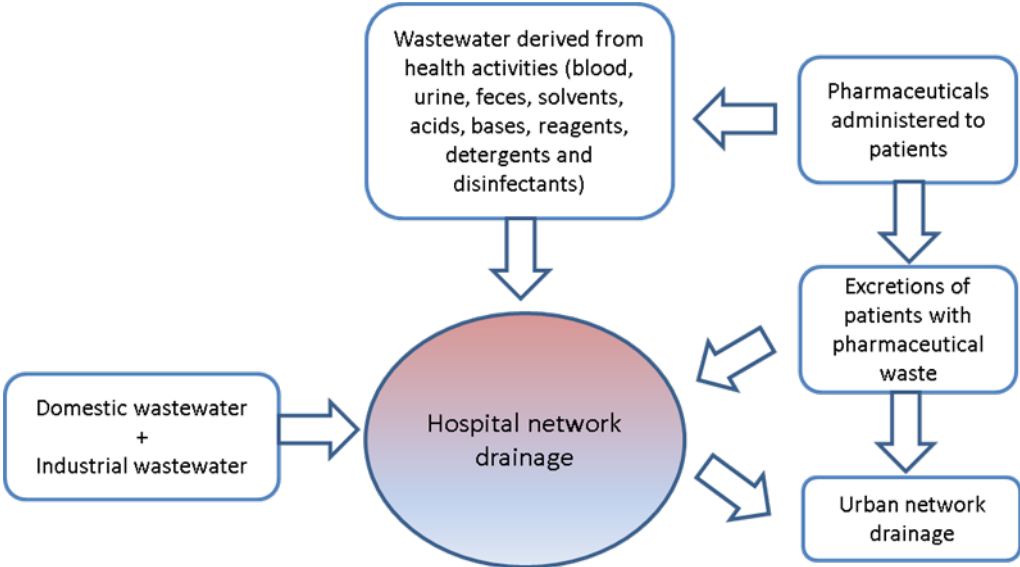
Figure 1.1 - The environmental problem of wastewater hospital (Emmanuel E. 2002)

Hospital pollutants entering aquatic ecosystems may cause toxic effects to organisms which can have potential negative effects on biological balance of natural environments (Emmanuel, Perrodin et al. 2005).

Table 1.I - Ecotoxicity of hospital wastewater (EC50 in % volume of effluent) (Boillot 2008)

Effect value		EC$_{50}$(%)				
Test Organism		*Pseudokirchneriella subcapitata*	*Daphnia magna*		Microtox®	
Duration of exposure		72 h	24 h	48 h	15 mn	30 mn
Hospitals	**A**	-	50.3	33,2	-	-
	B	-	0.7	0.4	-	-
	C	-	48.7 - >90	-	7.9 – 18.6	-
	D	-	2.1-8.1	-	2.1 – 5.7	-
	E	-	46.3 - >90	-	25 – 53.5	-
	F	1.8 – 11.9	0.8 - 10	1.4 – 1.9	23.8 - >76.2	21.7 - >76.2

With an EC$_{50}$ less than 0.8 % for *D. magna* mobility in 24 h, Table 1.I reveals that these effluents are highly toxic. The chronic ecotoxicity test performed with hospital wastewater using the algae *Pseudokirchneriella* reflects the same trend with an EC$_{50}$ of 1.8 %.

Most of the works that demonstrate high toxicity of hospital effluents, do not investigate which are the main components responsible for their toxicity. The most often accepted hypothesis concerns the presence of detergent and disinfectant products (Boillot, Bazin et al. 2008). But many studies have shown that pharmaceuticals have poor biodegradability and high ecotoxicity, which could contribute to the global ecotoxicity of these effluents. (Kümmerer, Steger-Hartmann et al. 1997; Cleuvers 2003; Ferrari, Paxéus et al. 2003). The latter hypothesis arises from the presence of iodinated contrast agents that lead to the formation of adsorbable organic halogens (AOX) in the drainage network (Kümmerer, Erbe et al. 1998).
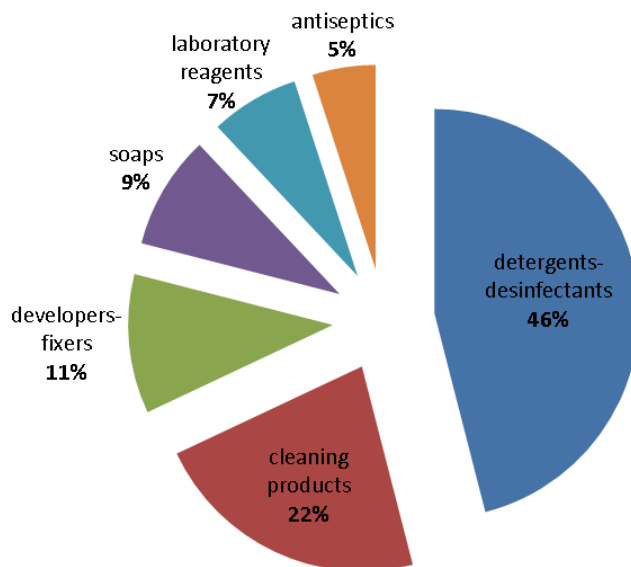
Figure 1.2 - Representation of purchases in volume of products used by Health Services and Hospital Laboratories of Havre (France) in 1996 (Boillot 2008)

However, by analyzing the compounds eliminated by hospitals, it can be seen that detergents and disinfectants are present in higher amounts that any other group of substances (Figure 1.2)

In fact, their presence in hospital wastewater, their ecotoxicity, effects on biological WWTP, and potential interaction with hundreds of other chemicals, may represent a real environmental problem. Then, it becomes necessary to characterize the ecotoxicological risk of hospital wastewater and study the fate of disinfectants and surfactants present in hospital effluents and their complex mixtures, while having care to include, on the ecotoxicological plan, the transference through the food chains.

The use of compounds like disinfectants and detergents is essential in hospitals and other health care settings for a variety of topical and hard-surface applications, but their discharge into wastewater is also a well-known problem, causing pollution of water resources and ecological risks for aquatic organism.

Detergents and disinfectants contribute with the largest portion of compounds eliminated from hospitals, and the qualitative effects of these compounds on

aquatic fauna have been now clearly demonstrated (Boillot, Bazin et al. 2008; Ivanković and Hrenović 2010).

The presence of surfactants in aquatic ecosystems represents a danger to aquatic life, as their toxicity to the three first organization levels of food chains (algae, crustacean, fish) has already been well established (Sütterlin, Alexy et al. 2008; Sütterlin, Alexy et al. 2008; Pérez, Fernández et al. 2009; Ivanković and Hrenović 2010)

1.2. Disinfectants

The term disinfection designates an operation aimed at preventing an infection. Disinfection is less lethal than sterilization, as it is the process that eliminates many or all pathogenic microorganisms, except bacterial spores, on inanimate objects by physical or chemical means.

The term antisepsis should be used to indicate the treatment of an infection by the use of a physical or chemical procedure that destroys all microorganisms including substantial numbers of resistant bacterial spores.

Disinfectant is a chemical agent used on inanimate objects (i.e., nonliving) (e.g., floors, walls, sinks) that kills all vegetative forms, but not necessarily all microbial forms (e.g., bacterial endospores). So, disinfectants are used in the decontamination process of patient-care devices and environmental surfaces (SCENIHR 2009). They are generally complex products or mixtures of active substances (Kümmerer 2001)

Large quantities of chemicals (eg, surfactants, detergents, biocides, disinfectants) are used in hospitals for cleaning and disinfection.

A wide variety of active chemical agents (or "biocides") are found in these products. A biocide is an active substance containing at least one active substance, intended to destroy, deter, render harmless, prevent the action of or exert some controlling effect on harmful/unwanted organisms by chemical or biological means. On the other hand, an active substance is a substance or micro-

organism having general or specific action on or against a harmful organism, i.e. an organism which needs to be controlled. Biocidal products have a very wide range of uses including disinfectants for home and industrial use; preservatives for manufactured and natural products; non-agricultural pesticides for use against insects, slugs and snails, rodents and other vertebrates. They also include a number of much specialised products such as embalming/taxidermist fluids and antifouling products.

Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets (McDonnell and Russell 1999).

It is important to note that many of these biocides may be used singly or in combination in a variety of products which vary considerably in activity against microorganisms. When combined, some compounds have better antiseptic/disinfectant or cleaning activity because their modes of action interact synergistically.

1.3. Surfactants

Surfactants are, referred in general as detergents and are all products that enable a cleaning operation. Large quantities of detergents are used in hospitals for cleaning which is often done prior to disinfection (Boillot and Perrodin 2008).

One of the active ingredients of detergents is a surfactant, which constitutes the largest organic portion of detergents. Surfactant molecules consist of both hydrophilic head group (water-attracting) and hydrophobic tail group (water-repelling) moieties in their structure that give detergents their tensioactive properties, and are thus referred to as amphiphilic/amphipathic molecule (Figure 1.3).
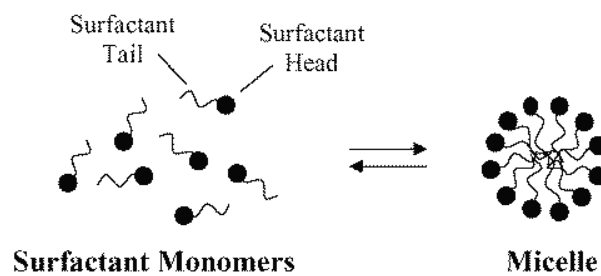
Figure 1.3- Surfactant structure (Yagui 2005)

The structure of surfactants generates specific physicochemical properties that are essential for the cleaning operation. When dissolved in water at low concentrations, surfactant molecules exists as monomers. At higher concentrations, surfactant molecules aggregate into micelles, reducing the system´s free energy. The concentration at which this property occurs is the Critical Micellar Concentration (CMC). CMC depends on temperature and the possible presence of other compounds in the preparation (Ivanković and Hrenović 2010). At low concentrations, detergents can change the conformation of the structures of membrane proteins and are thus able to make progressive cell permeabilisation and lysis. On the other hand, at high concentrations, they act by removing the layer of membrane phospholipids, which occurs along with the decrease of the cell´s biological activity (Panouillères, Boillot et al. 2007)

The action of detergents differs according to their class. Anionic, nonionic, and cationic surfactants are widely used in the production of cleaning products. These three main classes of surfactants correspond to the charge of the polar portion of the surfactant. Anionic surfactants are natural detergents widely used: soaps (R-COO-M) and salts of fatty acids. They are characterized by a hydrophilic negative charge, which can have a termination carboxylate (RCOOH), sulfate (R-O-SO$_3$-), sulfonate (R-SO$_3$) or phosphate, and are generally in the form of salts of alkalin metals or ammonium. The hydrophobic group is typically a hydrocarbon chain of C12 to C15 branched or linear. They can solubilize proteins until their denaturation. They can modify the activity of an enzyme by binding to it (Boillot and Perrodin 2008).

Nonionic surfactants have no charge groups over its head and they are also capable of solubilizing proteins but their action on enzymes is not as clear.

As for cationic surfactants, they have at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom, other alkyl groups such as methyl or benzyl groups acting as substituents. Quaternary ammonium compounds (QACs) are cationic surfactants containing a tetra-substituted ammonium salt and characterized by a positively charged quaternary nitrogen atom. Because of their positive charge, these compounds strongly adsorb to negatively charged surfaces of sludge, soil and sediments. It is also well documented that they bind to the fatty acids of cell membranes of organisms, which makes them useful as biocides (Boillot and Perrodin 2008). One commercially and toxicologically important representative of QACs was selected as model compound in the present study, namely benzalkonium chloride.

1.4. Importance of the study

The presence of complex mixtures in wastewater may represent a real environmental problem. In this context, it is very important to study the fate of hospital pollutants after their discharge into the environment as they are constituted not by single substances but by mixtures of substances that may interact.

This thesis focuses on the combined effects of some disinfectants and surfactants used in hospitals, as benzalkonium chloride (BKC), glutaraldehyde (GA), formaldehyde (FA) and ortho-phthalaldehyde (OPA) widely used in disinfection operations, for the model organism, *Danio rerio* and applying the more appropriate models to describe the toxicity of these chemicals binary mixtures.

Already proved the toxicity of detergents to the first three levels of living, this thesis will be useful for understanding the toxicity of mixtures of these substances with disinfectants. Since BKC is a widely used surfactant, we felt it necessary to

study the type of relation that this chemical and disinfectants may produce when present together. Due they are usually used in combination in hospitals, to improve antiseptics / disinfectants or cleaning activities.

1.5. Chemicals used in hospitals

1.5.1.Benzalkonium Chloride

Chemical and Physical Properties

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 [$C_6H_5CH_2N(CH_3)_2C_nH_{2n+1}\cdot Cl$] of various alkyl chain lengths, normally C12, C14, C16, and C18, creating homologues (BACs) (Tezel and Pavlostathis 2009).
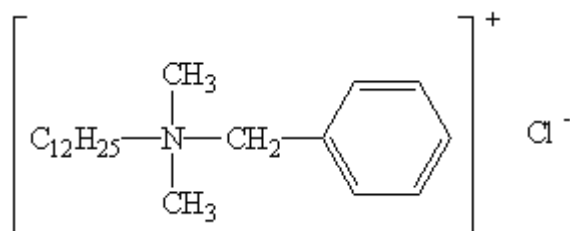
Figure 1.4 - Structural formula of Benzalkonium chloride (THWATER 2009)

Table 1.II - Physical and chemical properties of Benzalkonium chloride

Characteristic	Value
Molecular formula	$C_{21}H_{38}Cl$
Molecular weight	354.0127 g/mol
Melting point	-14 ºC
Boiling point	29 -34°C
Vapor pressure	0.6 Torr, at 25 ºC
Solubility	Easily soluble in water, ethanol and acetone. Aqueous solutions tend to foam strongly when shaken.
Log Kow	-0.11

Henry´s constant		1.1×10^{-7} atm/m^3 mol

Applications in Industry

It is used as disinfectant in households, medicine and industry. It is also used in fabric softeners, demulsifiers, emulsifiers, wetting agents, preservatives, and antiseptics in medicines and also as fungicides, spermicides, and virucides. In the last decade, BKC has been introduced in the formulation of most swimming pool algaecides and in cooling tower water treatment (Pérez, Fernández et al. 2009).

Environmental Fate

BACs are rapidly and strongly sorbed onto materials of environmental relevance, such as biomass, sediments, clays, and minerals. Biodegradation of aqueous phase (bioavailable) of BKC in aerobic biological systems has been demonstrated (Tezel, Pierson et al. 2006). However, BKC sorption is faster than biodegradation in aerobic systems leading to its transfer to anoxic/anaerobic compartments, such as anaerobic digesters and aquatic sediments. BKC concentrations in municipal primary and secondary sludge, digested sludge and aquatic sediments have been reported at levels typically 500 -fold higher than in sewage or surrounding aquatic system (Tezel and Pavlostathis 2009). In a recent study, microgram per liter concentrations of BACs were found in wastewater samples and samples downstream of wastewater treatment plants (Ferrer and Furlong 2002).

Effects on Aquatic Organisms

BKC is a substance toxic for aquatic organisms (Table 1.III).

According to Kummerer and co-workers (1997), the LC_{50} of BKC to fish is between 0.5 and 5.0 mg/l, and the toxicity to daphnids is even higher, with an LC_{50} from 0.1 to 1.0 mg/l (Kummerer et al., 1997).

Table 1.III - Toxicity of BKC in aquatic species

Test	Species	Result	Reference

			(mg/l)	
Algae	96-h algal growth inhibition	*Chaetoceros gracilis*	$Ec_{50}^{a}=87.3$	(Pérez, Fernández et al. 2009)
	96-h algal growth inhibition	*Isochrysis galbana*	$EC_{50}=66.4$	(Pérez, Fernández et al. 2009)
Crustacea	48-h acute	*Daphnia magna*	$EC_{50}= 0.02$	(FEF 2011)
Fish	96-h acute	*Danio rerio*	$LC_{50}^{b}=0.31$	(FEF 2011)
	96-h acute	*Oncorhynchus mykiss*	$LC_{50}=11.5$	(Pereira 2009)

[a] EC50 = effective concentration 50%

[b] LC50 = lethal concentration 50%

1.5.2. Formaldehyde

Physical and Chemical Properties

FA (CH_2O; CAS no. 50-00-0) is a flammable, colourless, reactive, and readily polymerized gas at normal temperature. The most common commercially available form is a 30-50% aqueous solution. Is readily soluble in water, alcohols, and other polar solvents, but has a low degree of solubility in non-polar fluids (IPCS 1989).
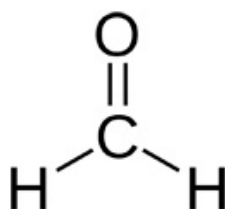
Figure 1.5 - Structural formula of formaldehyde (Indiamart 1996)

Table 1.IV - Physical and chemical properties of formaldehyde (IPCS 1989)

Characteristic	Value
Molecular formula	CH_2O
Molecular weight	30.03 g/mol
Melting point	-118 ºC
Boiling point	-19.2 ºC
Vapor pressure	Torr at 25 ºC

Solubility	soluble in water,
	alcohols, and other polar solvents
Log Kow	-0.65
Henry´s constant	0.02 Pa x m^3/mol

Applications in Industry

FA has a variety of uses, it has medical applications as a sterilant and is used as a preservative in consumer products, such as food, cosmetics, and household cleaning agents. Indoor areas of special importance are hospitals and scientific facilities where formaldehyde is used as a sterilizing and preserving agent.

FA is used as a preferred agent in disinfecting fluid pathways in dialysis patients. FA is sold and used principally as a water-based solution called formalin, which is 37% FA by weight (IPCS 1989).

Environmental Fate

FA is slightly persistent in water, with a half-life of 2–20 days. Complete degradation of FA within 30 hours (under aerobic conditions) and 48 hours (under anaerobic conditions) was observed in a stagnant lake (Environment 2006).

In air, FA has a short half-life of a few hours due to its reaction with sunlight and free radicals. Its half-life is approximately of 19 hours in clean air and 8 hours in polluted air. Besides being directly emitted to the atmosphere, FA is also formed as a result of photochemical reactions between other chemicals in already polluted air. These reactions may account for most of the FA in the air in some areas (Environment 2006).

Effects on Aquatic Organisms

Algae, protozoa, and other unicellular organisms are relatively sensitive to FA with acute lethal concentrations ranging from 0.3 to 22 mg/l. Aquatic invertebrates showed a wide range of responses. Some crustaceans are the most sensitive with median effective concentration (EC_{50}) values ranging from 0.4 to 20 mg/l. In 96 h

tests using several fish species, the LC_{50} of FA for adults ranged from a minimum of 10 mg/l to a maximum of several hundred mg/l; most species showed LC_{50} values in the range of 50-100 mg/l (Table 1.V). The responses of various species of amphibians are similar to those of fish with LC_{50} ranging from 10 to 20 mg/l for a 72 h exposure (IPCS 1989).

Table 1.V - Toxicity of formaldehyde in aquatic species

	Test	Species	Results	Reference
Algae	24-h	*Scenedesmus quadricauda*	Ec_{50}=14.7 mg/l	(Tišler and Zagorc-Končan 1997)
Crustacea	24-h acute	*Daphnia magna*	LC_{50}= 57 mg/l	(Martins, Oliva Teles et al. 2007)
Fish	96-h acute	*Danio rerio*	LC_{50}= 41 mg/l	(IPCS 1989)
	48-h acute	*Oncorhynchus mykiss*	LC_{50}= 50.0 (42.3-86.0) mg/l	(Tišler and Zagorc-Končan 1997)

1.5.3. Glutaraldehyde

Chemical and Physical Properties

GA (CHO-$(CH_2)_3$-CHO); CAS no. 111-30-8) is a saturated five-carbon aliphatic dialdehyde. GA is a colourless, oily liquid, with a pungent, aldehyde odour. GA is soluble in water and various organic solvents. Aqueous solutions up to 50% are not very volatile. GA is a reactive compound that readily reacts and cross-links proteins.
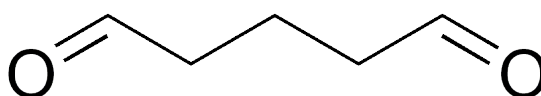
Figure 1.6 - Structural formula of glutaraldehyde (Wikipedia 2009)

Table 1.VI - Physical and chemical properties of glutaraldehyde (HSDB 1996)

Characteristic	Value
Molecular formula	$C_5H_8O_2$
Molecular weight	100.12 g/mol
Melting point	-14 ºC
Boiling point	188 ºC
Vapor pressure	0.6 Torr at 25 ºC
Solubility	soluble in water, alcohol, benzene
Log Kow	-0.18
Henry´s constant	$1.1e^{-7}$ atm/m^3 mol

Applications in Industry

It has a wide spectrum of industrial, scientific and biomedical applications. Currently, the largest application of GA is the medical and dental industries, where it is used primarily as a high-level disinfectant to clean heat-sensitive equipment (e.g., endoscopes, transducers, bronchoscopes, mirrors, etc). This chemical is also used as a tissue fixative in histology and pathology laboratories and as a hardening agent in the development of X-rays. It is also employed, to a lesser degree, for oil drilling applications and gas pipelines to reduce populations of sulfate bacteria and in the pulp and paper-mill industry to control populations of microorganisms (Sano, Krueger et al. 2005).

Environmental Fate

GA vapors are reported to undergo direct photochemical transformation in the troposphere, as well as photo-oxidative degradation (reaction with hydroxyl radicals). Any GA that may enter into receiving waters is likely to be rapidly diluted and undergo further biodegradation. Bioaccumulation of GA in aquatic organisms is precluded by its hydrophilicity and limited persistence.

Under aerobic conditions, GA was first biotransformed into the intermediate glutaric acid, which then underwent further metabolism ultimately to carbon

dioxide, without any intermediate metabolite. After 48 hours, there were no traces of either GA or glutaric acid and GA degradation was quite rapid under aerobic conditions (half-life of 10.6 h). In anaerobic conditions was also rapid (half-life of 7.7 h). Metabolism of GA under anaerobic conditions did not proceed ultimately to methane, but terminated with the formation of 1,5 - pentanediol via 5-hydroxypentanal as an intermediate (NICNAS 1994).

Aerobic System

Analysis by HPLC indicated that GA was oxidized rapidly to glutaric acid, which mineralizes.
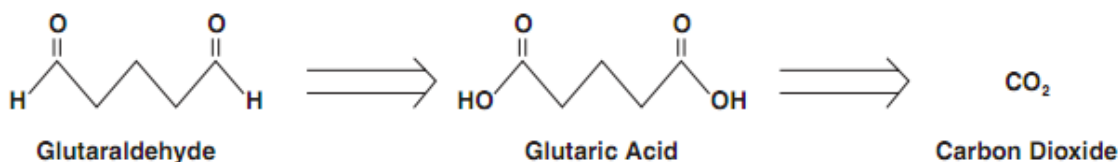
Figure 1.7 - Decomposition path of glutaraldehyde in aerobic systems (Bioshare 2002)

Anaerobic System

Anaerobic metabolism follows a completely different pathway, mainly involving reduction to 1,5-pentanediol (half-life is approximately one day).
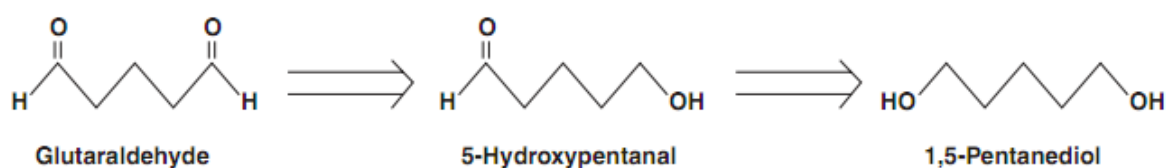
Figure 1.8 - Decomposition path of glutaraldehyde in aerobic systems (Bioshare 2002)

Effects on Aquatic Organisms

GA is acutely toxic to aquatic organisms at low doses. Its toxicity does not increase appreciably with repeated long-term exposure. Table 1.VII indicates that GA is slightly toxic to crabs, shrimp and sewage micro-organisms, slightly to moderately toxic to fish and Daphnia, moderately toxic to oyster larvae, and

moderately to highly toxic to algae. GA loses its biological activity below about 10 mg/L. GA effects on the natural species of the environment are noted for relatively weak concentrations, which prompted National Industrial Chemicals Notification and Assessment Scheme (NICNAS) to consider it as moderately toxic to aquatic fauna and highly toxic to algae (NICNAS 1994).

Table 1.VII - Toxicity of glutaraldehyde in aquatic species

	Test	Species	Result	Reference
Algae	96-h algal growth inhibition	*Scenedesmus subcapitatus*	EC_{50}=1 mg/l	(NICNAS 1994)
Crustacea	48-h acute	*Daphnia magna*	LC_{50}= 16.3 mg/l	(NICNAS 1994)
	96-h acute	*Green crabs*	LC_{50}=465 mg/l	(NICNAS 1994)
Fish	96-h acute	*Bluegill sunfish*	LC_{50}=11.2 mg/l	(NICNAS 1994)
	96-h acute	*Salmo gairdner*	LC_{50}=11 mg/l	(Hon-Wing 2001)

a LOEC = lowest observed effect concentration

1.5.4. Ortho-phthalaldehyde

Chemical and Physical Properties

OPA ($C_6H_4(CHO)_2$; CAS no. 643-79-8) is an aromatic compound with two aldehyde groups. This pale yellow solid is a building block in the synthesis of heterocyclic compounds and a reagent in the analysis of amino acids. OPA is well soluble in organic solvents (NICNAS 2005).
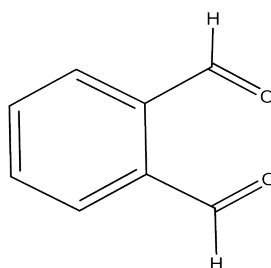
Figure 1.9 - Structural formula of ortho-phthalaldehyde (Wikipedia 2007)

Applications in Industry

OPA appears to have two broad areas of use. Firstly it is used as a chemical reagent in the analysis of amino acids due to its ability to fluoresce. Secondly, OPA has antimicrobial activity and it is used in a 0.55% solution as a high level disinfectant for surgical instruments such as endoscopes (NICNAS 2005). OPA is a new product that is claimed to have excellent microbiocidal, mycobactericidal and sporicidal activity (Simões, Pereira et al. 2003).

OPA, is a potent sporicidal and bactericidal activity and has been suggested as a replacement for the GA. Its trade name is Cidex-OPA® (McDonnell and Russell 1999). OPA has several potential advantages over GA. It has excellent stability over a wide pH range (pH 3-9), is not a known irritant to the eyes and nasal passages, does not require exposure monitoring, has a barely perceptible odor, and requires no activation. OPA, like GA, has excellent material compatibility. A potential disadvantage of OPA is that it stains proteins gray (including unprotected skin) and thus must be handled with caution (William A. Rutala 2008).

Environmental Fate

OPA was reported as a photodegradation product of 2-naphthoic acid in the presence of titanium dioxide (Muneer, Qamar et al. 2005) and also identified as one of the photodegradation products from irradiation of benz[a]anthracene in the presence of organic constituents (9,10-anthraquinone, 9-xanthone, and vanillin) of atmospheric aerosols (Jang and McDow 1997). OPA may also be formed by ozonolysis of remediated PAH-contaminated soils and wastewaters (Sarasa, Roche et al. 1998).

Effects on Aquatic Organisms

OPA is toxic to fish (*Oncorhynchus mykiss* (rainbow trout)) with an LC_{50} of 0,072 mg/l at 96 hours). Is also toxic to daphnia (*Daphnia magna*) with an Ec_{50} of 0,087 mg/l at 48 hours, and other aquatic invertebrates (MSDS 2006)

Basic toxicology data may not be sufficient to determine the potential effects of this new chemical on aquatic species.

1.5.5. Mode of Action

The mode of action of a chemical can be defined as "set of biochemical, physiological and behavioural signs that characterize an adverse biological response" in an organism exposed to a stress factor (McCarty and Borgert 2006).

Unlike antibiotics, biocides are multi-targeted antimicrobial agents. Several of the damaging effects reported to occur in the most widely studied organisms, bacteria, may also take place to varying degrees in other organisms. Thus, it is important to understand the reactions of different types of organisms to biocidal agents (Russell 2003).

Table 1.VIII - Mode of action of the compounds (McDonnell and Russell 1999)

Target	Antiseptic or disinfectant	Mechanism of action
Cell envelope (cell wall, outer membrane)	Glutaraldehyde	Cross-linking of proteins in cell envelope and elsewhere in the cell. The biocidal activity of glutaraldehyde results from its alkylation of sulfhydryl, hydroxyl, carboxyl, and amino groups of microorganisms, which alters RNA, DNA, and protein synthesis
Multi-target	o-Phtalaldehyde	Interact with amino acids, proteins, and microorganisms. Less potent cross-linking agent than glutaraldehyde.
Cytoplasmic (inner) membrane	QACs	Generalized membrane damage involving phospholipid bilayers
Cross-linking of macromolecules	Formaldehyde	Cross-linking of proteins, RNA and DNA. Inactivates microorganisms by alkylating the amino and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases.

1.6. Tested specie

The zebrafish (*D. rerio*, Hamilton-Buchanan 1822), formerly *Brachydanio rerio* is a small tropical fish native to the rivers of India and South Asia (Scholz, Fischer et al. 2008).

Zebrafish belongs to the family of freshwater fishes Cyprinidae and is originally from the Ganges and Brahmaputra basins in north-eastern India, Bangladesh and Nepal. In addition, zebrafish has also been reported in rivers throughout India, as well as in Pakistan, Myanmar, Sri Lanka and river basins draining into the Arabian Sea (Spence, Gerlach et al. 2008).

This species measures 3-5 cm as an adult and thrives in both soft and hard waters. At 26 ºC the zebrafish grows quickly and reaches maturity within three months (Nagel 2002). Males and females are of similar coloration, although males tend to have larger anal fins with more yellow coloration. Males are easily distinct

from females, under spawning conditions, since their body shape is more slender and females get swollen bellies (Figure 1.10) (Spence, Gerlach et al. 2008).
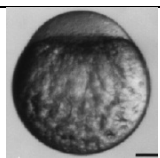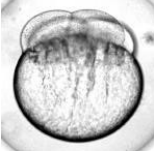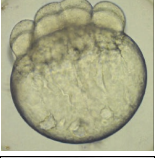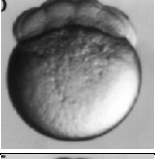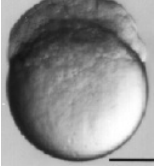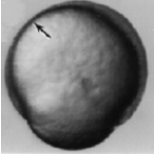
Figure 1.10 - Male and female zebrafish (Lab 2007)
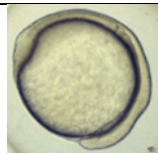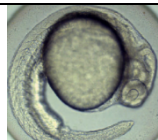
Zebrafish egg is telolecithal, and cleavage is meroblastic and discoidal. Shortly after fertilization, cytoplasm of the egg accumulates at the animal pole where it surrounds the nucleus of the zygote. Only this portion of egg cytoplasm, the so called blastodisc undergoes cleavage, whereas the yolk rich zone is excluded from cleavages (Nagel 2002). Zebrafish embryonic development has been well characterized (Kimmel, Ballard et al. 1995).

In Table 1.IX the stages of embryonic development of zebrafish embryos are summarized.

Table 1.IX - Stages of embryonic development of the *D. rerio* at 26±1°C (Kimmel, Ballard et al. 1995)

Time (h)	Stage	Characterization	
0	**Fertilisation**	Zygote	
0	**Zygote period**	Cytoplasm accumulates at the animal pole, one cell-stage	

Time (h)	Stage	Characterization	
¾	**Cleavage period**	Discoidal partial cleavage	
1		1. Vertical division: four-cell-stage	
1 ¼		2. Vertical and parallel to the plane of the first: eight-cell-stage	
1 ½		3. Vertical and parallel to the second plane of division: 16-cell-stage	
2	**Blastula period**	Start of blastula stage	
3		Late cleavage; blastodisc contains approximately 1024 blastomeres	
4		Flat interface between blastoderm and yolk	
5 ¼	**Gastrula period**	50% of epibolic movements, blastoderm thins and interface between periblast and blastoderm becomes curved	
8		75% of epibolic movement	
10		Epibolic movement ends, blastopore is nearly closed	
10 1/2	**Segmentation period**	First somite furrow	

Time (h)	Stage	Characterization	
12		Somites are developed, undifferentiated mesodermal component of the early trunk, tail segment or metamere	
20		Muscular twitches; sacculus; tail well extended	
22		Site to side flexures; otoliths	
24	**Pharyngula period**	Phylotypic stage, spontaneous movement, tail is detached from the yolk; early pigmentation	
30		Reduced spontaneous movements; retina pigmented, cellular degeneration of the tail end; circulation in aortic arch 1	
36		Tail pigmentation; strong circulation; single aortic arch pair; early motility; heart beating starts	
72-96	**Hatching period**	Heart beat regularly; yolk extension beginning to taper; dorsal and ventral stripes meet at tail; segmental blood vessels; thickend sacculus walls with two chambers; foregut developments	

1.6.1.Zebrafish as a model for toxicology

The zebrafish has been a prominent model vertebrate in a wide range of biological disciplines. The large amount of information from genetic research and evolutionary, with the completion of the zebrafish genome project next, has put zebrafish in an interesting position for use as a toxicological model, where the

objective is to identify adverse effects of chemical exposure (Hill, Teraoka et al. 2005).

To evaluate the toxicity of a chemical, it is essential to identify the endpoints of toxicity and their dose-response relationships, elucidate the mechanisms of toxicity, and determine the toxicodynamics of the chemical. It is known morphological, biochemical, and physiological information at all stages of early development and in juveniles and adults of both sexes. This makes using the zebrafish ideal for toxicology research where the objective is to identify adverse effects of chemical exposure (Hill, Teraoka et al. 2005).

1.6.2. Zebrafish´s advantage compared to other model organisms

The main benefits of using zebrafish as a toxicological model over other vertebrate species are with regards to their size, husbandry, and early morphology (Hill, Teraoka et al. 2005).

Its small size (approximately 1-1.5 inches long) greatly reduces housing space and husbandry cost, make it easily obtainable and inexpensive. Today there are several companies specializing in zebrafish tanks capable of supporting several thousands of fish (Hill, Teraoka et al. 2005).

Also zebrafish is readily maintainable and, under appropriate conditions, will provide a large number of non-adherent and transparent eggs. The transparent chorion enables the easy observation of development. Zebrafish have a very short reproductive cycle. They reach maturity at the age of about 3 months. One female can spawn about 100 eggs per day which are fertilized by sperm release of the male into the water (Scholz, Fischer et al. 2008).

They have a rapid development. Embryos hatch approximately 2–3 days post-fertilization and at 5 days post-fertilization, organogenesis of major organs is completed. Since the egg stage, zebrafish embryos can survive for several days in a single well of a 384 well plate through the absorption of yolk and can be visually assessed for malformation (Scholz, Fischer et al. 2008).

According to current European Union legislation for the protection of animals, used for experimental and other scientific purposes, the use of embryonic stages of vertebrates is not regulated. For that reason, experiments with embryos are considered as alternative to animal experiments (Scholz, Fischer et al. 2008).

The alternative to animal testing concept incorporates the 3 R's introduced by W. M. S. Russell and R. L. Burch (1958) in their book "The Principles of Humane Experimental Technique". The 3 R's represent: reduction of the number of animals used, refinement of techniques and procedures to reduce pain and distress, and replacement of animal with non-animal techniques.

The fish embryo toxicity test (FET) has advantages including the need for small amounts of test substances, shorter time periods of exposure, and the need for only breeding stock. These advantages will soon translate into reduced testing costs. Sublethal endpoints can be easily achieved in this testing framework which may translate into understanding prospects for chronic responses, teratogenicity, or other effects (Lammer, Carr et al. 2009)

The embryo test has the potential to be a substitute of fish test in routine waste water control and it could be also a model for testing chemicals in toxicology (Hill, Teraoka et al. 2005). *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).

1.7. Mixture toxicology

For understanding the mixture toxicity, fundamental concepts must be carefully defined along this thesis. A mixture can be defined as a combination of two or more component chemicals/compounds to which living organisms may be exposed, either simultaneously or sequentially (McCarty and Borgert 2006).

In aquatic toxicology, two different concepts, termed concentration addition (CA) and independent action (IA), have been used to describe general

relationships between the effects of single substances and the corresponding mixtures for similarly and dissimilarly acting chemicals, respectively, and allow calculation of an expected mixture's toxicity on the basis of known toxicities of the mixture's individual component (Barata, Baird et al. 2006).

CA model is thought to be applicable to mixtures composed of chemicals with a similar mode of action, and thus is most applicable for toxic substances that have the same molecular target site.

Mathematically the CA model can be expressed as:

$$\sum_{i=1}^{n} \frac{ci}{ECxi} = 1$$

Where ci is the concentration of chemical i in the mixture and $ECxi$ is the effect concentration of chemical i that results in the same effect (x%) as the mixture, so in the case of a 50% mixture effect insert $EC50_i$. For survival data, simply exchange EC_x with LC_x (lethal concentration). The quotient ci/ECx_i is also referred as the toxic unit (TU) that quantifies the contribution to toxicity of the individual chemical i in the mixture of n chemicals (Jonker, Svendsen et al. 2005).

The alternative model of independent action is applied to chemicals with diverse modes of action, interacting with different target sites (Barata, Baird et al. 2007).

Is described by the formula based on mathematical probabilities:

$$Y = u \max \prod_{i=1}^{n} qi(ci)$$

Where Y is the biological response, c_i is the concentration of toxic i in the mixture, and $qi(ci)$ is the probability of nonresponse (Jonker, Svendsen et al. 2005).

Mixture effects can be characterized by quantifying how observed data deviate from either reference model (Jonker, Svendsen et al. 2005). All combinations of a mixture caused a more severe (synergism) or less severe (antagonism) effect than calculated from either reference model.

When the effluent toxicity is greater than the sum of the toxicities of the individual constituents, synergism is indicated. When the toxicity of the effluent is less than the sum of the toxicities of the individual constituents that comprise the effluent toxicity, antagonism is implied (Calow 1997)

Sprague (1970) described a method by which the interactions of the 2-substances could be represented in two dimensions. It consists in representing an abscissa and a coordinate of the TU of substances A and B that composing the mixture. If the effects are additive, the curve is a straight line as shown in Figure 1.11 (a).

However as shown in Figure 1.11 (b), if the effect is synergistic, the isobole of the AB mixture is located below the additivity isobole, whereas, if the effect is antagonistic, the isobole of the mixture is located above the isobole of additivity (Panouillères, Boillot et al. 2007)
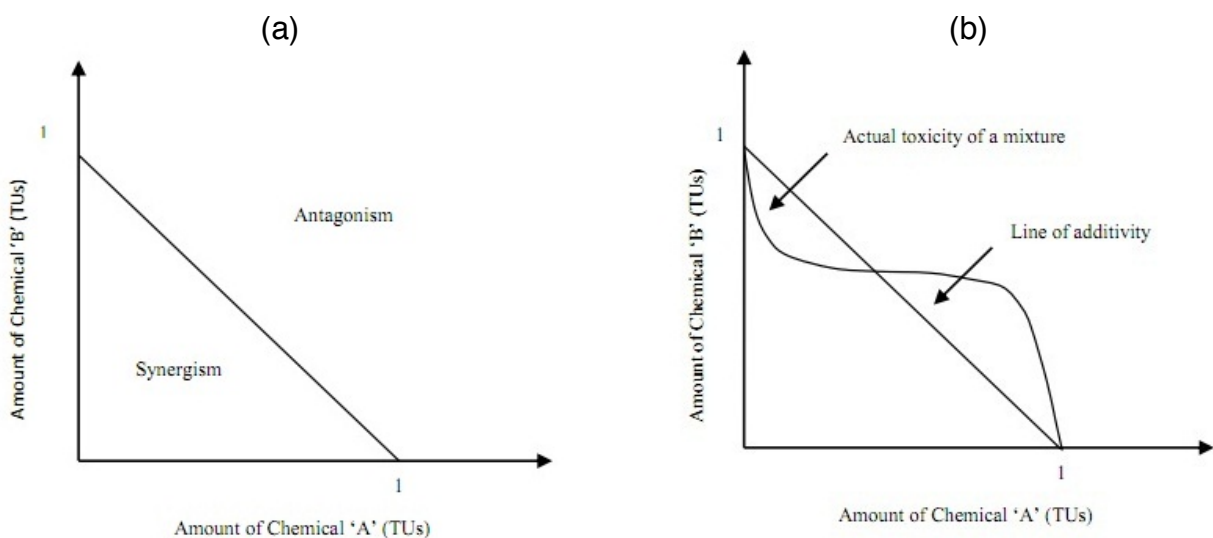
Figure 1.11 - Example of isoboles, showing additivity (a) and the domains of antagonism and synergism (b), highlighting a variation of interactions between two substances as a function of their ratio (Panouillères, Boillot et al. 2007)

Humans and all other organisms are typically exposed to multi-component chemical mixtures, present in the surrounding environmental media (water, air,

soil), in food or in consumer products. Because of this, in environment, combinations of substances of varying toxicity inevitably co-occur. However, the vast majority of available toxicity data deal with the effects of single pure chemicals (Environment 2009).

Aquatic organisms are thus constantly exposed to contaminant mixtures, whose individual components are likely to produce different life-history responses within the same organisms (Barata, Baird et al. 2006).

The study of mixtures is important because it permits understanding the combined effects between the substances present, for example, in effluents. The literature focuses two types of mixtures: simple mixtures and complex mixtures. Simple mixtures are composed of less than 10 substances with known qualitative and quantitative compositions. On the other hand, complex mixtures are composed of more than 10 substances with neither known qualitative nor quantitative compositions. To understand their toxicity it´s necessary to define 10 classes of substances that may be responsible for toxicity. Hospital effluents can be considered as complex mixtures in which detergents and disinfectants are the main sources of toxicity (Panouillères, Boillot et al. 2007).

Certain mixtures are synergic and constitute a real danger for the environment. Thus, detergents and disinfectants are mixed together in hospital effluents and could interact in synergy.

For binary mixtures, the ToxCalc spreadsheet built over Microsoft Excel permit to detect deviations (interactions) from the two reference models of CA and IA. This descriptive model not only allows evaluating if synergism and antagonism occurs in the binary mixture, but also the description of two more complex deviations, namely dose ratio (deviation is dependent of the ratio of the two components of the mixture) and dose level dependent deviation (deviation is dependent of the dose of each component in the mixture).

1.8. Objectives and structure of the thesis

This work was mainly aimed to study the ecotoxicological effects of the combination of a surfactant with three aldehydes on the embryos of the zebrafish. The surfactant selected for the study was the BKC, and aldehydes were the FA, GA and OPA. These products are routinely used in hospitals, and their concentration in aquatic ecosystems have been successively increasing to values which can cause toxic effects on living beings, as a result of increased water pollution of anthropogenic origin.

The specific objectives of work consisted of:

i)   to evaluate the acute toxicity of the FA and OPA 96 hours for the zebrafish embryo,

ii)   to evaluate and predict the acute toxicity of three binary mixtures of a surfactant with three aldehydes.

According to the objectives set, the first chapter of this dissertation is a general introduction to the issues of hospital waste contamination, and choice of test organism used.

The second chapter entitled **"Toxicity of hospital disinfectants mixture on zebrafish early life"** presents the results obtained in acute toxicity tests performed with the disinfectants FA and OPA and the test results of the toxicity assessment of a binary mixture between a surfactant with aldehydes, based on the theoretical model of independent action and addition of concentration.

Third chapter, presents a general discussion and conclusions from the results obtained in the work. Where, in addition to the comparison of results obtained in this work with some of the data already published by other authors, describes the main conclusions of chapters I and II.

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2. Toxicity of binary mixtures, used as hospital disinfectants, to zebrafish early life-stages

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Abstract

Large quantities of chemicals (e.g. detergents, biocides, disinfectants) are used in hospitals for cleaning and disinfection. Hospital effluents are a complex mixture that might cause serious environmental impacts.

This work assessed the effects on zebrafish early life-stages of binary mixtures of the surfactant benzalkonium chloride (BKC) with three aldehyde disinfectants commonly used in hospitals: glutaraldehyde (GA), formaldehyde (FA) and ortho-phthalaldehyde (OPA). The assays were based on the OECD guideline on Fish Embryo Toxicity (FET) Test. Over 96 hours the organisms were inspected daily with a stereomicroscope, using mortality as endpoint. The BKC, FA, GA and OPA showed high toxicity for zebrafish embryos presenting LC_{50} values at 96h of 3.77 mg/l, 546.8 mg/l, 27.64 mg/l and 64.9 μg/l, respectively. For mixtures it was used the independent action and concentration addiction models in order to determinate the more appropriate model to predict the mixture toxicity of this chemicals.

At 96 hours, the mixture toxicity of BKC and FA is best described by the concentration addition model with a dose level dependence (antagonism at low dose and synergism at high dose), concentration addition associated with

antagonistic effects best describes the mixture of BKC and GA and the Independent action model associated with synergistic effect best describes the mixture of BKC and OPA.

Hospital effluents are complex mixtures, including a wide range of disinfectants, which may represent an environmental problem when released into the environment.

2.1. Introduction

Disinfectants are highly complex products or mixtures of active substances (Kummerer 2002) widely used in hospitals to clean medical and surgical instruments from pathogenic organisms that cause nosocomial infectious diseases and to and detergents used to clean floors and surfaces that are widely used in hospitals to clean medical and surgical instruments from pathogenic organisms that cause nosocomial infectious diseases, and detergents used to clean floors and surfaces (Purohit, Kopferschmitt-Kubler et al. 2000). After use, these substances become part of hospital effluents which generally reach, together with the urban wastewater, the municipal sewer network without preliminary treatment, and are then directed to wastewater treatment plants (WWTP), which mostly employ biological treatment processes.

Hospital effluents generally have a low microbial load due to regular use of disinfectants. Many of them are bactericidal and can exert a negative influence on the biological processes of the WWTP. Even considering that these effluents are diluted after the WWTP discharge, the possibility of some substances generate, for a cumulative effect, a biological imbalance in the ecosystem cannot be discarded.

Beyond disinfectants and surfactants, pharmaceuticals, pigments, dyes, reagents, and drug components are widely used in hospitals. Certain substances, particularly organohalogens and partially metabolized pharmaceuticals, leave WWTPs mostly without any degradation. Researchers have detected chemotherapy drugs, antibiotics, and hormones in groundwater. This aquifer serves as a source of drinking water (Gautam, Kumar et al. 2007).

Due to the varied elements discharged, hospital effluents comprise three types of risk: toxic risk, infectious risk and radioactive risk. This study focuses exclusively on toxic risks. In the literature, we can find studies that focus the obvious ecotoxicity of these effluents. Societé Française d'Hygiène Hospitalière postulate that the origin of this toxicity is mainly due to the presence of disinfectants and detergents (Panouillères, Boillot et al. 2007).

The input of hospital pollutants into aquatic ecosystems constitutes a risk directly related to the existence of hazardous substances with potential negative effects on the biological balance of natural environments (Emmanuel, Perrodin et al. 2005). The fate of pharmaceuticals in the aquatic environment and the ecological risk of GA are examples already reported in the literature (Kümmerer, Steger-Hartmann et al. 1997; Jolibois, Guerbet et al. 2002). However, few studies deal with the risk resulting from the binary combination of pollutants present in the hospital effluents. In fact, detergents and disinfectants are mixed together in hospital effluents and could interact in a synergistic way. Also, the toxicity of BKC and aldehydes could vary as a function of their ratios. In view of this data, it is important to study the toxicity of BKC and different aldehydes to aquatic organisms.

The results of this study will elucidate the risks of the combined use of certain detergent and disinfectant products by studying their interactions.

The substances chosen for the study (glutaraldehyde, formaldehyde, ortho-phthalaldehyde, and benzalkonium chloride) are commonly used in hospitals. Glutaraldehyde (GA) has a widespread biomedical use for the cold sterilization of dental and medical instruments and endoscopes. GA is an aliphatic dialdehyde with carbonyl groups that interact readily with nucleic acids and proteins. This high

reactivity allows cross-linking of amine groups on the cell walls and cell membranes of microorganisms (Boillot and Perrodin 2008). GA is acutely toxic to aquatic organisms at low doses. GA has been detected between levels of 0.50 and 3.72 mg/l in hospital wastewaters (Jolibois, Guerbet et al. 2002).

Formaldehyde (FA) has a wide variety of uses in hospitals, in disinfectants, in tissue preservatives in pathology departments and in setting for cold sterilization of endoscopes. FA inactivates microorganisms by alkylating the amino and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases (William A. Rutala 2008). Aquatic organisms respond negatively to low concentrations of FA, which has been already found in hospital wastewater at levels of 0.07 mg/l (Kajitvichyanukul and Suntronvipart 2006).

Ortho-phthalaldehyde (OPA), commercially called Cidex®, is a new compound. In hospitals, it is a high level disinfectant with reduced exposure time, for flexible endoscopes. The disinfecting mechanism of OPA is thought to be similar to GA and is based on the powerful binding of the aldehyde to the outer cell wall of contaminant organisms (William A. Rutala 2008).

BKC is one of the most important quaternary ammonium compounds used for the disinfection of surfaces in medical care applications as well as in the food and glue industries. Its mode of action has been attributed to the inactivation of energy-producing enzymes, denaturation of essential cell proteins, and disruption of the cell membrane (William A. Rutala 2008). BKC consists of homologues of different alkyl chain length, and concentrations up to 6 mg/l have been measured in hospital effluents (Kümmerer, Eitel et al. 1997).

Two models are used to predict the effects of mixture of single compounds: concentration addition (CA), and independent action (IA), The CA model is founded on the assumption that mixtures components possess a similar pharmacological mode of action while IA assumes that mixture components possess dissimilar modes of action, interacting with different target sites (Faust, Altenburger et al. 2001; Barata, Baird et al. 2007).

Deviations from these two conceptual models have also been observed, probably due to interactions that may occur at toxicokinetics or toxicodynamics

levels and produce different behaviour patterns, according to a more severe effect (synergism), less severe effect (antagonism), dose level or dose ratio dependent.

The main goal of this study was to evaluate the effects of binary mixtures of BKC and three aldehydes (GA, FA e OPA) in the zebrafish embryos. This study drives attention to the problem of inappropriate use of chemicals in hospital systems, since its use is often combined, contributing to fulfill the data gap on ecotoxicological information necessary for ecological risk assessments of chemicals in the hospital units.

2.2. Materials and Methods

2.2.1. Chemicals

BKC (50% solution in water), FA (37 wt. % in H2O), GA (50% solution in water) and OPA (≥98.5% purity (HPLC)) were purchased from Sigma-Aldrich.

2.2.2. Test organisms

Zebrafish (*D. rerio*) from a culture established at the Department of Biology, University of Aveiro, are maintained in a semi-close recirculating system (ZebTech, Tecniplast), with osmosis filtered water at 28.0 ±0.5 °C under a 14 :8h light/ dark photoperiod cycle. Conductivity is kept at 750 ±100 μS/cm, 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.

2.2.3.Test conditions

The assays were based on the OECD guideline on Fish Embryo Toxicity Test (OECD 2006) and on the embryo test described by Oliveira et al (2009).

In the evening, adult male and female were put in the aquarium (proximally 2:1) with marbles on the bottom (spawning substrate), since adult zebrafish can be

predators of their eggs and larvae (Spence, Gerlach et al. 2008), so that the adults could not eat the eggs.

Zebrafish eggs were collected within 30 min after natural mating, rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon). Unfertilized eggs with irregularities during cleavage or injured were discarded (Figure 2.1). Only fertilized eggs between the 4- and 128-cell stages were used.

Test solutions of the selected concentrations for single tests and mixtures were prepared right before starting the test, by dilution of stock solution in fish water, with controlled pH (7.5 ±0.5) and conductivity (750 ±50 μS/cm). The temperature during the test was 26.0 ±1 $^{\circ}$C and the photoperiod was of 16 h light and 8 h dark.
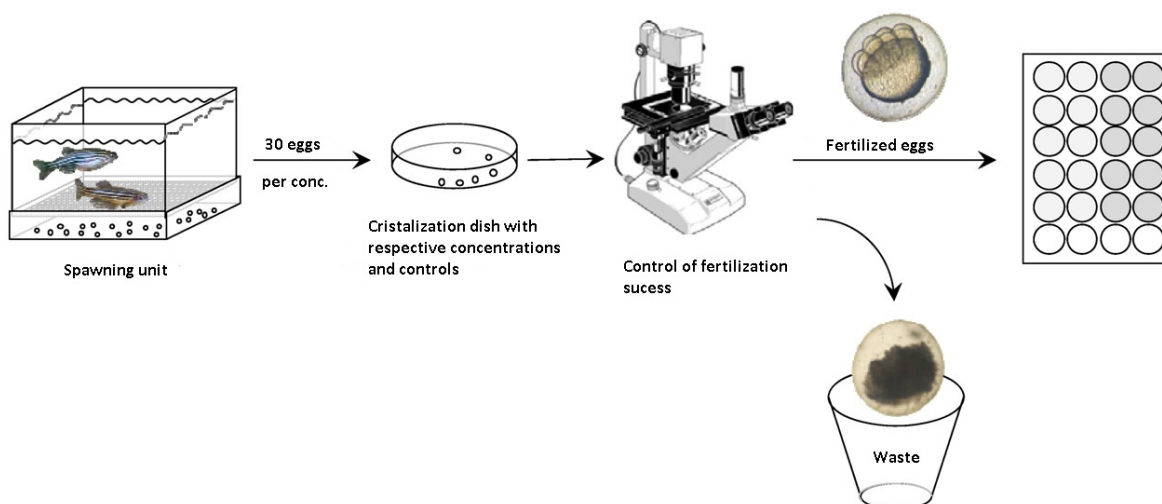
Figure 2.1 - Scheme of toxicity tests with embryos of zebrafish (Lammer, Carr et al. 2009).

2.2.4. Single compound toxicity tests

Toxicity tests with individual compounds were first performed to find the optimal concentration range (a range of concentrations leading from 0 to 100% of effect) to be used in the combined exposures.

GA and BKC toxicity was previously assessed by our group (Pereira, 2009). For FA the following nominal concentrations were tested: 0, 125, 250, 375, 750

and 1500 mg/l while for OPA the following nominal concentrations were tested: 0, 45, 50, 60, 75, 95, 120 and 150 μg/l. All compounds tested were soluble in water, except OPA which required a solubilizing agent; in this case the dimethylsulfoxide (DMSO) was used. An experimental design adapted from Lammer (2009) was set up using 24-well microplates according to Fig 2.2. Each concentration used 10 eggs set individually with 2 ml of the test solution, except for control which used 12 eggs. Three replicates of this experimental design were individually performed (Figure 2.2)

Figure 2.2 - Single tests experimental design: distribution of the different test concentrations (c1 to c5), control (c0) and solvent controls (cS) in the 24-wells plates. This scheme was performed in triplicate for each test.

Mortality was daily recorded. The examination of the organisms was carried out with the aid of a stereomicroscope using a magnification between x30 and x50. Embryos and larvae morphologic effects (edema, spine malformations, posture disturbance and mortality) were observed in the test of FA, according to their period of occurrence. The posture disturbance is characterized by an impossibility of larvae in keeping an upright posture, either swimming or stopped. Spine malformations were characterized by a curved tail.

2.2.5. Mixture toxicity tests

In the mixture experiments, 25 binary combinations for BKC and FA mixture and BKC and GA mixture were made and 30 binary combinations for BKC and OPA mixture, based on the LC_{50} calculated in the individual tests, simultaneously with five concentrations of each compound (BKC, GA and FA) and six for OPA due the use of control solvent.

For BKC and FA mixture, BKC concentrations ranged from 1.5 to 5.9 mg/l, for FA single ranged from 250 to 1500 mg/l. In the mixture, concentrations for FA ranged 160 to 520 mg/l. For GA concentration used ranged from 1.2 and 5.9 mg/l.

For BKC and GA, the concentrations for BKC single compound ranged from 1.7 to 5.9 mg/l, and for GA ranged 1.8 to 100 mg/l. In mixture tests, concentrations for BKC ranged from 0.9 to 3.6 mg/l and 6.25 to 50 mg/l for GA.

For BKC and OPA, concentrations for BKC single compound used ranged 1.5 to 5.9 mg/l and 45 to150 μg/l for OPA. For mixture tests, the BKC concentration ranged from 1.5 to 4.5 mg/l and 45 to 65 μ/l for OPA.

For all combinations, the experimental design consisted of single exposures each chemical and combinations of both chemicals, building a fixed ray design, where the mixture ratio is kept constant throughout the studies and the overall concentration of the mixture is systematically varied. The combinations used were planned to characterize the best possible concentration-response, taking into account possible effects dependent on the level of concentration and ratio of the mixture components, according to the scheme shown in Figure 2.3. The preparation of this plan was based on the concept of toxic unit (TU). This dimensionless concept is defined as the ratio of a given concentration (C) of a substance and the concentration required causing a 50% effect (EC_{50}) on the criterion of toxicity studied (Jonker, Svendsen et al. 2005).

The TU values of the binary mixture are then plotted on an isobologram which facilitates characterizing the combined effects of binary mixtures and has the advantage of being illustrative. An isobologram is a two dimensional chart with the TU of each chemical as its axes. The plots drawn on an isobologram represent the

response contour and are called isoboles. Each isobole represents a set of conditions resulting in similar responses.
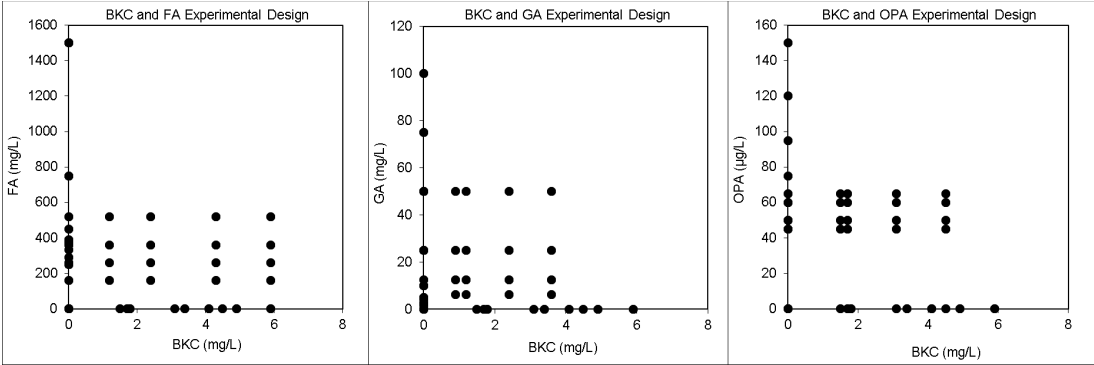
Figure 2.3 - Plan adopted in experimental toxicity test of the three binary mixture, indicating the combinations of concentrations used

Experimental design according to Figure 2.4, using 5 eggs per treatment, each plate was filled with a different BKC concentration solution, with a row per FA, GA or OPA concentration solution. In each plate the column on the right was left without toxics, only control water. Experimental design was performed in triplicate.

Figure 2.4- Mixtures experimental design .Distribution of the different test concentrations and controls: Each plate with 5 wells (in row) of a given concentration (1 to 4). Negative controls (dilution water; c0).

The tests were performed in an acclimatized chamber, with a photoperiod of 16 hours of light and 8 hours of dark, during 96 hours. The temperature was 26 ±1 ^{o}C.

Embryos and larvae were observed daily with the help of stereomicroscopy. Magnification used for observations of eggs was ×70 and was ×40 for larvae. Endpoint was mortality identified by immobilization.

2.2.6. Statistical analysis

In the mixture assay, the concentration-response relationship for compounds was studied using the simple fit to the data obtained from a logistic function:

$$R_i = R_{Max} \left[\frac{1}{1 + \left(\frac{[Chem_i]}{EC50_i} \right)^{\beta_i}} \right] = R_{Max} \left[\frac{1}{1 + TU_i^{\beta_i}} \right],$$ where R_i is the expected

response (mortality) for a given exposure concentration of compound i, [$Chem_i$], β_i is the slope of the sigmoid function and EC_{50i} is the median for the lethal concentration that kills 50% of the individuals.

The function was fitted to the experimental data by minimizing sum of squared deviations (SS) with the Solver add in for Excel within the ToxCalc spreadsheet (Nogueira, in prep.). The models used to fit the data were IA and CA, as presented in Barata et al.(2006) (Table 2.I) Binary mixture data was used to identify possible deviations from each model using functions, adapted from Jonker et al. (2005) (Table 2.II). The standard errors of the regression parameters were calculated with the macro SolvStat version 2.0 (Billo 2001) that was integrated, with adaptations, into ToxCalc.

Table 2.I- Mixture toxicity functions used in ToxCalc spreadsheet functions. (*) Deviation functions from baseline models were adapted from Jonker et al. (2005)

Concentration Addition	$$R_{mix} = R_{Max}\left[\cfrac{1}{1+\cfrac{\left(\sum\limits_{i=1}^{n} TU_i\right)^{\beta_\kappa}}{e^{G(TU_1,..,TU_n)}}}\right]$$
To calculate β_κ	$$\tau = \sum_{i=1}^{n} TU_i$$ $$\beta_\kappa = \sqrt[\tau]{\prod_{i=1}^{n}\left(\beta_i\right)^{TU_i}}$$
Independent Action	$$R_{mix} = R_{Max}.\phi\left\{\phi^{-1}\left[\prod_{i=1}^{n}\left(\frac{1}{1+TU_i^{\beta_i}}\right)\right]+G(TU_1,..,TU_n)\right\}$$
Deviations from baseline model	$$G(TU_1,..,TU_n)$$
Synergism / Antagonism (*)	$$a\prod_{i=1}^{n}\left(\frac{TU_i}{\sum\limits_{j=1}^{n}TU_j}\right)$$
Dose Ratio (*)	$$\left[a+\sum_{i=1}^{n}b_i\left(\frac{TU_i}{\sum\limits_{j=1}^{n}TU_j}\right)\right]\prod_{i=1}^{n}\left(\frac{TU_i}{\sum\limits_{j=1}^{n}TU_j}\right)$$
Dose Level (*)	$$a\left[1-\sum_{i=1}^{n}b_i\left(\sum_{j=1}^{n}TU_j\right)^{\beta_\kappa}\right]\prod_{i=1}^{n}\left(\frac{TU_i}{\sum\limits_{j=1}^{n}TU_j}\right)$$

Table 2.II- Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from concentration addition (CA) and independent action (IA). Adapted from Jonker (2005)

Deviation Pattern	Parameter a	Parameter b_i (CA)	(IA)
Synergism/antagonism (S/A)	**a>0**: antagonism **a<0**: synergism		
Dose-ratio dependent (DR)	**a>0**: antagonism except for those mixture ratios where negative b value indicate synergism	$b_i>0$: antagonism where the toxicity of the mixture is caused mainly by toxicant *i*	
	a<0: synergism except for those mixture ratios where a positive b value indicate antagonism	$b_i<0$: synergism where the toxicity of the mixture is caused mainly by the toxicant *i*	
Dose level dependent (DL)	**a>0**: antagonism at low dose level and synergism at high dose level	$b_i>1$ change at lower dose level than the EC50	$b_i>2$
		$b_i=1$ change at the EC50 level	$b_i=2$
		$0<b_i<1$ change at higher EC50 level	$1<b_i<2$
	a<0: synergism at low dose level and antagonism at high dose level	$b_i<0$ no change, but the magnitude of synergism / antagonism is dose level dependent	$b_i<1$ effect level dependent

2.3. Results

2.3.1. Single tests

The results shown in Table 2.III summarize the LC_{50} values of the three individual substances calculated for the 96 hours, based on which the final concentrations tested for the mixture toxicity bioassays were calculated. The model parameters for each compound are presented with the 95% confidence limits.

OPA is the most toxic compound (LC_{50} = 64.9 µg/l) followed by BKC (LC_{50} = 3.77 mg/l) (Pereira 2009), GA (LC_{50} = 23.97 mg/l) (Pereira 2009) and FA (LC_{50} = 546.0 mg/l).

Table 2.III - Lethal concentration (LC_{50}) obtained after 96 hours of exposure to acute toxicity tests of BKC, GA, FA and OPA for zebrafish embryos, with 95% confidence limit.

	BKC (mg/l)	GA (mg/l)	FA (mg/l)	OPA (µg/l)
LC_{50}	3.9 ±3.75	23.97 ±2.50	546.8 ±84.4	64.9 ±1.3

The results of the single exposure study of FA and OPA can be seen in Fig. 2.5 and Fig. 2.6, respectively. Proportion of embryos that died along the experiment (red bars), alive embryos (orange bars), hatched embryos (yellow bars) and larvae that died (green bars) is presented as stacked bars.
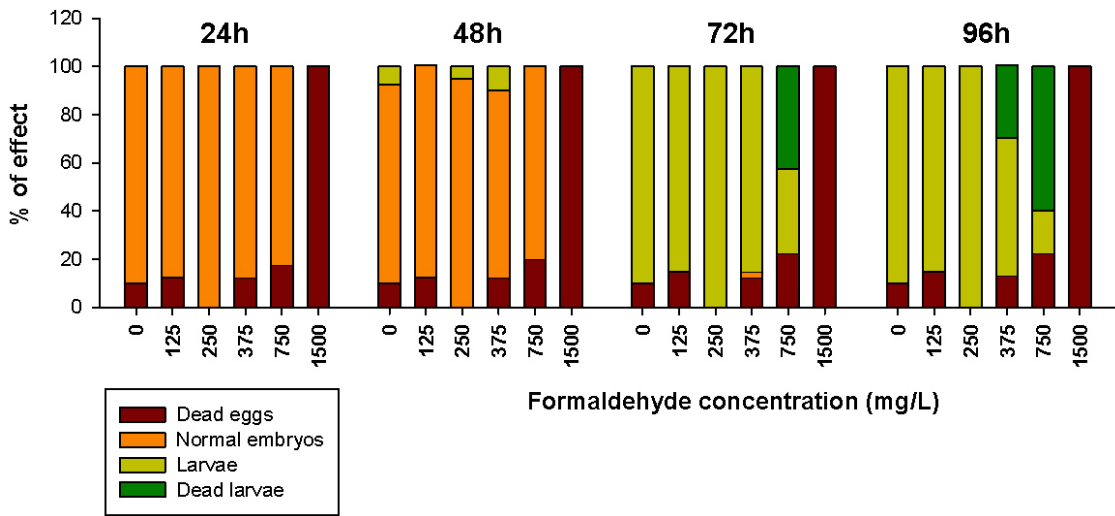
Figure 2.5 - General overview of FA effects on *D.rerio* embryo and larvae during 96h of exposure.
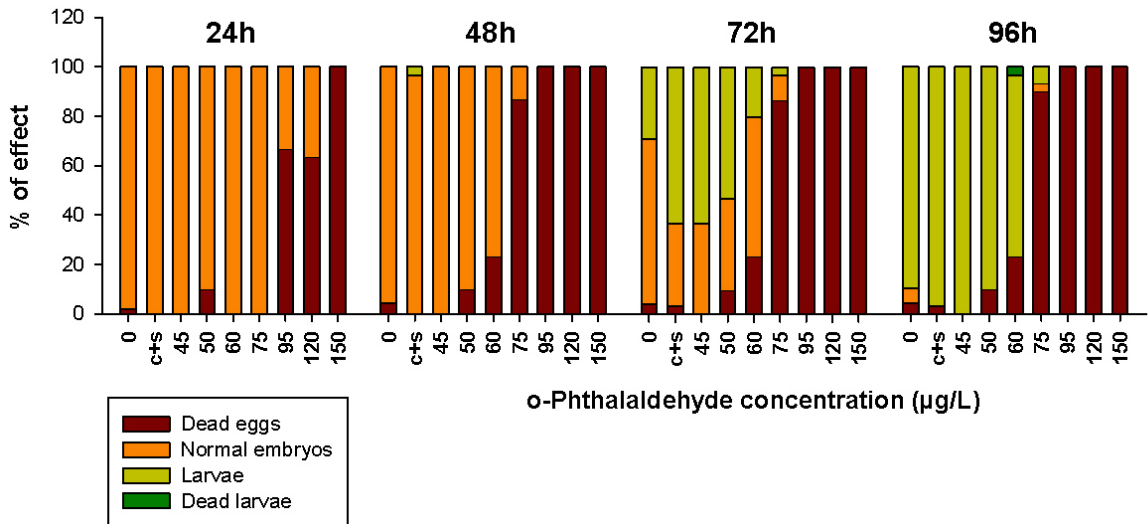
Figure 2.6 - General overview of OPA effects on *D.rerio* embryo and larvae during 96h of exposure.

The control group presented a normal embryo development as described by (Kimmel, Ballard et al. 1995), showing low mortality. The control group had low mortality for the two compounds, respectively, 10% for FA, 4.2% and 3.3% (solvent control) for OPA, at 96h, fulfilling the requirements for validation of the test. In the first 24 h of FA assay, all embryos exposed to 1500 mg /l died.

Embriotoxicty
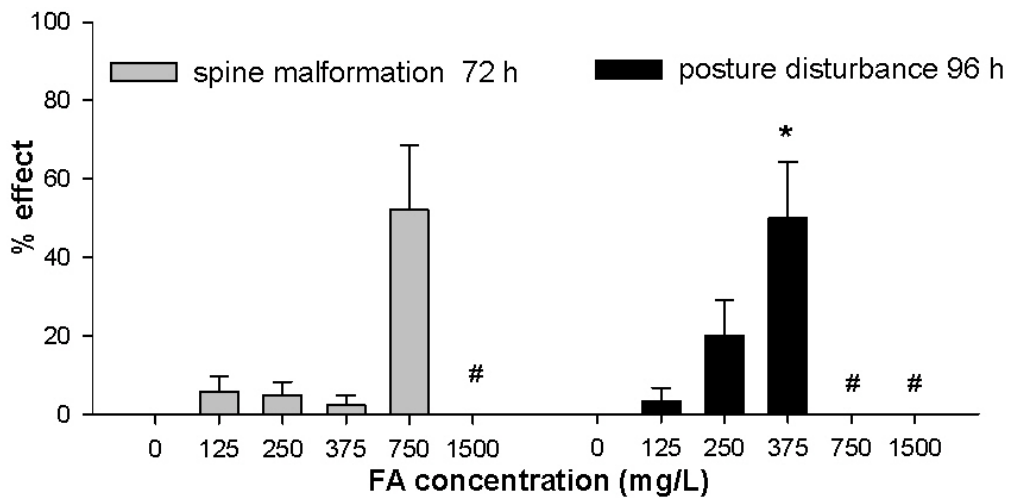
Figure 2.7 - Effect of FA on the endpoints spine malformation as curved tail and posture disturbance (lack of equilibrium) at 72 and 96 hours respectively. Asterisks means statistically significant difference among the concentrations, while sign cardinal shows concentrations without live embryo or not enough to measure the effect.

Among the embryo development parameters evaluated along the four days of exposure to FA, spine malformations and posture disturbance (lack of equilibrium) were the only affected (Fig 2.7). Considerable spine malformations were observed at 72 h (Kruskal-Wallis H = 14,327, P <0.006), although no differences from control were observed – as depicted in Figure 2.7 and 2.8. Posture disturbance (Kruskal-Wallis H = 12,738, P = 0.005) were observed at 96 h, although statistical significant differences were only observed at concentration of 375 mg/l.
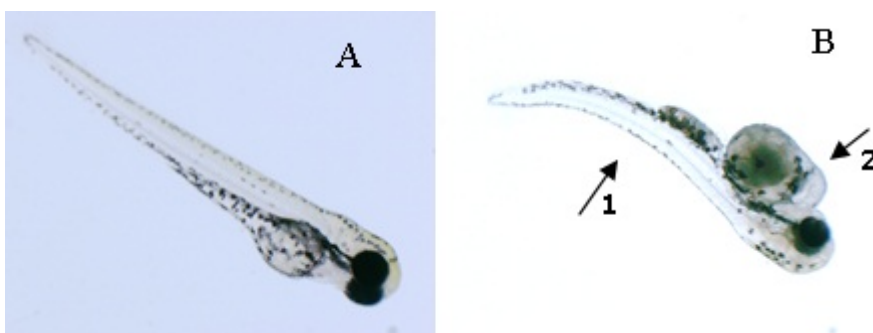
Figure 2.8 - A: Control *Danio rerio* larva at 72 h; B: *Danio rerio* larva response to FA (125 mg/l) at 72 h, with spine malformation (1) and edema (2).

2.3.2. Combined effects

After 96 hours of exposure, there was no mortality in the control group or the group exposed to the solvent of OPA, similarly to what was found for single chemicals. The logistic parameters for individual chemicals used to parameterize the mixture toxicity models show, as expected, increased toxicity with time (Tables 2.IV, 2.V, 2.VI).

Table 2.IV - Model parameters for *Danio rerio* mortality test, presented with the correspondent 95% confidence limits, obtained for the single measured simultaneously with the mixture test

BKC and FA

	24h		48h		72h		96h	
	BKC (mg/l)	FA (mg/l)	BKC (mg/l)	FA (mg/l)	BKC (mg/l)	FA (mg/l)	BKC (mg/l)	FA (mg/l)
EC_{50}	4.3±0.3	788.2±128.2	4.3±0.3	675.9±131.4	4.1±0.5	399.5±48.0	3.8±0.4	288.3±28.8
β	8.2±4.2	3.1±1.0	7.8±4.7	2.6±1.0	6.1±4.4	4.3±2.4	6.6±4.3	4.1±2.0
r^2	0.737		0.658		0.549		0.626	
n	109							

BKC and GA

	24h		48h		72h		96h	
	BKC (mg/l)	GA (mg/l)	BKC (mg/l)	GA (mg/l)	BKC (mg/l)	GA (mg/l)	BKC (mg/l)	GA (mg/l)
EC_{50}	4.4±0.3	60.8±6.6	4.5±0.3	52.2±33.1	4.3±0.4	49.4±94.5	4.0±0.3	37.2±6.3
β	9.1±5.2	10.0±5.4	10.0±7.1	17.0±250.0	7.8±5.7	20.3±3088.8	8.5±5.4	4.1±2.0
r^2	0.714		0.667		0.610		0.693	
n	111							

BKC and OPA

	24h		48h		72h		96h	
	BKC (µg/l)	OPA (µg/l)	BKC (µg/l)	OPA (µg/l)	BKC (µg/l)	OPA (µg/l)	BKC (µg/l)	OPA (µg/l)
EC_{50}	4.3±0.3	76.6±11.7	4.3±0.3	57.7±5.0	4.0±0.4	57.7±5.4	3.7±0.3	56.8±4.6
β	7.9±5.0	3.4±1.7	7.4±4.3	5.1±2.7	5.7±3.4	5.1±2.9	6.1±3.3	5.4±2.7
r^2	0.634		0.710		0.654		0.706	
N	74							

BKC LC$_{50}$ value does not vary much during the 96 hours of exposure. Moreover values calculated in the three mixtures seem to agree.

The toxicity of FA increased significantly over time, and at 96 hours, LC$_{50}$ was about 2.7 times lower than at 24 hours.

The toxicity of GA also increased but not so evident and OPA suffered the least toxicity increase over time.

There also differences in shape of the dose-response (β) relationships of the separate compounds during the exposure.

Deviations such as synergism/antagonism or dose ratio/dose level dependence, were also fitted to each model, by the addition of two parameters (*a* and *b*). Statistical comparisons between CA and IA, and within each of them were used to identify the most suitable effects associated with the mixture (Table 2.VII)

Table 2.V - Summary of the analysis of fitting parameters of the effect mixtures responses of embryos of *Danio rerio*

Mixture	Best Baseline Model	Intera ction Effect	a	b	r^2	Goodness of fit
	Time (hours)					
BKC and FA	24	CA	DL	62.333	0.010	0.146	$F_{(2,45)}$=3.830, P<0.029
	48	CA	DL	66.915	0.016	0.464	$F_{(2,45)}$=19.500, P<0.001
	72	CA	DL	28.281	0.009	0.881	$F_{(2,45)}$=166.150, P<0.001
	96	CA	DL	61.167	0.008	0.775	$F_{(2,45)}$=77.420, P<0.001
BKC and GA	24	CA	A	14.732	-	0.366	$F_{(1,46)}$=26.600, P<0.001
	48	CA	A	11.851	-	0.847	$F_{(1,46)}$=254.420, P<0.001
	72	CA	A	13.411	-	0.860	$F_{(1,46)}$=281.750, P<0.001
	96	CA	A	11.430	-	0.701	$F_{(1,46)}$=107.750, P<0.001
BKC and OPA	24	IA	S	-6.398	-	0.428	$F_{(1,46)}$=34.430, P<0.001
	48	IA	S	-4.633	-	0.742	$F_{(1,46)}$=131.960, P<0,001
	72	IA	S	-7.233	-	0.319	$F_{(1,46)}$=21.570, P<0,001
	96	IA	S	-7.315	-	0.294	$F_{(1,46)}$=19.120, P<0,001

r^2 coefficient of determination, a and b parameters of the deviation functions, CA concentration addition, IA independent action, A antagonism, S synergism, DL dose level deviation from the reference, $F=\frac{SSR}{SSE}$

24h   48h   72h   96h

% survival
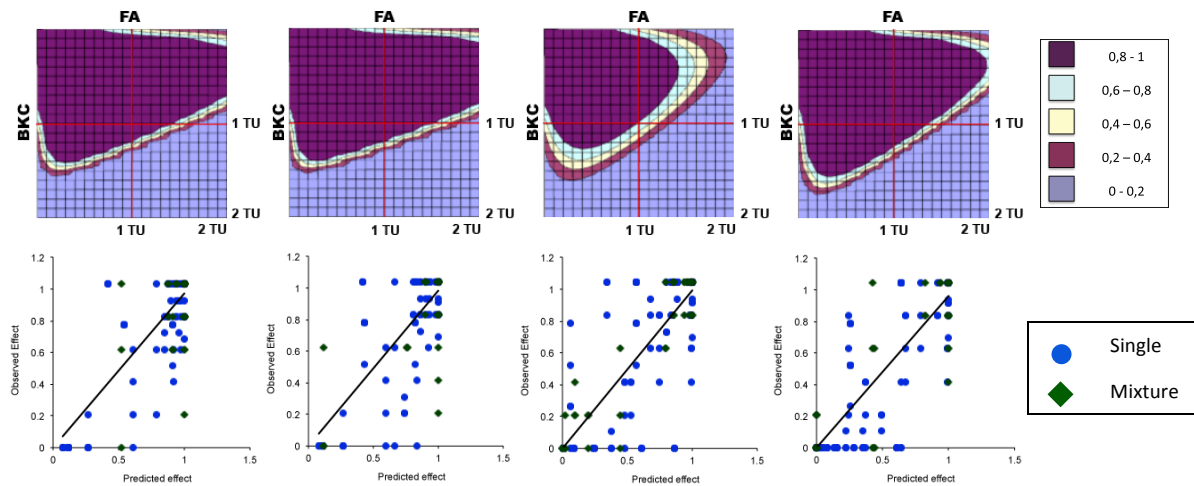
Figure 2.9 - Mortality expected response of zebrafish to the mixture of BKC and FA for 24, 48, 72 and 96 hours, respectively. Upon the isobolograms. Below, the graphics of Observed and Predicted Effects.

Data from combined effects of BKC and FA (Figure 2.9) showed a good fit to the CA model, but when assessing deviations, a dose level dependence was detected. In this case, an antagonism was observed when concentrations of both chemicals were low, and a synergism was verified when concentrations were high at 96 hours (a=61.167; b=0.008; P<0,001; r^2=0.775).
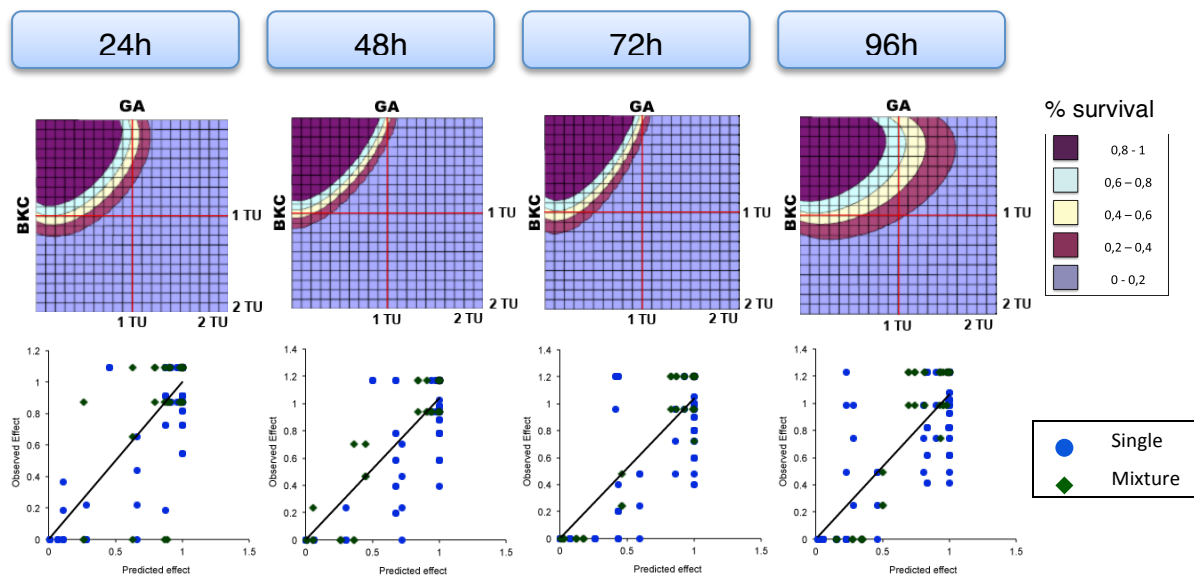
Figure 2.10 - Mortality expected response of zebrafish to the mixture of BKC and GA for 24, 48, 72 and 96 hours, respectively. Upon the isobolograms. Below, the graphics of Observed and Predicted Effects.

Regarding the exposure of BKC and GA, a good fit the CA model was obtained (Figure 2.10), but when changing the functions to assess deviations a antagonism was detected (a=11.430; P<0.001; r^2=0.701)
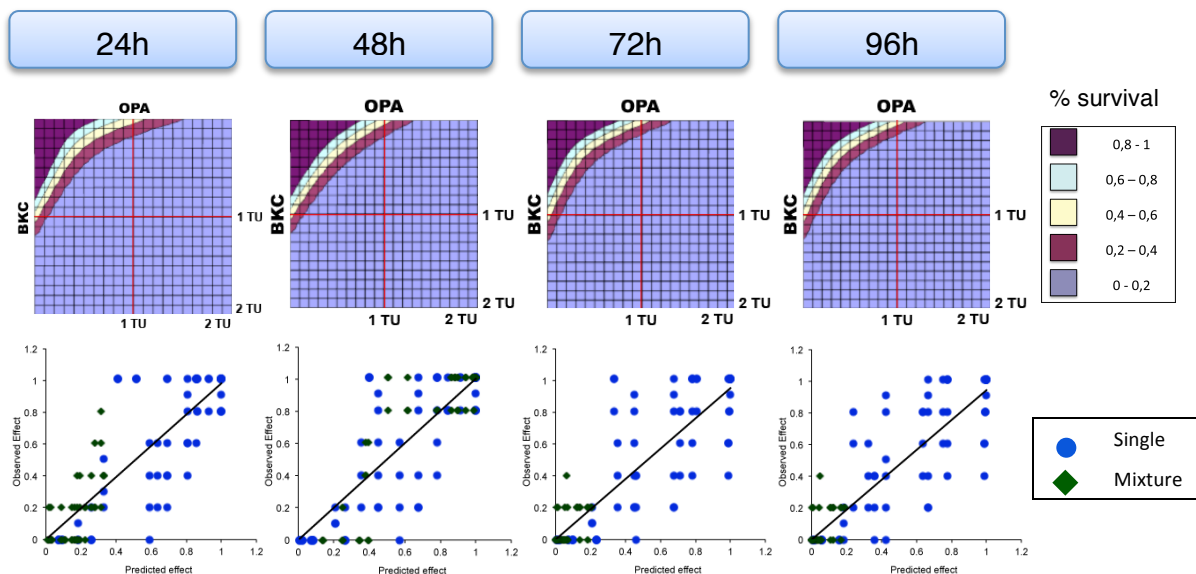
Figure 2.11 - Mortality expected response of zebrafish to the mixture of BKC and OPA for 24, 48, 72 and 96 hours, respectively. Upon the isobolograms. Below, the graphics of Observed and Predicted Effects.

Data from combined effects of BKC and OPA showed a good fit to the IA model (Figure 2.11). However, when assessing deviations, a synergism pattern was obtained (a=-7.315; P<0.001; r2=0.294)

2.4. Discussion

2.4.1. Single toxicity

The results obtained from this study indicate that the chemicals used are, in general, acutely toxic to aquatic organisms. Embryos mortality was correlated with FA concentration, and a 96h-LC_{50} of 546.8 mg/l was calculated. FA showed lower toxicity to *D.rerio* embryos compared to the literature (96 h-LC_{50} = 41.0 mg/l; (HSDB 2006). Is the less toxic of all compounds studied in this work.

The disparity in the value found in the literature may be due to the possible use of different clones in studies of this species with different sensitivities, or the possible use of culture conditions and different test. FA is degraded in the atmosphere, with very small amounts being transferred to water. When released to water or soil, FA undergoes various biological and physical degradation processes. FA is not bioaccumulative or persistent in any compartment of the environment (Chénier 2003).

GA has a lower 96 h-LC_{50} value (27.64 mg/l) reported by Pereira (2009). This was an high LC50 value compared with other embryotoxicity studies performed with fish species: a 96 h LC_{50} of 11 mg/l was found for *Lepomis macrochirus* (UCC 1978) and a 96 h LC_{50} of 0.0239 mg/l was found for *Oncorhynchus mykiss* (EPA 2000). Concentrations of GA ranging from 0.50 to 3.72 mg/l have been detected in hospitals wastewater. The toxicity of GA is not appreciably increased with repeated long-term exposures, it is readily biodegradable in the freshwater environment and has the potential to biodegrade in the marine environment. Aquatic metabolism studies suggest that GA, under aerobic conditions, is metabolized to CO_2 via glutaric acid as an intermediate. Under anaerobic conditions, GA is metabolized to 1,5-pentanediol (Hon-Wing 2001). Assuming that the effluent discharge occurred mostly under anaerobic conditions, and absence of light, then this compound probably has the tendency to form 1,5-pentanediol. Although, this type of reaction is generally believed to occur in nature, GA presents a certain degree of hazard to various organisms. At the broadest level, GA may affect marine life when released

into the environmental via hospital wastewater, although less toxic to saltwater fish than freshwater fish (Smith and Wang 2006)

Embryos mortality was correlated with BKC concentrations, and a 96 h-LC$_{50}$ of 3.9 mg/l was calculated by Pereira (2009). However in another work with *D.rerio* (FEF 2011), BKC toxicity (96 h-LC$_{50}$ =0.31 mg/l) was much higher compared to these studies. Pereira (2009) demonstrated that the toxicity of BKC is considerably higher than for GA. Concentrations of up to 6 mg/l have been measured in hospital effluents (Sütterlin, Alexy et al. 2008). Its resistance to biodegradation, in anaerobic biological systems, results in its environmental persistence. The presence of surfactants in aquatic ecosystems may reach harmful levels to aquatic life, especially to invertebrates and crustaceans which seem to be of the most sensitive groups. As biocides, QACs bind to cytoplasmic membranes and disorganize them via long alkyl chain. Regarding their mode of action, the primary target site appears to be the cytoplasmic (inner) membrane of bacteria. (Sütterlin, Alexy et al. 2008)

D. rerio was much more sensitive to OPA (64.9 µg/l) than to any other compounds. A similar LC$_{50}$ value was reported to *Oncorhynchus mykiss* (rainbow trout) (72.0 µg/l at 96 h.) (Aldrich 2010). OPA is a newly introduced aromatic dialdehyde, and there are few studies showing the toxicity of this compound to aquatic organisms. In micro-organisms, OPA interacts strongly with amino acids. Interestingly, GA does not interact with histidine, whereas OPA does. A possible reason for this is the formation of Van der Waals interaction of the two aromatic components benzene (in OPA) and imidazole, (in histidine). This could be an explanation for the high toxicity of OPA (Simons, Walsh et al. 2000)

The chorion of the egg, considered as a barrier, protect embryo from the surrounding environment but might allow different pollutants to penetrate. No studies were found in literature relating toxicity of FA and OPA with embryo development in *D. rerio* but studies dealing with GA and BKC teratogenic effects on embryo development could be found (Pereira 2009). In this study, we found some teratogenic effects of FA on the zebrafish embryos, like spine malformation

and posture disturbance.

2.4.2.Mixture toxicity

The analysis of adverse effects of chemical mixtures can be performed using two main conceptual models based on the effect of individual compounds: CA and IA. These classic models for the prediction of mixture toxicity are based on simple assumptions on the mode of toxic action. However, the mode of action has already proved to be irrelevant after being demonstrated that toxicological interactions, (namely synergism or antagonism), can occur irrespectively of the primary mode of action (Chou 2006).

The evaluation of the type of interactions existent between the surfactant and the disinfectant compounds, tested using embryos of zebrafish, showed several patterns of response, after the mathematical modeling, ranging between antagonistic to synergistic interactions.

Some authors also found differences between the tests to determine EC50s and single assessments during the test mixture, sometimes more than one order of magnitude, although in most cases less than two times. Usually, estimated EC50 values were nearly similar but in some cases still differed sufficiently to support the need for simultaneous collection of single-compound and mixture data in order to avoid erroneous identification of interactions as a result of between test sensitivity shifts (Martin, Svendsen et al. 2009).

With reference to BKC and FA mixture, data agree with the model IA, with a dose level deviation. The positive value of parameter a indicated an antagonistic behaviour at low stress levels, and synergism at high stress levels. Switching between antagonism and synergism occurs at mixture doses that cause a specific level of effect, indicated by the value of parameter b_i. In fact, a dose level deviation has not serious implications in terms of impact on aquatic ecosystems. Since that in lower doses, the effect is less severe (antagonism). However, these doses are

more likely to be found in the environment.

With CA, individual toxicants act upon the same or a very similar biological system and contribute to a common response in proportion to their respective toxicities. This model best fitted the data of the BKC and GA mixture test, and all combinations of the mixture caused a less severe (antagonism) effect than calculated from either reference model. In our study, the antagonism was more evident at 96 hours. We can relate with the hatching period that usually occurs between 72 and 96 hours. When the larvae hatch, we can suppose, that has not suffered so much toxicity probably because what was left of the decomposition of GA was CO_2 due the conditions of the test with oxygen. In antagonism, the compounds in combination have an overall effect that is less than the sum of their individual effects. In terms of ecological risk antagonism between the compounds is not so worring. However, in this study, which involves products used in hospital, there must be a concern to not use these chemicals together once they are antagonistic, where one agent acts against the effectiveness of another. They inactivate each other, and in turn, disinfection will not be as effective. These can develop resistance microbiological, instead of decreasing it.

In the literature, the effects of antagonistic interactions between GA and surfactants on *Daphnia magna* were identified (Emmanuel, Hanna et al. 2005). But in another study an additive interaction (no interaction) was obtained for the interaction of GA and CTAB, a cationic surfactant (Emmanuel, Hanna et al. 2005). This result is probably due the fact that both compounds are antimicrobial agents. However, it has been demonstrated that solutions of GA can be inactivated by ammonium compounds.

Relatively to the acute exposures of BKC and OPA, the reference model IA and possible deviations were assessed due to dissimilarity on chemicals mode of action. But deviations from the IA conceptual model were found, indicating a more severe combined effect (synergism), due the negative value of a (-6,398). The U.S. EPA defines synergism as "when the effect of the combination is greater than that

suggested by the component toxic effects" (U.S.EPA 2000). While interacting with each other, these pollutants can produce greater impacts on ecological environments. However, is what is intended by the combinations of cleaning or disinfecting hospital. The mode of action of OPA on the molecular structure is also very harmful. It has been described that OPA binds to membrane receptors due to cross-linkage; impairs the membrane functions allowing the biocide to enter through the permeabilized membrane; it interacts with intracellular reactive molecules, such as RNA, compromising the growth cycle of the cells and, at last, with DNA (Simões, Simões et al. 2007).

In general, no attempt has been made by previous researchers to explore the combined effects of such organic mixtures on zebrafish.

Very little is known about mixture toxicity, especially about molecules which might interact due to their chemical properties. In the case studied here interactions between the compounds may take place and affect the toxicity. Our analyses show that different relative toxicity relationships can be observed when different aldehydes with the same surfactant are applied. We obtained an antagonism, a synergism and a dose level dependence. It has been stated also that the interaction of surfactants and chemicals affects different functions and multiple cellular response targets. Such interaction, generates a complex cascade of events in biological systems. As a consequence, synergism or antagonism may occur independently of a similar or dissimilar mode of action.

This work shows that mixtures of surfactants and aldehydes do not always have the same behavior, since we obtained an antagonism, a synergism and a dose level dependence. The results obtained for interactions between these aldehydes and surfactant could be helpful for assessing the real environmental risk and life cycle of these hospital pollutants. We can predict, also, about their effects in aquatic ecosystems and highlights the importance of caution in the use and combination of these compounds in the hospital.

3. Conclusions and final remarks

Toxicity data from single pure chemicals tests provide an essential input to scientific assessments of chemical risks to aquatic life. Aquatic organisms, however, are rarely exposed to only one single contaminant, but commonly to mixtures of numerous man-made-chemicals with varying constituents in varying concentrations and concentration ratios (Faust, Altenburger et al. 2003). Nowadays the interpretation of the combined effects is difficult with a lack of data for comparison, because the chemical mixture toxicity assessment is not yet a routine in ecotoxicology.

The effect of the mixture of thousands of organic pollutants in hospitals wastewater effluent on receiving water bodies is difficult to assess accurately, due to the multiplicity of the chemical structures, and the formation of metabolites. Because of the low levels and structural variability, mostly chronic and interactive effects - antagonism, and synergism - will occur.

The main purpose of this study was to highlight the potential combined effects in binary mixtures of a surfactant and three aldehydes. The first conclusion that can be addressed from these results is that deviations from reference models (IA or CA) were found in all combinations studied (synergism, antagonism and dose level dependency).

Antagonistic interactions in mixtures of compounds could be an advantage in environmental management. This is because antagonism implies that interaction between the constituents results in the lowering of the toxicity of one or all the constituents of a mixture against living species (Ince, Dirilgen et al. 1999). In our work, we obtained an antagonism in the mixture of BKC and GA. In hospitals, there are products that fulfill its function well, others that associated have better disinfectant/antiseptic activity or even better cleaning, because their characteristics are associated synergistically. For example, the use of quaternary ammonium in the disinfection of surfaces, based on the fact that the chemical disinfectant has

low activity (but useful for a vast majority of microorganisms) and simultaneously has a very acceptable power of detergency, conditioning a cleaning/disinfection (low level) in a single act. Some manufacturers have joined to this family, chemicals, like alcohols, that improve their ability to disinfecting and degreasing. For example, Kohrsolin® is a new disinfectant for surfaces based on the synergistic combination of aldehydes and quaternary ammonium compounds. In this study, a synergism between BKC and OPA was verified.

It is not possible to determine all products used in hospital environment, only the classes, because each day brings further one with a "miracle mixture" that solves all the problems that the previous did not solve. In disinfection programs, some aspects should be considered. Mixtures could be avoided because this procedure can cause negative effects such as the neutralization of the disinfecting power, chemical reaction producing toxic byproducts, and still be able to increase the resistance of certain microorganisms.

Standardized tests with embryos of zebrafish are quite useful and commonly used in ecotoxicological studies, and are also necessary for assessing the ecotoxicity of new chemical compounds, recommended by international organizations such as EPA and OECD.

The models developed by Jonker (2005) were particularly useful for evaluating the toxicity of mixtures. Finally, the evaluation of toxic effects of these mixtures for other aquatic species, as well as other binary mixtures in which other disinfectants combined with this surfactant or vice versa (used in hospitals), will be useful both for evaluation and prediction toxic effects for the better understanding of mechanisms of toxicity involved. It is important, also, to consider working on more complex mixtures that can occur in effluents.

The study emphasizes the importance of repeating mixture toxicity experiments, especially for test systems with large variability, and using caution when drawing biological conclusions from the test results.

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4. Annexes

Annex 1 - Confidence interval of standard of Ec_x estimates of BKC and FA mixture

	Chemical I BKC	95% CI (±)	Chemical II FA	95% CI (±)
	24 h			
EC_1	2,474	0,739	181,613	83,878
EC_5	3,025	0,600	307,711	92,607
EC_{10}	3,313	0,511	390,664	92,049
EC_{20}	3,656	0,400	506,177	90,448
EC_{50}	4,328	0,263	788,173	128,200
EC_{80}	5,124	0,526	1227,271	312,800
EC_{90}	5,655	0,831	**1590,157**	**522,267**
EC_{95}	**6,194**	**1,180**	2018,832	809,375
EC_{99}	**7,571**	**2,200**	3420,560	1943,388
	48 h			
EC_1	2,396	0,879	116,403	66,934
EC_5	2,959	0,721	218,973	78,892
EC_{10}	3,256	0,617	291,485	79,368
EC_{20}	3,611	0,485	397,588	78,111
EC_{50}	4,312	0,322	675,935	131,364
EC_{80}	5,148	0,651	1149,150	385,558
EC_{90}	5,711	1,032	**1567,451**	**685,724**
EC_{95}	**6,283**	**1,473**	2086,500	1117,628
EC_{99}	**7,760**	**2,774**	3925,043	2978,490
	72 h			
EC_1	1,919	1,078	136,974	77,991
EC_5	2,511	0,940	201,199	72,059
EC_{10}	2,835	0,829	239,451	63,691
EC_{20}	3,235	0,673	289,236	50,354
EC_{50}	4,053	0,458	399,479	47,545
EC_{80}	5,079	0,948	551,742	140,548
EC_{90}	5,795	1,556	666,457	236,156
EC_{95}	**6,544**	**2,288**	793,164	356,235

	Chemical I BKC	95% CI (±)	Chemical II FA	95% CI (±)
EC_{99}	**8,560**	**4,588**	1165,067	771,785

	Chemical I BKC	95% CI (±)	Chemical II FA	95% CI (±)
	96 h			
EC_1	1,895	0,913	94,338	53,253
EC_5	2,430	0,788	140,922	53,052
EC_{10}	2,720	0,694	168,996	49,554
EC_{20}	3,074	0,566	205,827	42,506
EC_{50}	3,787	0,386	288,324	28,763
EC_{80}	4,667	0,726	403,886	69,339
EC_{90}	5,274	1,174	491,908	125,508
EC_{95}	5,902	1,714	589,904	198,950
EC_{99}	**7,569**	**3,380**	881,202	462,086

Values shown in bold are extrapolations (i.e. they fall outside the experimental range)

Annex 2 - Confidence interval of standard of Ecx estimates of BKC and GA mixture

	Chemical I BKC	95% CI (±)	Chemical II GA	95% CI (±)
	24 h			
EC1	2,668	0,787	38,394	8,652
EC5	3,198	0,624	45,284	6,775
EC10	3,471	0,525	48,796	5,945
EC20	3,794	0,404	52,918	5,398
EC50	4,417	0,261	60,785	6,619
EC80	5,142	0,529	69,822	10,975
EC90	5,621	0,828	75,719	14,665
EC95	**6,101**	**1,165**	81,593	18,741
EC99	**7,312**	**2,125**	96,234	30,154
	48 h			
EC1	2,846	0,941	39,768	134,708
EC5	3,357	0,731	43,847	85,200
EC10	3,618	0,608	45,829	59,095
EC20	3,924	0,461	48,081	27,918
EC50	4,508	0,298	52,190	33,142
EC80	5,178	0,616	56,650	104,647
EC90	5,616	0,953	59,434	151,955
EC95	**6,052**	**1,328**	62,120	199,434
EC99	**7,139**	**2,375**	68,492	318,812
	72 h			
EC1	2,390	1,064	39,382	1428,801
EC5	2,952	0,873	42,715	1022,380
EC10	3,248	0,748	44,314	813,031
EC20	3,603	0,588	46,118	566,434
EC50	4,302	0,391	49,374	94,540
EC80	5,137	0,786	52,860	446,835
EC90	5,698	1,247	55,012	798,649
EC95	**6,269**	**1,779**	57,072	1147,483
EC99	**7,743**	**3,351**	61,901	2008,726

	Chemical I BKC	95% CI (±)	Chemical II GA	95% CI (±)
	96 h			
EC1	2,351	0,859	12,162	7,159
EC5	2,853	0,704	18,180	7,398
EC10	3,114	0,607	21,808	7,178
EC20	3,424	0,486	26,570	6,701
EC50	4,027	0,331	37,240	6,342
EC80	4,737	0,575	52,195	11,349
EC90	5,209	0,893	63,591	18,262
EC95	5,686	1,263	76,282	27,601
EC99	**6,898**	**2,344**	**114,026**	**61,657**

Values shown in bold are extrapolations (i.e. they fall outside the experimental range)

Annex 3 - Confidence interval of standard of Ecx estimates of BKC and OPA mixture

	Chemical I BKC	95% CI (±)	Chemical II FA	95% CI (±)
	24 h			
EC1	2,400	0,915	19,430	12,433
EC5	2,957	0,750	31,799	12,841
EC10	3,251	0,642	39,742	12,006
EC20	3,602	0,505	50,624	10,293
EC50	4,293	0,335	76,565	11,739
EC80	5,116	0,669	115,798	34,906
EC90	5,669	1,059	147,504	60,774
EC95	**6,231**	**1,510**	**184,352**	**95,483**
EC99	**7,679**	**2,840**	**301,708**	**228,501**
	48 h			
EC1	2,297	0,847	23,533	11,065
EC5	2,868	0,703	32,486	9,896
EC10	3,172	0,606	37,591	8,714
EC20	3,538	0,480	44,043	6,930
EC50	4,264	0,322	57,739	5,020
EC80	5,139	0,651	75,694	13,361
EC90	5,731	1,037	88,685	22,413
EC95	**6,338**	**1,488**	102,621	33,469
EC99	**7,915**	**2,834**	141,663	69,729
	72 h			
EC1	1,779	0,886	23,533	11,850
EC5	2,376	0,790	32,486	10,598
EC10	2,709	0,703	37,591	9,332
EC20	3,124	0,576	44,043	7,422
EC50	3,984	0,389	57,739	5,376
EC80	5,081	0,822	75,694	14,308
EC90	5,857	1,373	88,685	24,002
EC95	**6,678**	**2,047**	102,621	35,843
EC99	**8,921**	**4,206**	141,663	74,673
	96 h			
EC1	1,749	0,764	24,162	10,518
EC5	2,294	0,675	32,843	9,294
EC10	2,594	0,600	37,739	8,151
EC20	2,964	0,494	43,882	6,469
EC50	3,724	0,330	56,786	4,581

	Chemical I BKC	95% CI (±)	Chemical II FA	95% CI (±)
EC80	4,678	0,634	73,484	11,810
EC90	5,345	1,051	85,445	19,698
EC95	**6,045**	**1,560**	98,181	29,255
EC99	**7,931**	**3,169**	133,456	60,142

Values shown in bold are extrapolattions (i.e. they fall outside the experimental range)